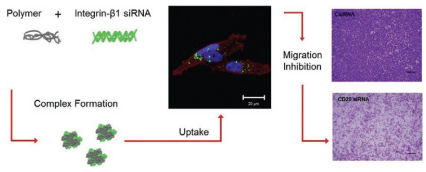
Polymeric Delivery of siRNA against Integrin-β1 (CD29) to Reduce Attachment and Migration of Breast Cancer Cells

Daniel Nisakar Meenakshi Sundaram, Cezary Kucharski, Manoj B. Parmar, Remant Bahadur KC, Hasan Uludağ*

Cell surface integrins, which play important roles in the survival, proliferation, migration, and invasion of cancer cells, are a viable target for treatment of metastatic breast cancer. This line of therapy still remains challenging due to the lack of proper identification and validation of effective targets as well as the lack of suitable therapeutic agents for treatment. The focus is on one such molecular target for this purpose, namely integrin-β1, and effective lowering of integrin-β1 levels on a breast cancer model (MDA-MB-231 cells) is achieved by delivering a dicer-substrate short interfering RNA (siRNA) targeting integrin-β1 with lipid-modified low molecular weight polyethylenimine polymers. Reduction of integrin-β1 levels leads to reduced adhesion of MDA-MB-231 cells to extracellular matrix component fibronectin as well as to human bone marrow cells. A reduced migration of the breast cancer cells is also observed after integrin-β1 silencing in "scratch" and "transwell" migration assays. These results highlight the importance of integrin-β1 for the migration of metastatic breast cancer cells by effectively silencing this target with a practical dose of siRNA.



1. Introduction

Metastasis of breast cancer to various organs such as brain, liver, lung, and bone makes the disease almost incurable. Metastatic breast cancer is the second leading cause of cancer deaths following lung cancer.[1] The process of metastasis is initiated with (i) epithelial-to-mesenchymal transition (EMT) of the tumor at the primary site, (ii) extracellular matrix degradation, (iii) intravasation, (iv) migration, (v) evasion from host cell defense mechanism, and (vi) extravasation followed by attachment to the distant metastatic site.[2] The cell surface integrins expressed on tumor cells are vital for attachment of metastasizing cells to other organs. The integrins are cell surface receptors that are essential for cell–cell and cell–extracellular matrix interactions, and play a pivotal role in migration, invasion, proliferation, survival of tumor cells, and also in growth factor receptor signaling.[3] In mammals, there are 18 α-subunits and 8 β-subunits in the integrin family which can form 24 different integrin receptors and bind to various extracellular matrix components such as fibronectin, collagen, laminin, and vitronectin.[4,5] Overexpression of integrins and their involvement in cancer progression has been demonstrated in different cancer types.

In breast cancer, integrin-β1 (CD29) activation contributes to dormancy to metastatic shift in in vitro and in vivo models,[6,7] and its overexpression is shown to

D. N. Meenakshi Sundaram, M. B. Parmar, Prof. H. Uludağ
Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta
Edmonton, AB T6G 2H1, Canada
E-mail: hasan.uludag@ualberta.ca
C. Kucharski, Dr. R. B. KC, Prof. H. Uludağ
Department of Chemical and Materials Engineering
University of Alberta
Edmonton, AB T6G 2V2, Canada
Prof. H. Uludağ
Department of Biomedical Engineering
University of Alberta
Edmonton, AB 2V2, Canada

DOI: 10.1002/mabi.201600430

be associated with invasive potential of the cancer cells and a significant decrease in the overall disease-free survival.[8–11] Antibodies targeting integrin-β1 were shown to result in induction of apoptosis as well as decrease in tumor growth. One such promising antibody, PF-04605412 targeting α5β1, displayed acute-infusion-related reaction, and it failed to inhibit the tumor growth in clinical trials.[12] The use of CRISPR/Cas9 to study the importance of integrin-β1 on epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (gefitinib) resistance revealed reduced migration of cells and negative regulation of EGFR activation upon integrin-β1 knockout.[13] The use of CRISPR/Cas9 for therapeutic intervention, however, may not be desirable, given the permanent deletion of integrin-β1. Resistance to trastuzumab, a monoclonal antibody targeting human EGFR2 was addressed by targeting integrin-β1 using a combination of a blocking antibody (AIIB2) and specific short interfering RNA (siRNAs; delivered by electroporation).[14] In addition to antibodies, ATN-161, an integrin-β1-binding peptide, reduced the metastasis and tumor growth in animal models, and its clinical trials resulted in prolonged stable disease in patients.[15,16] The importance of integrin-β1 during tumor progression was further emphasized by ablating integrin-β1 in a mouse model, which interfered with the proliferation of cancer cells, and mice deficient in integrin-β1 exhibited a drastic reduction in mammary lesion.[17] Evidence of integrin-β1 as the major factor for the attachment of breast cancer cells has been shown by binding studies to fibronectin. Attachment to fibronectin activates various intracellular signaling pathways, which enhances cancer cell proliferation, migration, survival, and also helps to confer increased drug resistance.[18–20]

These studies supported the importance of integrin-β1 as a potential therapeutic target to reduce breast cancer metastasis. Though several integrins have been identified and their inhibitors interrupted breast cancer metastasis, the preclinical and clinical outcomes of such drugs had little impact to improve the survival rate of the patients.[21] There is a strong need to develop more efficient approaches to overcome integrin-mediated metastasis of breast cancer. An alternative approach to target integrin-β1 is to employ an RNA interference (RNAi) mechanism where a target protein can be endogenously silenced post-transcriptionally. It seems possible to silence any protein target at will using RNAi. A recent study focused on integrin-β1 by using lentiviral short hairpin RNA (shRNA); integrin-β1 knockdown resulted in reduced attachment of MDA-MB-231 and MDA-MB-231 BO (bone metastatic) cells to human osteoblastic cell-derived matrices and led to reduced cellular migration without affecting the proliferation of cells.[22] Employing viral mechanisms to implement RNAi, however, is not desirable in a clinical setting. A more acceptable approach is to employ a pharmacological mediator of RNAi, namely siRNA, with nonviral carriers to achieve silencing.

In this study, we further explored the therapeutic prospect of integrin-β1 using a simpler and efficient intervention based on the polymeric delivery of a dicer-substrate siRNA targeting integrin-β1. As the successful entry of intact siRNA into the cells is plagued by barriers such as the anionic cell membrane and degradation by RNase A, a safe nonviral delivery system was employed to protect and deliver it into the cells. For this purpose, we utilized polyethylenimine (PEI) polymers of low molecular weight (1.2 kDa; 1.2PEI) which have been substituted with lipids to improve the interaction of the cationic PEI/siRNA complexes with the anionic cell membrane. The use of dicer-substrate siRNA (rather than conventional 21 nt double-stranded RNA) previously showed improved silencing by being incorporated into dicer enzyme in the RNA-induced silencing complex (RISC) complex[23] and was additionally demonstrated in a previous study of our group.[24] We successfully silenced integrin-β1 with minimal siRNA concentration which in turn reduced the migration of breast cancer cells as well as its binding ability to human bone marrow stromal cells and fibronectin-coated surface. This treatment had very minimal effect on the proliferation of the breast cancer cells, thus providing a promising as well as a specific approach for breast cancer metastasis.

2. Experimental Section

2.1. Materials

The 1.2PEI, fetal bovine serum (FBS), anhydrous dimethyl sulfoxide, fibronectin, bovine serum albumin (BSA), formaldehyde, chloroform, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Hank's balanced salt solution (HBSS), trypsin/Ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified Eagles medium (DMEM), penicillin, streptomycin, and UltraPure DNase/RNase-free dH_2O were obtained from Fisher Scientific (Ottawa, Canada). Phycoerythrin (PE)-labeled mouse antihuman CD29 was from BD Biosciences (Oakville, Canada). Dicer-substrate siRNA (CD29_1 sense: 5′-AGUUAACAGUGAAGACAUGGAUGCT-3′, antisense: 5′-AGCAUCCAUGUCUUCACUGUUAACUUC-3′; CD29_2 sense: 5′-GCAAAUUCUAGCAAUGUAAUUCAGT-3′, antisense: 5′-ACUGAAUUACAUUGCUAGAAUUUGCAG-3′) and primers for polymerase chain reaction (PCR) analysis were from IDT (Coralville, USA). Trizol used for total RNA extraction was from Invitrogen (Carlsbad, CA). Corning Costar transwell plates (6.5 mm inserts with 8.0 μm pore size Polyester (PET) membrane) were from Fisher Scientific. Cell lysis buffer (BML-KI117-0030) and caspase-3 substrate (Ac-DEVD-AFC) were purchased from Enzo Life Sciences (Farmingdale, NY).

