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siRNA therapy in cutaneous T-cell lymphoma cells using polymeric carriers

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ABSTRACT

Cutaneous T-cell lymphomas (CTCLs) arise from specific molecular aberrations that lead to uncontrolled cell proliferation. RNA interference (RNAi) with short interfering RNAs (siRNAs) is a feasible approach to interrupt aberrant signal processing in CTCL cells, but functional biomaterial carriers are needed to effectively deliver siRNAs intracellularly. Towards this goal, we explored the utility of lipid-substituted polyethylenimines (PEI) carriers in a cell model of CTCL. Using caprylic and linoleic acid substituted 2 kDa PEI (PEI-CA and PEI-LA, respectively), we showed effective delivery of siRNA to T-lymphocyte Hut78 and Jurkat cells, but silencing of a model protein (Green Fluorescent Protein, GFP) was possible only in the Hut78 cells. To enhance siRNA delivery to Hut78 cells, a high siRNA: carrier ratio used to assemble the complexes and centrifugation of cells in the presence of complexes were found effective. The toxicities of PEI-CA and PEI-LA were significantly lower than other commercial carriers, 25 kDa PEI and Lipofectamine® RNAiMAX. This might have contributed to reduced siRNA delivery efficiency of the latter carriers. Screening several endogenous targets led us to identify phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and cyclin-dependent kinase 18 (CDK18) as viable targets to induce siRNA-mediated cell growth inhibition. The results of this study identified promising polymeric carriers and molecular targets that could control proliferation of CTCL cells based on RNAi therapy.

1. Introduction

Cutaneous T-cell lymphoma (CTCL) is among the subtypes of peripheral T-cell lymphomas that arise from clonal accumulation of T-lymphocytic neoplasms in the skin. Sézary syndrome is one of the most common CTCLs that is characterized by circulating, atypical, malignant T-cells with cerebriform nuclei (Sézary cells), erythroderma and often lymphadenopathy [1,2]. Although numerous drugs are currently available for chemotherapy for Sézary syndrome and partial/complete remission could be achieved for a significant fraction of patients, subsequent relapses are common and Sézary syndrome is still largely incurable with an average survival of 2–4 years [3,4]. Thus, there is a significant need for developing new and alternative therapies. Cytogenetic analysis of Sézary syndrome indicates chromosomal abnormalities such as the loss of 1p, 17p, 10q/10 and gain of 4q/4, 18 and 17q/17 [5]. Molecular abnormalities are beginning to be elucidated in connection with such changes; for example, deletion of the tumor suppressor TNFAIP3 [6], and constitutively activated forms of intracellular signaling molecules STAT3 [7], PDCD10 [8] and NFkB [9] have been linked to unregulated proliferation of the malignant cells. With emerging molecular underpinnings of CTCL, it is becoming possible to devise more precise molecular therapies for specific control of malignant cell growth, instead of relying on broadly active and non-specific chemotherapy to curb uncontrolled T-cell proliferation.

The endogenous RNA interference (RNAi) based post-transcriptional gene silencing is a promising approach in this

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regard. In RNAi, regulatory microRNA molecules (miRNA) are incorporated into RNA-Induced Silencing Complex (RISC) that facilitates cleavage of complementary mRNAs. The pharmacological intervention based on RNAi utilizes synthetic double-stranded RNA (short interfering RNA; siRNA) comprised of 21–23 nucleotides in length with a characteristic and highly specific 2–3 nucleotide 3′ overhang. The mRNA cleavage at the central location of complementary binding site is facilitated by the endonuclease argonaute found in the RISC [10]. This process silences the targeted gene at the translational level and provides a highly specific and broadly applicable approach to molecular therapy as long as the target mRNA sequence is available [11]. However, delivery of therapeutic siRNAs into cells has been a major obstacle [12]; siRNAs are highly unstable in serum due to the presence of endogenous nucleases, and are unable to enter the cells on their own due to their large size (13 kDa) and polyanionic nature [10,13,14]. Complexing the siRNA with synthetic biomaterial carriers that are able to mask its anionic charges, protect it from the nucleases and package it into nanoparticles suitable for cellular uptake has been found indispensible for effective siRNA delivery.