2.2. Cell Model

The metastatic breast cancer cell line, MDA-MB-231, were kindly provided by Dr. Judith Hugh (Faculty of Medicine and Dentistry,

University of Alberta, Edmonton) and confirmed to be mycoplasma free. The cells were maintained in DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C and 5% CO_2. Cells were passed after reaching 80% confluency, using 0.25% trypsin/EDTA for 2 min at room temperature. Cells were collected by adding complete DMEM and centrifuged at 600 rpm for 5 min. They were allowed to grow for 24 h prior to treatment. Human bone marrow stromal cells (hBMSCs) were isolated from patients (between 25 and 50 years of age) based on a procedure[25] approved by the Research Ethics Board of University of Alberta. These hBMSC cells were maintained in DMEM with 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 5 ng mL^{-1} basic fibroblast growth factor at 37 °C and 5% CO_2.

2.3. Polymer Synthesis and siRNA–Polymer Complex Preparation

The 1.2PEI modified with thiol–ester containing α-linoleic acid (tαLA, 2.73 αLA substitutions/PEI; 1.2PEI-tαLA), amide-linked lauric acid (Lau, 4.6 Lau substitutions/PEI; 1.2PEI-Lau), and amide-linked linoleic acid (LA, 6 LA substitutions/PEI; 1.2PEI-LA) were synthesized based on a previously published protocol,[26–28] and the degree of substitution was determined through ^{1}H-NMR. The polymer–siRNA complexes were prepared in serum-free DMEM and incubated for 30 min at room temperature before adding to the cells (in 10% FBS). Complexes were prepared at 4:1 and 8:1 polymer-to-siRNA weight/weight ratios (corresponding N:P ratios were 15:1 and 30:1). 40×10^{-9} and 80×10^{-9} M of siRNA concentrations were used in culture treatments for dose optimization experiments. All other experiments were carried out at 4:1 polymer-to-siRNA (weight/weight) ratio with 40×10^{-9} M siRNA concentration. Lipofectamine 2000-siRNA complexes were prepared at 2:1 lipid-to-siRNA (weight/weight) ratio (as suggested by the manufacturer) with similar siRNA concentrations and incubated for 30 min at room temperature.

2.4. Flow Cytometry for Integrin-β1 Analysis

The MDA-MB-231 cells were seeded 24 h prior to the experiments. The cells were treated with the polymer/siRNA complexes for 72 h (day 3) following which they were collected using Accutase (1:1 diluted with HBSS), washed using HBSS, and stained with PE-anti-CD29 for 1 h at room temperature. The cells were washed twice with HBSS to remove excess antibody. For study groups involving day 6 and day 9 assessment, the medium was replenished with fresh complete DMEM medium on day 3 and were incubated further, which was followed by staining as mentioned above. Cells were fixed with 2% formaldehyde prior to analysis with BD LSRFortessa (Becton-Dickinson, San Jose, USA). The extent of integrin-β1 expression was expressed as either mean levels per cell (in arbitrary fluorescent units) or as percentage of cell population positive for integrin-β1. Cells with no treatment (NT; unstained) were designated as 1% positive population.

2.5. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To quantify the integrin-β1 mRNA levels, qRT-PCR was performed for which cells were seeded 24 h prior to treatment. Complexes prepared, as mentioned earlier, were added to the MDA-MB-231 cells. Later, cells were collected at three different time points (days 3, 6, and 9) as mentioned in the previous section, and total RNA was extracted using Trizol reagent based on the manufacturer's instruction. 2 μg of total RNA was reverse transcribed to synthesize cDNA by using 2 μL of mix-1 containing 0.5 μL random hexamer primer, 1 μL (10×10^{-3} M) dNTPs, and 0.5 μL Oligo (dT), and heated at 65 °C for 5 min. Following which 8 μL of mix-2 containing 4 μL synthesis buffer (5×), 2 μL DTT (0.1 M), 1 μL RNase out, and 1 μL M-MLV RT enzyme was added and incubated at 25 °C for 10 min, 37 °C for 50 min, and 70 °C for 15 min. Real-time PCR was carried out on a StepOnePlus RT-PCR system with human β-actin (forward: 5′-GCG AGA AGA TGA CCC AGA T-3′ and reverse: 5′-CCA GTG GTA CGG CCA GA-3′) as the endogenous housekeeping gene and for integrin-β1 (forward: 5′-CCG CGC GGA AAA GAT GAA T-3′ and reverse: 5′-TGA GCA AAC ACA CAG CAA ACT-3′). 10 μL of reaction mixture containing 5 μL master mix SYBR Green, 2 μL of 10×10^{-6} M primers, and 3 μL of 5 ng μL^{-1} cDNA template was added in triplicates to the MicroAmp Fast Optical 96-well reaction plate. The reaction mixtures were heated at 95 °C for 10 min before proceeding through 40 cycles of the denaturation step, 95 °C for 15 s, and annealing/elongation step, 60 °C for 1 min. ΔC_T, $\Delta\Delta C_T$, and relative quantity of mRNA were calculated with endogenous gene and the NT group as reference points.

2.6. Scratch Assay

MDA-MB-231 cells were plated in 48-well plates with four replicates and cultured for 24 h. The prepared 1.2PEI–LA polymer/siRNA complexes were added to the cells and incubated for 48 h. After this scratches were made using a 200 μL pipette tip, following which cells were washed to remove the floating cells. Images were obtained before incubating (0 h) the cultures at 37 °C for 24 h, after which images were obtained once again. The open wound area was measured using TScratch software (available from http://cse-lab.ethz.ch/software/) and the percentage of migration was calculated by subtracting values of 0 h from 24 h and values were plotted relative to the no-treatment group.

2.7. Transwell Migration Assay

The MDA-MB-231 cells were grown on six-well plates, 24 h prior to the treatment. 1.2PEI–LA polymer/siRNA complexes were added to the cells and incubated at 37 °C for 24 h. The cells were then washed gently with HBSS to remove the serum content and fresh serum-free medium was added and further incubated for 24 h. This was followed by collection of cells using trypsin and ≈$1–2 \times 10^5$ cells were resuspended in 100 μL of serum-free medium and were added to transwell inserts. The lower bottom of the wells containing the inserts was filled with medium containing 20% serum as a chemoattractant. This setup was incubated for an additional 24 h at 37 °C. The cells present on the upper surface of the inserts (the cells that did not migrate) were removed gently using a cotton swab. The inserts were then fixed with 3.7% formaldehyde for 20 min and stained with 0.1% crystal violet for 1 h. The inserts were provided with three to four washes with HBSS before imaging under a microscope. The dye was subsequently solubilized with 10% acetic acid, and the

absorbance was measured at 570 nm using the EL_x800 Universal Microplate reader (Bio-Tek Instruments).

2.8. Fibronectin Binding Assay

The binding assay was carried out based on a published protocol[29] with minor modifications. Briefly, fibronectin was coated onto 96-well flat bottom plates at 4 °C overnight with 50 μL of 5 μg mL^{-1} of fibronectin. The plates were then blocked with 2% BSA in phosphate buffered saline (PBS) for 1 h at room temperature. MDA-MB-231 cells treated with polymer/siRNA complex for 72 h were collected by trypsinization, and an equal number of cells were added in triplicates to the fibronectin-coated plates and incubated at 37 °C for 1 h. The plates were subsequently inverted and further incubated for 3 h at 37 °C. Cells were fixed with 3.7% formaldehyde followed by staining with 0.1% crystal violet for 1 h and washed with HBSS. The dye was solubilized with 10% acetic acid, and the absorbances were measured at 570 nm using the EL_x800 Universal Microplate reader (Bio-Tek Instruments).

2.9. hBMSC Adhesion Assay

Human bone marrow stromal cells were seeded in 96-well flat bottom plates and were maintained for 2–3 d to reach confluency.[30] MDA-MB-231 cells were treated with desired siRNA/polymer complexes, harvested after 72 h of treatment (i.e., siRNA complexes incubated continuously with cells during this time), and stained with DiI (carbocyanine dye) for 20 min. These cells were added to the confluent hBMSC monolayers in 96-well plates and incubated for 1 h at 37 °C followed by which the plates were placed upside down and further incubated for 3 h at 37 °C. The nonadherent cells were removed by washing, and the fluorescence was recorded at E_x (549 nm)/E_m (565 nm), using the Fluoroskan Ascent plate reader (Thermolab systems).

2.10. siRNA Uptake

FAM (6-carboxyfluorescein) labeled control siRNA was used to study the uptake of siRNA in MDA-MB-231 cells. The cells were allowed to grow for 24 h, following which FAM–siRNA/polymer complexes (polymer/siRNA ratio of 4:1 and 40×10^{-9} M in solution; prepared as above by replacing unlabeled/specific siRNAs with FAM-labeled siRNA) were added to the cells and incubated for 24 h. Cells were trypsinized, washed twice with HBSS, and fixed with 3.7% formaldehyde. Cells treated with unlabeled control siRNA were used as negative population, and the uptake was quantified using BD LSRFortessa (Becton-Dickinson, San Jose, USA). The extent of siRNA uptake was expressed as either mean siRNA levels per cell (in arbitrary fluorescent units) or as percentage of cell population positive for FAM-labeled siRNA. Cells treated with unlabeled siRNA complexes were designated as 1% positive population.

The siRNA uptake was also investigated by confocal microscopy as a complementary tool to flow cytometry analysis. After treating the cells grown on coverslips with siRNA complexes for 24 h, the cells were washed thoroughly with HBSS and were fixed with 4% paraformaldehyde for 20 min at 37 °C. Cells were once again washed with ddH_2O and stained with 1 μg mL^{-1} wheat germ agglutinin (WGA)–Texas Red conjugate for 5 min at room temperature and were washed three times. 6 μL of mounting medium with 4,6-diamino-2-phenylindole (DAPI) was used to mount the coverslip onto the slides. Samples were imaged under 40 × 1.3 plan-apochromat lenses in laser scanning confocal microscopy (LSM710, Carl Zeiss AG, Oberkochen, Germany). The captured images were analyzed using the software ImarisCell v 8.3, BITPLANE.

2.11. Caspase Activity

Following 3 d of treatment with polymer/siRNA complex, cells were collected, counted, and lysed using a cell lysis buffer (50×10^{-3} M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 5×10^{-3} M 1,4-Dithiothreitol (DTT), 0.1×10^{-3} M EDTA; pH 7.4) with incubation on ice for 5 min. The supernatants were centrifuged, and 50 μL of reaction buffer prepared by mixing 10 μL of Ac-DEVD-AFC substrate (20×10^{-3} M) with 1 mL of HEPES buffer was added to the cell lysate at 0.2×10^{-3} M (final concentration). Fluorescence was recorded at E_x (400 nm)/E_m (505 nm) using a Fluoroskan Ascent plate reader (Thermolab systems) for different time points (0, 30, 60, 90, and 120 min).

2.12. Statistical Analysis

The results were summarized as mean ± standard deviation (SD), and the unpaired Student's *t*-test was used to assess the statistical differences between the group means with *p*-value <0.05 considered as statistically significant. Where specified, the number of independent experiments used to generate the data (*n*) is indicated.

3. Results

3.1. Low Concentration of siRNA is Sufficient to Reduce Integrin-β1 Levels

To undertake effective delivery of siRNA across the cellular membrane, we employed three in-house prepared PEI polymers that were modified with (i) tαLA (2.73 substitutions per PEI):1.2PEI–tαLA, (ii) Lau (4.6 substitutions/PEI): 1.2PEI–Lau, and (iii) LA (6 substitutions/PEI): 1.2PEI–LA. These polymers were selected based on their silencing efficiency from an initial library screening where an anti-integrin-β1 siRNA was used (Figure S1, Supporting Information) to lower the cell surface integrin-β1 levels. The size of the polymer:siRNA complexes was in the range of 300–350 nm with positive zeta potentials and conferred complete siRNA protection that has been reported previously.[28]

The siRNA uptake studies using flow cytometry with the polymers displayed similar levels of internalization (≈50% of cell population) with all three polymers, which was equivalent to siRNA delivery with Lipofectamine 2000 (Figure 1A). However, the mean fluorescence, corresponding to a mean

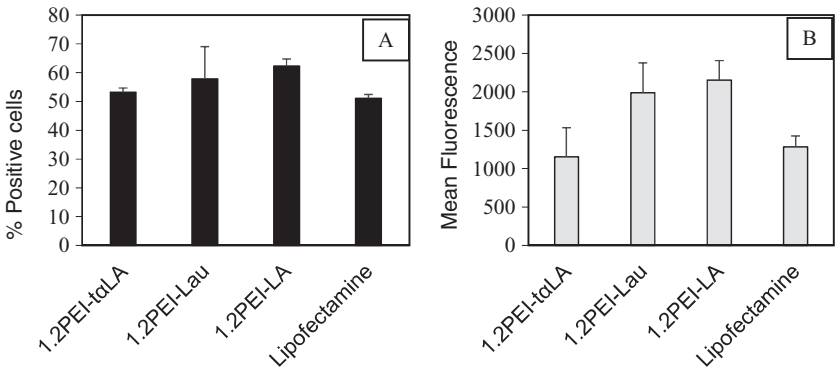


Figure 1. The uptake of FAM-labelled siRNA with 1.2PEI–tαLA, 1.2PEI–Lau, 1.2PEI–LA, and Lipofectamine 2000 after 24 h of treatment. A) The percentage of positive cells for FAM–siRNA/polymer complex and B) its corresponding mean fluorescence.

amount of siRNA associated per cell, was approximately twofold higher for 1.2PEI–LA and 1.2PEI–Lau polymers, followed by similar levels of fluorescence by the 1.2PEI–tαLA polymer and Lipofectamine 2000 (Figure 1B).

The localization of polymer/siRNA complex inside the cell was analyzed using confocal microscopy. The cytoplasmic localization was higher with 1.2PEI–LA polymer when compared to 1.2PEI–Lau and Lipofectamine 2000 ($p \leq 0.0001$) with relatively few complexes in the nucleus (Figure 2A,B). The overall internalization was slightly lower with 1.2PEI–Lau but it still exhibited higher cytoplasmic localization than in nucleus unlike Lipofectamine 2000, which exhibited equivalent localization between the cytoplasm and the nucleus. The overall internalization of siRNA delivered with 1.2PEI–Lau and Lipofectamine 2000 appeared to be similar. Thus, the higher cytoplasmic localization of 1.2PEI–LA/siRNA complexes was considered beneficial since the site of action of siRNA delivery is cytoplasmic.

Since optimal silencing depends on siRNA concentration as well as polymer:siRNA ratio used in making the complex formulations, we performed a dose optimization study for reducing cell surface integrin-β1 levels with two dicer-substrate siRNAs (CD29_1 and CD29_2) targeting two different regions in the integrin-β1 gene. The siRNA concentrations were 40×10^{-9} and 80×10^{-9} M, while the complexes were formed at 4:1 and 8:1 weight/weight ratios (polymer-to-siRNA) (Figure 3A–C[i]). The CD29_2 siRNA was not effective in silencing integrin-β1 with any of the polymers as the integrin-β1 levels remained similar to the control scrambled siRNA (CsiRNA) treatment. On the other hand, CD29_1 siRNA displayed significant silencing at the lowest ratio of 4:1 with 40×10^{-9} M siRNA in complexation with all polymers. Further increasing the concentration of siRNA or the polymer:siRNA ratio did not influence the silencing effect. Lowering the siRNA concentration as low as 10×10^{-9} M was also effective in silencing and with gradual increase in concentration, the effect was maximum between 20×10^{-9} and 40×10^{-9} M (Figure 3C[ii]). The lower doses of siRNA are advantageous as they reduce the possibility of off-target effects.[31] Failing to observe any increase in silencing after adding higher doses of siRNA or polymer:siRNA ratio, further studies were conducted at 4:1 polymer:siRNA ratio and with 40×10^{-9} M of CD29_1 siRNA.

Using direct cell counts (Figure 4), integrin-β1 siRNA delivery with 1.2PEI–tαLA and 1.2PEI–LA polymers gave significant decrease in cell numbers after 3 d of siRNA treatment. The cell number was drastically reduced for both integrin-β1 siRNA and CsiRNA-treated sample of 1.2PEI–Lau, which was indicative of nonspecific toxicity of the complexes on the cells.

3.2. Polymer-Mediated siRNA Delivery Sustains the Silencing Effect

We examined the duration of silencing over a period of 9 d after treating the MDA-MB-231 cells with polymer/siRNA complexes. All three polymers exhibited similar extent of silencing (40%–50% based on mean integrin-β1 levels) on day 3 when compared to the CsiRNA-treated cells (Figure 5). With 1.2PEI–tαLA, the integrin-β1 levels relapsed back to the levels observed for CsiRNA-treated samples at days 6 and 9. The 1.2PEI–Lau polymer was able to sustain the silencing until day 6, but the effect was not significant on day 9. The cells treated with 1.2PEI–LA polymer exhibited prolonged and significant silencing from day 3 to day 9. The reference reagent, Lipofectamine 2000, was also capable of achieving significant reduction in integrin-β1 levels, but the silencing effect was not significant on day 9.

3.3. Polymer-Mediated siRNA Delivery Provides Strong Knockdown of Integrin-β1 mRNA

The reduction of integrin-β1 mRNA levels was assessed through qRT-PCR after treating the cells with siRNA/polymer complexes for 3 d. All three polymers displayed significant reduction in the mRNA levels after day 3, but the knockdown efficacy was much higher with the 1.2PEI–LA delivered siRNA (≈90%). Though all the polymers expressed similar levels of silencing in the flow cytometry analysis on day 3 (Figure 5), the integrin-β1 mRNA levels were reduced to a different extent (Figure 6A). The silencing was significantly higher with siRNA delivered with 1.2PEI–LA (90%) than 1.2PEI–tαLA (70%) and 1.2PEI–Lau (40%). It was interesting to note that treatment with 1.2PEI–Lau complexes resulted in elevated integrin-β1 levels with CsiRNA and this increase could be due to the presence of lauric acid.