Polymeric systems are being increasingly pursued for siRNA-mediated cancer therapy since optimizing the physicochemical features of polymers can be easily undertaken to improve the siRNA delivery. Polyethylenimine (PEI) based polymers and especially non-toxic low molecular weight PEIs were modified with lipids and other hydrophobic moieties in order to improve cell membrane compatibility [15,16]. We and others have previously shown that lipid-substituted PEIs were capable of effectively delivering siRNA to breast cancer cells [17] and leukemia cells [18]. The chemical nature of lipophilic substituents was critical for the delivery; for certain breast cancer cells, caprylic acid-substituted PEI2 (PEI-CA) was found to be effective in silencing mediators important for proliferation and drug-resistance (e.g., survivin) [19], whereas linoleic acid-substituted PEI2 (PEI-LA) was found effective in other

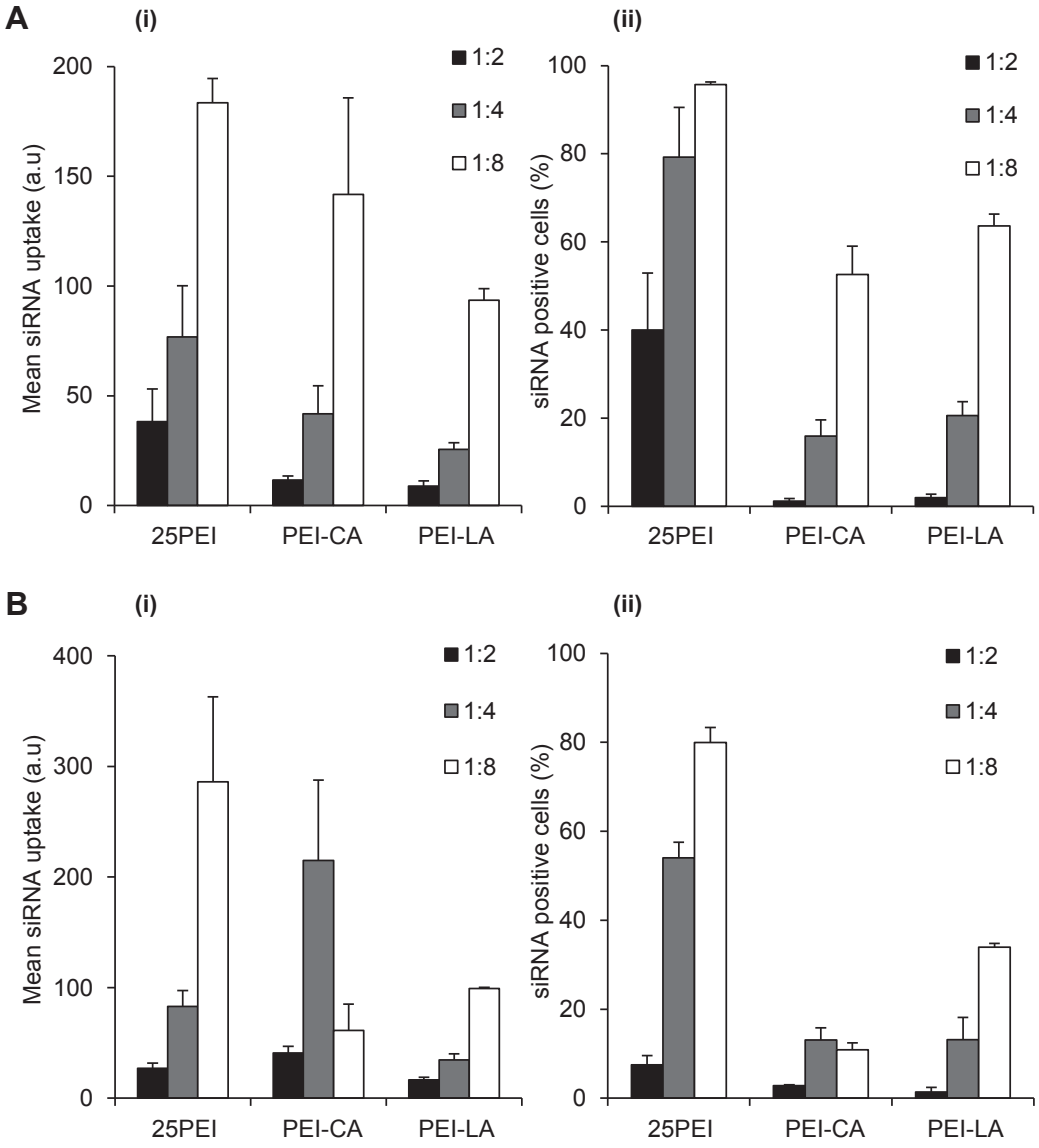

Fig. 1. siRNA uptake by Hut78 (A) and Jurkat cells (B) treated with the FAM-siRNA complexes of 25PEI, PEI-CA and PEI-LA at siRNA: carrier ratios 1:2, 1:4 and 1:8. The siRNA and polymer concentrations were 25 nM and 0.70 μg/mL (1:2), 1.40 μg/mL (1:4) and 2.80 μg/mL (1:8), respectively. The cells were analyzed for FAM fluorescence using flow cytometer after 24 h of incubation with the complexes. The results (mean ± SD) are summarized in terms of mean FAM-siRNA fluorescence per cell (i) and percentage of cells displaying FAM-siRNA (ii).

breast cancer cells [17]. PEI-LA and PEI-CA in particular were more effective in siRNA delivery in anchorage-independent acute myeloid leukemia (AML) cells [18], but their effectiveness was diminished in leukemic cells as compared to more differentiated breast cancer cells. Polymer-mediated siRNA delivery to CTCL cells, which also display anchorage-independent growth, has not been explored before and it is not known whether siRNA delivery could be effectively undertaken in this disease by polymeric carriers.