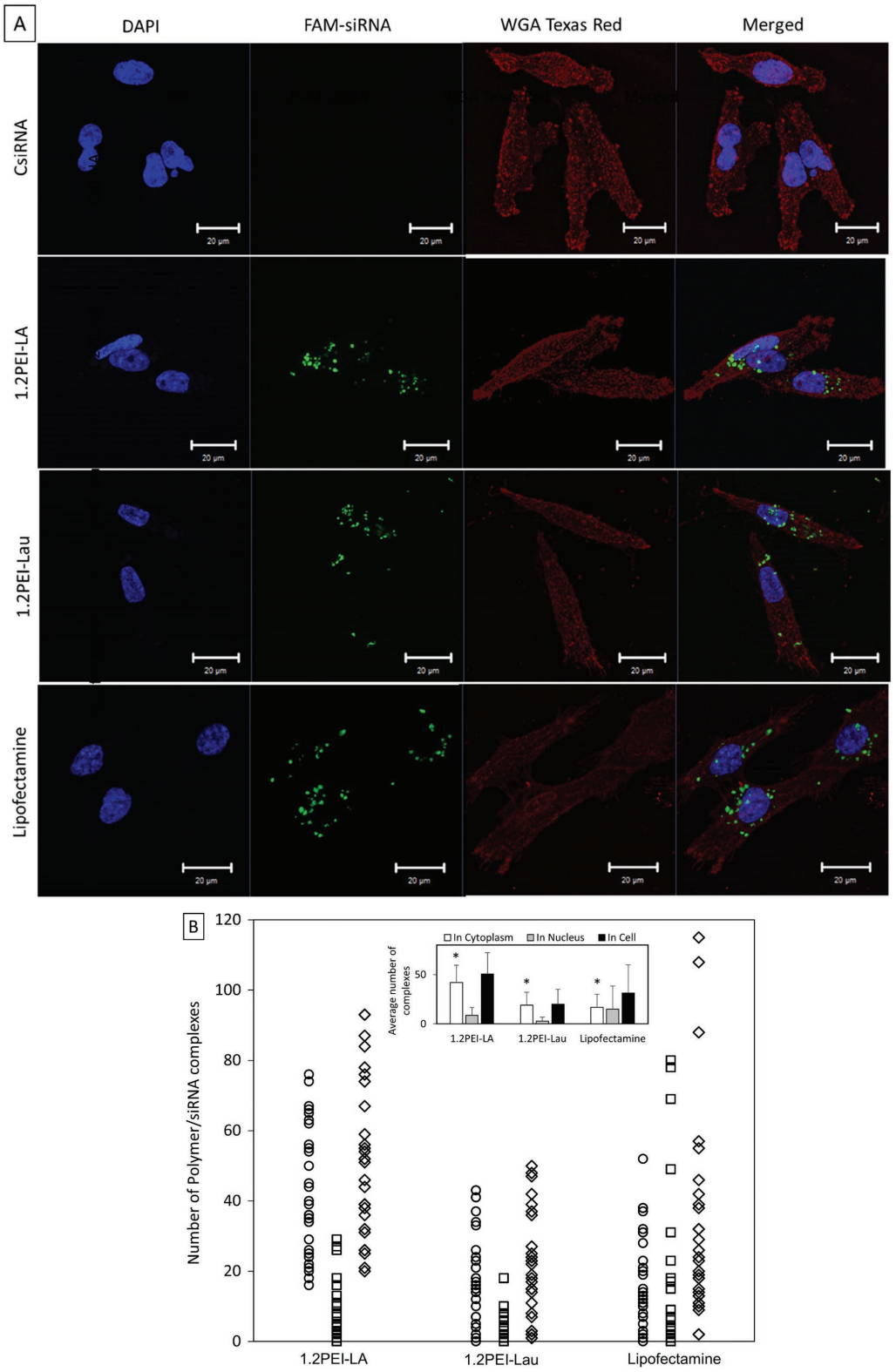


Figure 2. A) The uptake of FAM-labelled siRNA with 1.2PEI–LA, 1.2PEI–Lau, and Lipofectamine 2000 after 24 h of treatment. Representative pictures are shown where the siRNA particles were visualized as green, cytoskeleton as red, and nuclei as blue. Unlabeled CsiRNA was used as a negative control. B) The number of polymer/siRNA complexes in cytoplasm (circle), nucleus (square), and total cell (diamond) was analyzed using the Imaris software. The inset represents the average number of polymer/siRNA complexes in each cell (+ SD). *, $p \leq 0.0001$ ($n = 31$ for each group).

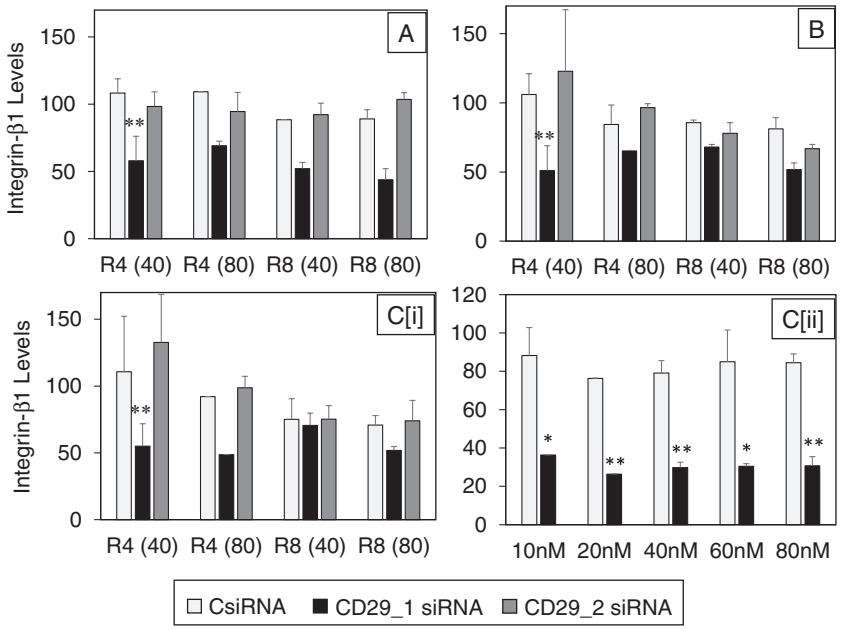


Figure 3. Integrin-β1 silencing with siRNA delivery. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no-treatment group) were measured after 3 d of siRNA treatment for polymers A) 1.2PEI–tαLA, B) 1.2PEI–Lau, and C[i]) 1.2PEI–LA. The polymer:siRNA (weight/weight) ratios of 4 (R4) and 8 (R8) were used at 40×10^{-9} and 80×10^{-9} M of siRNA concentration with two different siRNAs (designated as CD29_1 and CD29_2 siRNAs). C[ii]) Dose response curve for integrin-β1 levels with CD29_1 siRNA delivered with 1.2PEI–LA polymer with increasing siRNA concentrations. *, $p \leq 0.05$; **, $p \leq 0.01$ ($n = 2$). CsiRNA: Control scrambled siRNA.

Lauric acid (at very high concentrations) was shown to regulate the expression of NF-κB in macrophages[32] and in colon epithelial cells.[33] The transcription factor NF-κB binds to integrin-β1 promoter region and could account for our observation in this experiment. We selected 1.2PEI–LA polymer to study the silencing duration; siRNA delivery with this polymer gave strong integrin-β1 knockdown until day 9 (Figure 6B), which was consistent from day 3 to day 9.

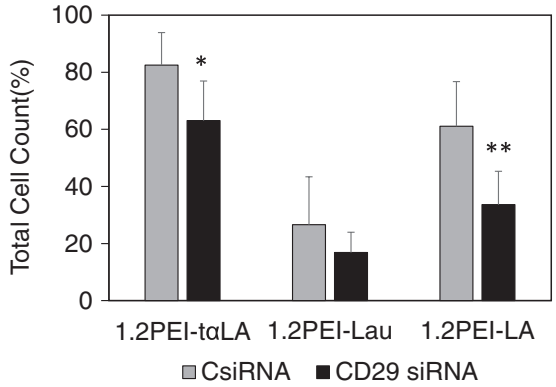


Figure 4. Effect of integrin-β1 silencing on cell number. Cell counts were obtained after 3 d of treatment with the 3 polymers at a ratio of 4 and at 40×10^{-9} M CD29 siRNA concentration (given by percentage of cell count relative to no-treatment group). *, $p \leq 0.05$; **, $p \leq 0.007$ ($n = 3$). CsiRNA: Control scrambled siRNA.

3.4. Integrin-β1 Silencing Reduces Binding of Breast Cancer Cells to Fibronectin and hBMSCs

Following successful knockdown of integrin-β1, we checked the functionality of treatment by assessing the binding of siRNA-treated cells to primary integrin-β1 receptor fibronectin. After treatment with the polymer/siRNA complexes for 3 d, all polymers showed significant reduction in cell binding, consistent with integrin-β1 silencing. The cells treated with 1.2PEI–LA/siRNA complexes, which presented the highest knockdown efficiency among the polymer complexes, showed higher decrease in binding to fibronectin (Figure 7A). Treatment with increasing siRNA concentrations showed significant and steady decrease in fibronectin binding starting from 10×10^{-9} to 40×10^{-9} M, but additional increase in siRNA concentration (60×10^{-9} and 80×10^{-9} M) had no further effect on binding (Figure 7B).

Binding to hBMSCs was additionally explored for siRNA-treated MDA-MB-231 cells, where the treated cells were collected and allowed to adhere to a monolayer of hBMSC. Significant reduction in hBMSC binding was observed with 1.2PEI–LA/siRNA complex treatment (≈20%), whereas other polymers did not inhibit the binding of treated cells to hBMSCs (Figure 7C).

3.5. Integrin-β1 Silencing Inhibits Migration of Breast Cancer Cells

The migration of MDA-MB-231 was studied using the well-established "scratch" assay following the silencing of integrin-β1. The percentage of open wound area was calculated using image analysis after allowing the cells to recover for 24 h. We employed only 1.2PEI–LA polymer to deliver the integrin-β1 siRNA as it showed strongest silencing at the protein and mRNA levels (data from Figures 5 and 6). We observed ≈60% migration inhibition with the integrin-β1 siRNA delivery as compared to the CsiRNA-treated samples (Figure 8A,B[i]). A small but significant decrease in cell numbers (assessed by the MTT assay) was noted for integrin-β1 siRNA-treated cells (Figure 8B[ii]), similar to results in Figure 4. A dose effect study with different siRNA concentrations (low to high) showed a gradual and steady drop in the migration of cells with increase in the concentration of siRNA (Figure 9; Figure S2, Supporting Information).

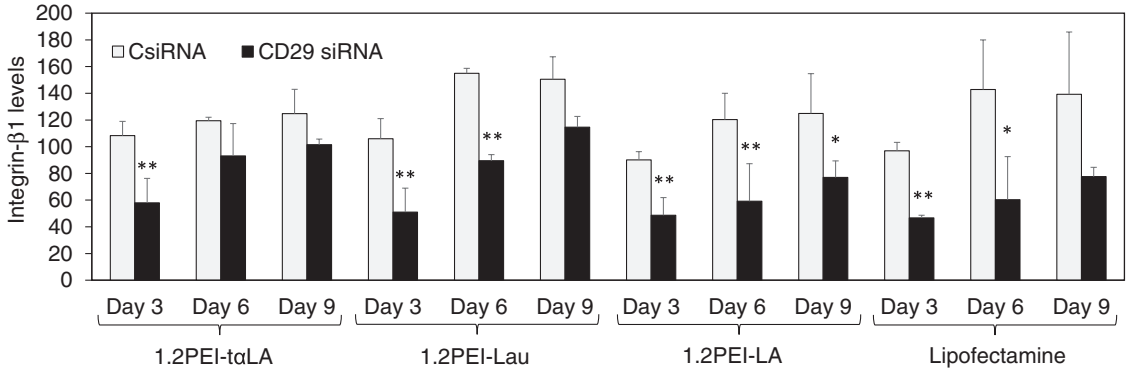


Figure 5. Integrin-β1 silencing with siRNA delivery at longer durations. The integrin-β1 levels (given by percentage of mean fluorescence intensity relative to no-treatment group) were measured after 3, 6, and 9 d of siRNA treatment for polymers 1.2PEI–tαLA, 1.2PEI–Lau, 1.2PEI–LA, and Lipofectamine 2000. A polymer:siRNA (weight/weight) ratio of 4 with 40×10^{-9} M of siRNA concentration was used. *, $p \leq 0.04$; **, $p \leq 0.01$ ($n = 2$).

To further validate the scratch assay results, we performed a transwell migration assay under the same conditions as above. Using crystal violet staining, representative images showed a decline in cells that have migrated to the lower surface of the insert upon integrin-β1 siRNA treatment (Figure 10A). Upon quantitating the cell numbers, the CsiRNA-treated cells in the absence of serum displayed ≈26% migration, whereas the CsiRNA-treated cells in complete medium (i.e., with 20% serum) exhibited ≈85% migration relative to no treatment (Figure 10B). The siRNA delivery against integrin-β1 resulted in ≈50% reduction in migration, compared to CsiRNA-treated cells.

4. Discussion

The primary interactions between the cancer cells and their microenvironment, hence their propensity to metastasize and establish distant colonies, are significantly influenced by cell surface integrins.[2,3] Cellular interactions mediated by integrin-β1 were, therefore, explored as a potential therapeutic approach to prevent metastasis of breast cancers.[34] Peptides and antibodies were designed to block this receptor-mediated tumor growth and metastatic invasiveness in several xenograft studies.[35] The blocking antibodies were able to inhibit the adhesion of breast cells to human osteoblast cells and also to ECM proteins by targeting integrins β1, α1, α2, and α3.[36,37] Antibodies targeting integrins have advanced to clinical trials, but have numerous limitations such as high production costs and possible adverse interaction(s) with the immune system, and their efficacy has not been proven. Hence, we envisioned to employ siRNA as a promising approach to target integrin-β1. We have shown for the first time that a simpler approach, based on the delivery of integrin-β1 siRNA with lipid-modified PEI polymers, is effective in reducing the migration and adhesion of the breast cancer cells to extracellular proteins. The use of dicer-substrate siRNA leads to the direct interaction of the siRNA with the dicer enzyme before incorporating into RISC assembly which helps to increase the silencing effect by employing the natural siRNA silencing pathway.[38] Three different 1.2PEI polymers, each modified with a unique lipid (i.e., 1.2PEI–tαLA, 1.2PEI–Lau, and 1.2PEI–LA) were functional in undertaking siRNA delivery against integrin-β1, although significant differences in their performance and nonspecific effects were observed.

We successfully reduced the cell surface integrin-β1 levels for relatively long durations (9 d) with a single treatment of siRNA on MDA-MB-231 cells. We could observe the silencing to be much more effective at the mRNA level, which

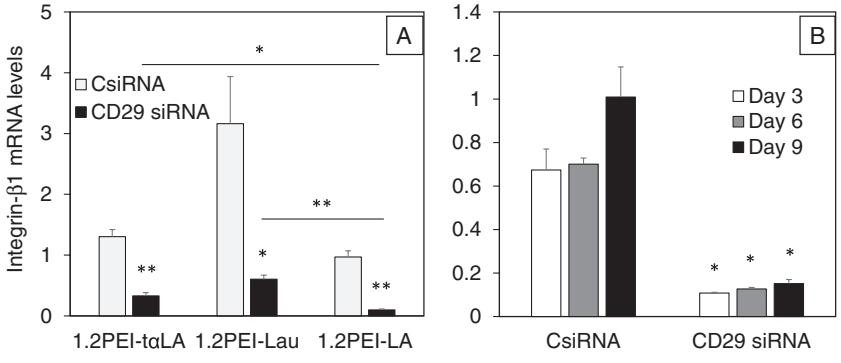


Figure 6. Integrin-β1 mRNA levels after siRNA treatment. A) The mRNA levels of integrin-β1 after 3 d of siRNA treatment with the polymers 1.2PEI–tαLA, 1.2PEI–Lau, and 1.2PEI–LA were quantified through qRT-PCR (values are plotted relative to no-treatment group). B) Integrin-β1 mRNA levels after treatment with 1.2PEI–LA polymer for 3, 6, and 9 d. *, $p \leq 0.005$; **, $p \leq 0.0002$. CsiRNA: Control scrambled siRNA.

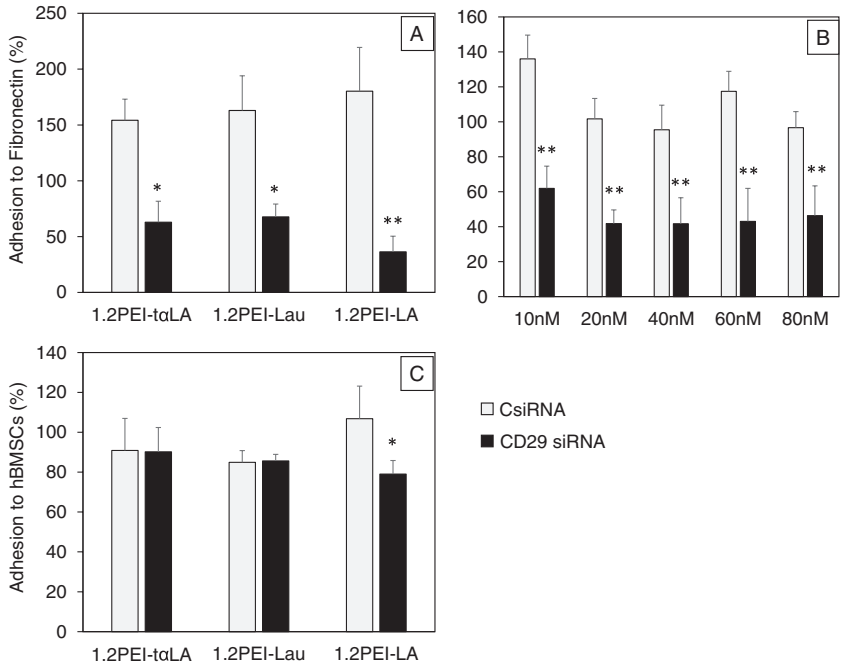


Figure 7. Effect of integrin-β1 silencing on cell adhesion. A) Adhesion on fibronectin-coated surfaces. Percentage of MDA-MB-231 cells binding to fibronectin after 3 d of treatment with siRNA using the polymers 1.2PEI–tαLA, 1.2PEI–Lau, and 1.2PEI–LA ($n = 2$). B) Adhesion to fibronectin after treatment with 1.2PEI–LA at different siRNA concentrations ($n = 2$), and C) adhesion toward hBMSCs: percentage of MDA-MB-231 cells binding to human bone marrow stromal cells (hBMSCs) after 3 d of siRNA treatment with all three polymers. *, $p \leq 0.03$; **, $p \leq 0.001$ ($n = 5$). CsiRNA: Control scrambled siRNA.