In this study, the established cell model for Sézary syndrome, Hut78 cells (derived from a 53 year old Caucasian male) [20], and another T-cell line, Jurkat cells (derived from a 14-year old boy with acute lymphoblastic leukemia [21]) were used to explore the possibility of delivering siRNA for T-cell malignancies. We first used green fluorescent protein (GFP) expressing Hut78 and Jurkat cells to identify a suitable polymeric carrier for siRNA delivery based on GFP silencing as a model system. After selecting the most appropriate carriers, and optimizing the siRNA delivery, several therapeutic targets were explored with specific siRNAs to investigate the possibility of reducing cell growth.

2. Materials and methods

2.1. Materials

Unmodified branched PEIs (2 and 25 kDa; 2PEI and 25PEI, respectively), Trypan Blue solution (0.4%) and formaldehyde (37%) were purchased from Sigma–Aldrich (St Louis, MO). The 25PEI [22] was used (as received) as a reference delivery reagent throughout this study. RPMI 1640 medium, penicillin (10,000 U/mL), streptomycin (10 mg/mL), Ultrapure water, AlamarBlue®, FAM-labeled siRNA and specific siRNAs against indicated intracellular targets were purchased from Life Technologies (Burlington, ON). Fetal bovine serum (FBS) was purchased from PAA Laboratories Inc. (Etobicoke, ON). FlowTACS™ Apoptosis Detection Kit was purchased from Trevigen (Gaithersburg, MD). GFP siRNA (GFP-22; Cat#: 1022064), RNeasy Mini Kit and QIAshredders were purchased from Qiagen. A scrambled (non-specific) siRNA (Silencer® Negative Control #1 siRNA, Cat #: AM4635) was obtained from Ambion. Clear Hank's balanced salt solution (HBSS) for non-sterile procedures was prepared in house. Reagents needed for RNA isolation and digital PCR analysis was obtained from Invitrogen (Burlington, ON).