displayed 75%–90% silencing up to 9 d. This silencing of integrin-β1 was attained using a relatively low siRNA concentration of 40×10^{-9} M (in fact, as low as 10×10^{-9} M siRNA was also effective, albeit to a lower extent) and at a polymer-to-siRNA ratio (4:1) that was lower than other targets utilized in our laboratory.[39] We have previously reported that hydrophobic lipids on low molecular weight PEI helped with the better interaction of polymers with siRNA as well as DNA in multiple studies, which in turn increased the transfection efficiency when compared to the unmodified PEI.[39–41] An initial library of PEI polymers having different lipid modifications and varying levels of substitution was screened by looking into the surface integrin-β1; only 3 polymers (1.2PEI–tαLA, 1.2PEI–Lau, and 1.2PEI–LA) out of 32 polymers showed promising effect, indicating that the details of polymer design (such as molecular weight of backbone, the nature of lipid substitution, and the extent of lipid substitution) were crucial in ultimate silencing efficiency. Minimal use of siRNA for cell treatment is crucial as lower siRNA concentration is known to display lower off-target or nonspecific effects.[42,43] We consider the 40×10^{-9} M of siRNA used here to be sufficiently low enough to perform significant silencing. siRNA CD29_1 was effective whereas CD29_2 was not functional as these sequences were selected from a pre-existing library of commercial siRNAs targeting the same gene at different sites. Clearly, the silencing efficiency was dependent on the target site, and it might be possible to further improve the efficacy of integrin-β1 silencing by targeting different regions of the mRNA. The levels of integrin-β1 started to relapse with time, for 1.2PEI–tαLA and 1.2PEI–Lau polymers, but the silencing effect was significant and stable for the 1.2PEI–LA treatment. The presence of linoleic acid on the PEI is shown to improve the interaction of polymer with siRNA/DNA, which, in turn, has helped in better interaction with the cell membrane resulting in higher cellular uptake.[28,40,44–46] In addition to this, better cytoplasmic localization as well as higher intracellular release of siRNA could have contributed for the effective silencing. As the aim of our study was to validate the therapeutic potential of integrin-β1, we did not explore in detail the role of different substituents in this work. We note that published work from our laboratory has previously explored mechanistic insight into the beneficial effect of LA substitution in the context of delivery of siRNAs against other oncotargets.[30]

Migration ability of breast cancer cells after silencing integrin-β1 was investigated in vitro as a model of metastatic response. The well-established scratch assay was performed after treating the cells with the 1.2PEI–LA/siRNA complex, which showed 60% inhibition in the migration of the MDA-MB-231 cells after 24 h of scratch formation. It was earlier reported that integrins β1 and β4 were critical during the process of tumor formation and also during epithelial-to-mesenchymal transition in the initial steps of metastasis.[47,48] The knockout of integrin-β1 using CRISPR/Cas9 reported 50% inhibition in migration through scratch assay in a previous study,[13] so that the siRNA-mediated approach in this study yields a similar level of efficiency. While CRISPR/Cas9 causes a permanent deletion of the target gene, its high potency is worrisome if the target gene is silenced in other tissues. A transwell migration assay was additionally employed to evaluate the impact of integrin-β1 silencing on migration, which supported the scratch assay results with 50% inhibition, and provided a stronger confirmation of the therapeutic potential of silencing integrin-β1 with specific siRNAs. A cyclic peptide targeting integrin-$\alpha_2\beta_1$ showed a relatively lower ≈25% inhibition in wound healing

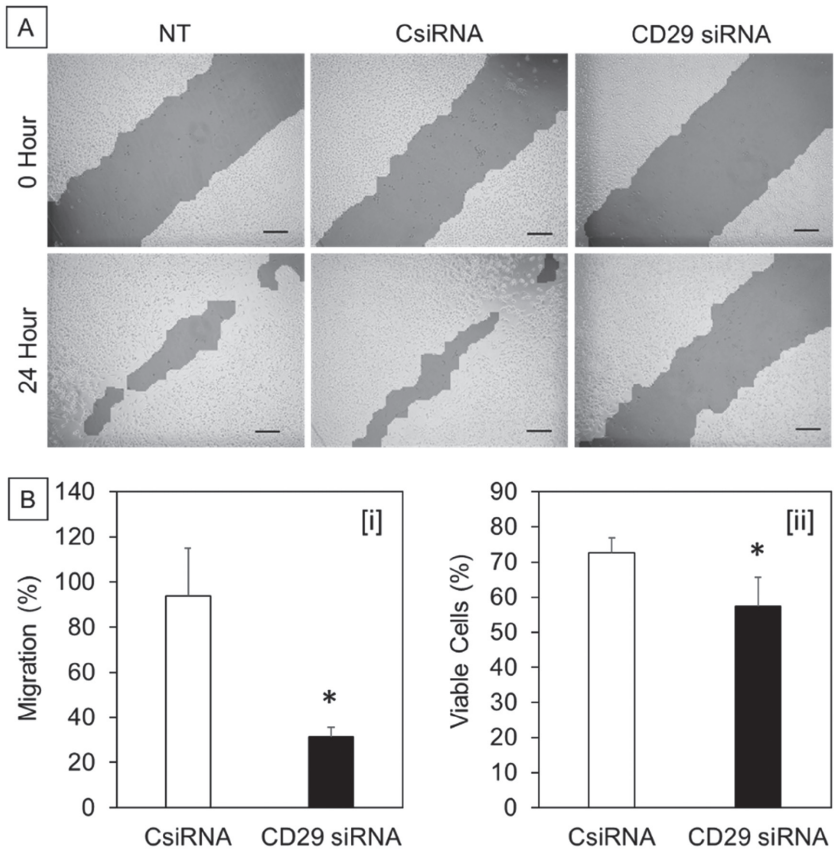


Figure 8. Effect of integrin-β1 silencing on cell migration. Scratch assay was performed after 2 d of siRNA treatment with 1.2PEI–LA polymer. A) The representative images of scratches captured at 0 and 24 h and the inhibition in migration of cells. Scale bar: 200 μm. B[i]) Percentage of migration relative to no treatment (NT) was calculated using TScratch software and B[ii]) its corresponding cell viability through MTT assay. *, $p \leq 0.0003$ ($n = 3$). CsiRNA: Control scrambled siRNA.

(scratch assay) as well as in migration assays, while its inhibition in tumor growth and bone metastasis in vivo was significantly higher.[49] The benefit of siRNA therapy using lipid-modified polymers could be more significant in vivo, albeit this issue remains unexplored in the current study.

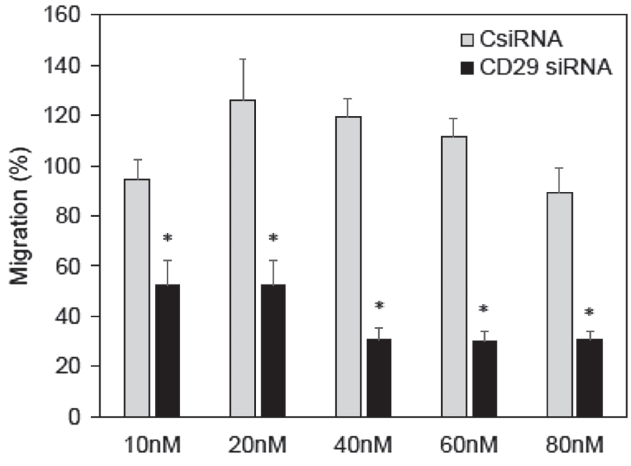


Figure 9. Percentage of migration after 3 d of treatment with 1.2PEI–LA polymer at different siRNA concentrations was calculated using TScratch software following scratch assay. *, $p \leq 0.0005$.