2.2. Preparation and characterization of polymeric carriers

Lipid modified PEIs were synthesized by using a modification of previously published procedures [15,16]. Caprylic acid (CA) and linoleic acid (LA) was used as the specific lipids for N-acylation of 2PEI (purified by freeze-drying of manufacturer

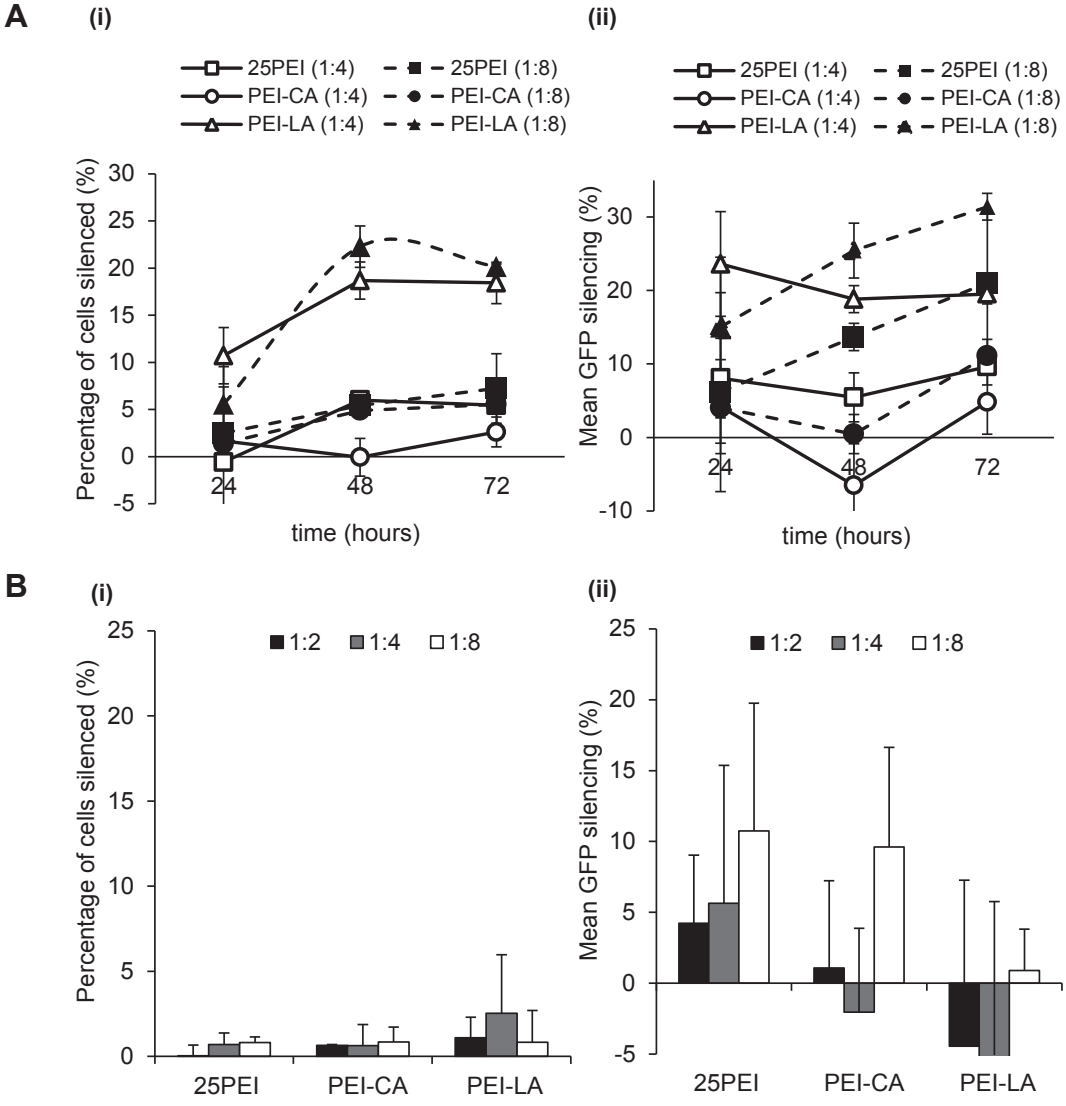

Fig. 2. GFP silencing in Hut78 (A) and Jurkat (B) cells. The results (mean ± SD) are summarized in terms of percentage of cells displaying reduced GFP expression (i) and percent decrease in mean GFP fluorescence (ii). The results were normalized against control (scrambled siRNA) treatment groups. The siRNA and polymer concentrations were 25 nM and 0.70 μg/mL (1:2), 1.40 μg/mL (1:4) and 2.80 μg/mL (1:8), respectively. The Hut78 cells were analyzed after 24, 48 and 72 h of treatment while Jurkat cells were analyzed after 72 h of treatment. The siRNA: carrier ratios were 1:4 and 1:8 for Hut78 cells and 1:2, 1:4 and 1:8 for Jurkat cells. The siRNA concentration was 25 nM and polymer concentration in medium was 1.40 μg/mL (1:4) and 2.80 μg/mL (1:8).

supplied product). In a typical reaction, lipid chlorides individually dissolved in 5 mL of anhydrous dichloromethane were added drop-wise to 100 mg of PEI in 15 mL of dichloromethane (containing 50 μL of triethylamine). Four different lipid:PEI amine ratios (ranging from 0.1 to 0.4, corresponding to lipid:PEI ratios of ~0.7–~4.0) were used during synthesis to control the level of lipid substitutions. After 24 h at room temperature under nitrogen, the solutions were concentrated by a rotary evaporator, polymers precipitated in ethyl ether and then washed with excess ethyl ether. After dissolving the precipitated polymers in ultrapure water, the solutions were freeze-dried at room temperature to obtain polymer powders. The polymers were analyzed by ^{1}H NMR in D_2O using the characteristic proton shifts of lipids (δ ~ 0.8 ppm; $-C\underline{H}_3$) and PEI (δ ~ 2.5–2.8 ppm; NH–$C\underline{H}_2$–$C\underline{H}_2$–NH-) to calculate the lipid substitution levels. The levels of substitution were 6.9 CA/2PEI and 2.1 LA/2PEI in initial studies (Figs. 1 to 5), and 6.0 CA/2PEI and 2.6 LA/2PEI in subsequent studies (Figs. 6 to 9).