As the colonization of secondary tumor is initiated with the attachment of cancer to distant organs, we checked the ability of integrin-β1-silenced MDA-MB-231 cells to adhere to fibronectin for which integrin-β1 is a primary receptor. The knockdown of the cell surface integrin-β1 using 1.2PEI–LA led to 65% inhibition in the binding of MDA-MB-231 cells to fibronectin, while the other polymers displayed lower (≈40%) inhibition of fibronectin binding. As low as 10×10^{-9} M siRNA treatment was sufficient to influence the fibronectin binding. It is likely that other receptors might have participated in fibronectin binding, so that complete inhibition could not be achieved solely by the integrin-β1 siRNA. We further examined the adhesion ability by allowing the treated breast cancer cells to interact directly with hBMSCs, which express a wide range of adhesion proteins on cell surface and aid in tumor cell interactions leading to the adhesion of cells and colonization.[50] Only the most effective polymer (1.2PEI–LA) treatment displayed significant inhibition of binding. However, this reduction in binding to BMSC was relatively low (≈20%). Such a difference in the adhesion studies between fibronectin and hBMSCs could be attributed to the involvement of other adhesion molecules including other members of the integrin family of proteins. A recent study (retroviral transduction to stably express shRNA targeting integrin-β1 RNA) with MDA-MB-231 cells also reported similar levels of binding inhibition toward human-osteoblast-derived matrices. Our study indicates the possibility of achieving similar functional outcomes by targeting integrin-β1, but without the need for viral carriers.[22] Thus, silencing of integrin-β1 solely in the metastatic MDA-MB-231 cells could be more beneficial to reduce cellular migration rather than adhesion of cells to other bone marrow resident cells.

Treating the cells with AIIB2 antibody led to a drastic decrease in the total cell number along with decreased proliferation and increased apoptosis without affecting nonmalignant cells.[50,51] A combinational treatment of AIIB2 and ionizing radiation enhanced the apoptosis in cells.[34] Earlier reports on the inhibition of paclitaxel-induced apoptosis due to the presence of integrin-β1

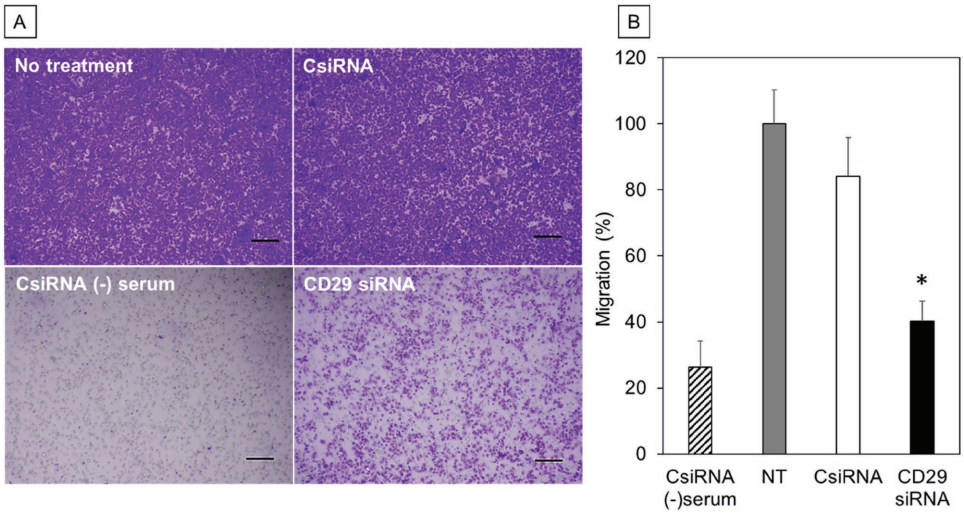


Figure 10. Effect of integrin-β1 silencing on cell migration. Transwell migration assay was performed after 24 h of siRNA treatment with 1.2PEI–LA polymer. A) Representative images of the crystal-violet-stained cells that have migrated and B) the corresponding absorbance of the migrated cells after solubilizing the crystal violet in 10% acetic acid (absorbance values are relative to no treatment (NT) group). *, $p \leq 0.012$ ($n = 2$). Scale bar: 200 μm.

explain its critical roles on cell viability.[50] In this study, we also evaluated the effect of integrin-β1 silencing on the cell numbers; we observed a small but significant effect when compared to CsiRNA-treated cells. The induction of apoptosis (by caspase assay) was also not evident in our hands (Figure S3, Supporting Information). Previous studies with antibodies and peptides intended to block integrin-β1 have reported apoptosis in breast cancer cells,[19,50] but another approach using integrin-β1 lentiviral shRNA had no significant difference in apoptosis when compared to control treatment.[22] Similarly, the CRISPR/Cas9 approach to study the role of integrin-β1 did not report its involvement in apoptosis; rather, a strong dependency in the cell growth at lower cell density and suppression of cell growth at higher cell density were observed.[13] Such contrasting observations could be explained by the different approaches used for targeting integrin-β1 and different cell types used in the studies. Perhaps, the model of inhibition, i.e., post-transcriptional versus post-translational, rather than the extent of integrin-β1 levels on cell surfaces, might be a causative factor for apoptotic response. This issue needs to be clarified in future studies.

The downstream integrin-β1 signaling via phosphatidylinositol 3′-kinase (PI3K) and serine/threonine kinase AKT provides resistance toward drug-induced apoptosis and is a key survival pathway in drug-resistant breast cancer.[52] Integrin-β1 adhesion to ECM proteins upregulated FAK, ERK, p38 MAPK, and JNK expressions in MDA-MB-231 cells, which also induced expression of matrix metalloproteinase-13.[37] The knockout of integrin-β1 comprehensively decreased the expression levels of FAK and AKT in MDA-MB-231 cells, affected cell migration, and revealed a negative effect on the expression of EGFR, suggesting an inverse relationship between integrin-β1 and EGFR.[13,53] It is likely that siRNA-mediated integrin-β1 silencing in MDA-MB-231 cells also downregulated FAK, PI3K, or AKT in this study.

The importance of integrin-β1 during the EMT, angiogenesis, migration, proliferation, and reattachment at distant sites has been explored in various studies. As the process of metastasis starts with EMT followed by migration and later reattachment, our study that demonstrated reduced cell attachment might curtail initial propensity to migrate along with reduced adhesion toward fibronectin, and hBMSC could be a promising approach. During the execution of these studies, we also focused on the possibility of cell detachment from the tissue culture plates; we did not observe any significant difference in the number of floating cells in wells between the CsiRNA and integrin-β1 siRNA-treated cells (Figure S4, Supporting Information), so that silencing integrin-β1 does not seem to alter the initial adhesiveness once the cells are attached to a substrate. The siRNA-treated cells in circulation can consequently fail to adhere to fibronectin/hBMSC, and might pose less threat to form a secondary tumor, as they have lost the ability to bind to target sites as a result of integrin-β1 silencing. It appears that the possibility of increased metastasis from the primary site (due to inhibition of ECM binding after siRNA silencing) should not be a concern with our approach. This was also not the case in antibody and peptide inhibition studies involving integrin-β1, where no incidence of increased metastasis was observed as the in vivo studies with these systems showed reduced metastasis to various organs and a decrease in the tumor volume which was also

reported by retroviral-shRNA and CRISPR/Cas9 knockout studies.[13–15,50]

Thus, for the first time, we have shown that therapeutic silencing of integrin-β1 was possible by using linoleic-acid-modified PEI polymers and using dicer-substrate siRNA delivery. Such an approach reduces the migration of metastatic breast cancer to a large extent, in addition to significant inhibition of cell adhesion to fibronectin and human bone marrow stromal cells. Little effect on cell viability and cell numbers was observed after integrin-β1 silencing. Thus, silencing of a single integrin subunit can have multiple inhibitory effects on the breast cancer cells, and careful selection of additional integrin subunits could be more beneficial to inhibit its attachment to different metastatic sites.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements: This study was supported by an Innovation Grant from the Canadian Breast Cancer Foundation (CBCF), a Discovery Grant from Natural Sciences and Engineering Council of Canada (NSERC) and a joint Canada-China Initiative Grant provided by the University of Alberta Office of Research. The authors acknowledge the technical help provided by Bindu Thapa while performing the qRT-PCR studies.

Received: October 14, 2016; Revised: December 23, 2016; Published online: February 3, 2017; DOI: 10.1002/mabi.201600430

Keywords: breast cancer therapy; dicer-substrate siRNA; integrin-β1; lipid-substituted polyethylenimine; migration; siRNA therapy

[1] Canadian Cancer Society's Advisory Committee on Cancer Statistics. Canadian Cancer Statistics 2015. Canadian Cancer Society, Toronto, ON, www.cancer.ca/statistics (accessed: July 2016).
[2] J. Y. Krzeszinski, Y. Wan, *Trends Pharmacol. Sci.* **2015**, *36*, 360.
[3] J. S. Desgrosellier, D. A. Cheresh, *Nat. Rev. Cancer* **2010**, *10*, 9.
[4] R. O. Hynes, *Cell* **2002**, *110*, 673.
[5] C. K. Miranti, J. S. Brugge, *Nat. Cell Biol.* **2002**, *4*, E83.
[6] D. Barkan, L. H. El Touny, A. M. Michalowski, J. A. Smith, I. Chu, A. S. Davis, J. D. Webster, S. Hoover, R. M. Simpson, J. Gauldie, J. E. Green, *Cancer Res.* **2010**, *70*, 5706.
[7] D. Barkan, H. Kleinman, J. L. Simmons, H. Asmussen, A. K. Kamaraju, M. J. Hoenorhoff, Z. Y. Liu, S. V. Costes, E. H. Cho, S. Lockett, C. Khanna, A. F. Chambers, J. E. Green, *Cancer Res.* **2008**, *68*, 6241.
[8] F. Wang, R. K. Hansen, D. Radisky, T. Yoneda, M. H. Barcellos-Hoff, O. W. Petersen, E. A. Turley, M. J. Bissell, *J. Natl. Cancer Inst.* **2002**, *94*, 1494.
[9] P. B. dos Santos, J. S. Zanetti, A. Ribeiro-Silva, E. I. Beltrao, *Diagn. Pathol.* **2012**, *7*, 104.
[10] E. S. Yao, H. Zhang, Y. Y. Chen, B. Lee, K. Chew, D. Moore, C. Park, *Cancer Res.* **2007**, *67*, 659.
[11] H. Lahlou, W. J. Muller, *Breast Cancer Res.* **2011**, *13*, 229.
[12] J. Mateo, J. Berlin, J. S. de Bono, R. B. Cohen, V. Keedy, G. Mugundu, L. Zhang, A. Abbattista, C. Davis, C. Gallo Stampino, H. Borghaei, *Cancer Chemother. Pharmacol.* **2014**, *74*, 1039.
[13] S. Hou, T. Isaji, Q. Hang, S. Im, T. Fukuda, J. Gu, *Sci. Rep.* **2016**, *6*, 18430.
[14] D. Lesniak, Y. Xu, J. Deschenes, R. Lai, J. Thoms, D. Murray, S. Gosh, J. R. Mackey, S. Sabri, B. Abdulkarim, *Cancer Res.* **2009**, *69*, 8620.
[15] P. Khalili, A. Arakelian, G. Chen, M. L. Plunkett, I. Beck, G. C. Parry, F. Doñate, D. E. Shaw, A. P. Mazar, S. A. Rabbani, *Mol. Cancer Ther.* **2006**, *5*, 2271.
[16] M. E. Cianfrocca, K. A. Kimmel, J. Gallo, T. Cardoso, M. M. Brown, G. Hudes, N. Lewis, L. Weiner, G. N. Lam, S. C. Brown, D. E. Shaw, A. P. Mazar, R. B. Cohen, *Br. J. Cancer* **2006**, *94*, 1621.
[17] D. E. White, N. A. Kurpios, D. Zuo, J. A. Hassell, S. Blaess, U. Mueller, W. J. Muller, *Cancer Cell* **2004**, *6*, 159.
[18] O. Pontiggia, R. Sampayo, D. Raffo, A. Motter, R. Xu, M. J. Bissell, E. B. Joffé, M. Simian, *Breast Cancer Res. Treat.* **2012**, *133*, 459.
[19] J. M. Nam, Y. Onodera, M. J. Bissell, C. C. Park, *Cancer Res.* **2010**, *70*, 5238.
[20] A. Nista, C. Leonetti, G. Bernardini, M. Mattioni, A. Santoni, *Int. J. Cancer* **1997**, *72*, 133.
[21] P. S. Steeg, *Nat. Rev. Cancer* **2016**, *16*, 201.
[22] L. Thibaudeau, A. V. Taubenberger, C. Theodoropoulos, B. M. Holzapfer, O. Ramuz, M. Straub, D. W. Hutmacher, *Oncotarget* **2014**, *6*, 332.
[23] M. Amarzguioui, J. J. Rossi, *Methods Mol. Biol.* **2008**, *442*, 3.
[24] M. B. Parmar, H. M. Aliabadi, P. Mahdipoor, C. Kucharski, R. Maranchuk, J. C. Hugh, H. Uludağ, *Front Bioeng. Biotechnol.* **2015**, *3*, 1.
[25] H. Jiang, C. Secretan, T. Gao, K. Bagnall, G. Korbutt, J. Lakey, N. M. Jomha, *Stud. Health Technol. Inform.* **2006**, *123*, 467.
[26] K. C. Remant Bahadur, B. Landry, H. M. Aliabadi, A. Lavasanifar, H. Uludağ, *Acta Biomater.* **2011**, *7*, 2209.
[27] K. C. Remant Bahadur, H. Uludağ, *J. Biomater. Sci. Polym. Ed.* **2011**, *22*, 873.
[28] H. M. Aliabadi, B. Landry, R. K. Bahadur, A. Neamnark, O. Suwantong, H. Uludag, *Macromol. Biosci.* **2011**, *11*, 662.
[29] M. J. Humphries, *Methods Mol. Biol.* **2009**, *522*, 203.
[30] H. Gul-Uludağ, J. Valencia-Serna, C. Kucharski, L. A. Marquez-Curtis, X. Jiang, L. Larratt, A. Janowska-Wieczorek, H. Uludağ, *Leuk. Res.* **2014**, *38*, 1299.
[31] X. Wang, X. Wang, R. K. Varma, L. Beauchamp, S. Magdaleno, T. J. Sendera, *Nucleic Acids Res.* **2009**, *37*, e152.
[32] L. Wei-Siong, G. Mary-Shi-Ying, O. Melissa-Hui-Ling, C. Choy-Hoong, *Asian Pac. J. Reprod.* **2015**, *4*, 217.
[33] L. Zhao, M. J. Kwon, S. Huang, J. Y. Lee, K. Fukase, N. Inohara, D. H. Hwang, *J. Biol. Chem.* **2007**, *282*, 11618.
[34] D. E. White, W. J. Muller, *J. Mammary Gland Biol. Neoplasia* **2007**, *12*, 135.
[35] C. C. Park, H. J. Zhang, E. S. Yao, C. J. Park, M. J. Bissell, *Cancer Res.* **2008**, *68*, 4398.
[36] A. Lundström, J. Holmbom, C. Lindqvist, T. Nordström, *Biochem. Biophys. Res. Commun.* **1998**, *250*, 735.
[37] S. Ibaragi, T. Shimo, N. M. Hassan, S. Isowa, N. Kurio, H. Mandai, S. Kodama, A. Sasaki, *Anticancer Res.* **2011**, *31*, 1307.
[38] D. H. Kim, J. J. Rossi, *Nat. Rev. Genet.* **2007**, *8*, 173.
[39] K. C. Remant Bahadur, C. Kucharski, H. Uludağ, *J. Mater. Chem. B* **2015**, *3*, 3972.
[40] C. Y. M. Hsu, H. Uludağ, *Biomaterials* **2012**, *33*, 7834.
[41] H. M. Aliabadi, B. Landry, P. Mahdipoor, C. Y. M. Hsu, H. Uludağ, *Eur. J. Pharm. Biopharm.* **2012**, *81*, 33.

[42] S. P. Persengiev, X. Zhu, M. R. Green, *RNA* **2004**, *10*, 12.
[43] D. Semizarov, L. Frost, A. Sarthy, P. Kroeger, D. N. Halbert, S. W. Fesik, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6347.
[44] B. Landry, H. Gül-Uludağ, S. Plianwong, C. Kucharski, Z. Zak, M. B. Parmar, O. Kutsch, H. Jiang, J. Brandwein, H. Uludağ, *J. Controlled Release* **2016**, *224*, 8.
[45] C. Sun, T. Tang, H. Uludag, *Biomaterials* **2013**, *34*, 2822.
[46] A. Falamarzian, H. M. Aliabadi, O. Molavi, J. M. Seubert, R. Lai, H. Uludağ, A. Lavasanifar, *J. Biomed. Mater. Res., Part A* **2014**, *102*, 3216.
[47] A. Vassilopoulos, C. Chisholm, T. Lahusen, H. Zheng, C. X. Deng, *Oncogene* **2014**, *33*, 5477.
[48] P. Nisticò, F. Di Modugno, S. Spada, M. J. Bissell, *Breast Cancer Res.* **2014**, *16*, 459.
[49] N. Rucci, M. Capulli, O. K. Olstad, P. Önnerfjord, V. Tillgren, K. M. Gautvik, D. Heinegård, A. Teti, *Cancer Lett.* **2015**, *358*, 67.
[50] D. Van der Velde-Zimmermann, M. A. Verdaasdonk, L. H. Rademakers, R. A. De Weger, J. G. Van den Tweel, P. Joling, *Exp. Cell Res.* **1997**, *230*, 111.
[51] C. C. Park, H. Zhang, M. Pallavicini, J. W. Gray, F. Baehner, C. J. Park, M. J. Bissell, *Cancer Res.* **2006**, *66*, 1526.
[52] F. Aoudjit, K. Vuori, *Oncogene* **2001**, *20*, 4995.
[53] L. Huck, S. M. Pontier, D. M. Zuo, W. J. Muller, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 15559.