2.3. Cell lines and culture

Hut78 cells were used as a model of cutaneous T-cell lymphoma. The cells produce IL-2 and TNF-α and display IL-2, CXCR4 and T-cell receptors, and CD4 on the cell surface [20,23–25]. The related Jurkat cells, derived from an acute lymphoblastic leukemia (ALL) patient, also produce IL-2 and display the same surface markers [25–28]. For the optimization of the transfection, retrovirally-modified GFP expressing Hut78 and Jurkat cells were used along with their wild-type phenotype. Both cell types were cultured in RPMI-1640 media with 10% FBS (v/v), 100 U/mL (1%) penicillin and 100 U/mL (1%) streptomycin, and maintained between 10^5–10^6 cells/mL by routine subculturing. Subculturing was performed by simply diluting the cells in fresh media (10–25 fold) without trypsin treatment. The cells were cultured for a maximum of 4–5 weeks for more reproducible results, since they start losing GFP expression after 4–5 weeks of culture. For siRNA treatments, the cells were seeded at 10,000 cells/well in 48-well plates (for dose-response studies in target validation), and 15,000 cells/well in 24-well plates (for all other studies) 24 h prior to the treatment.

2.4. siRNA complex formation and cell treatments

The complexes of siRNA and polymer were formed by incubating siRNAs with the polymers at room temperature for 30 min in the presence of 150 mM NaCl. The siRNAs used were FAM-labeled siRNA, anti-GFP siRNA, scrambled siRNA as the control and other specific siRNAs as additional treatments as described below. Both siRNA (in RNAse free water; stored at −20 °C) and polymer solutions (in nuclease-free Ultrapure water; stored at +4 °C) were allowed to equilibrate to room temperature and vortexed. A desired volume of siRNA stock solution (10 μM) was added to a specific volume of 150 mM NaCl solution in microcentrifuge tubes, after which desired volume of polymer solutions (1 mg/mL) was added, followed by a brief

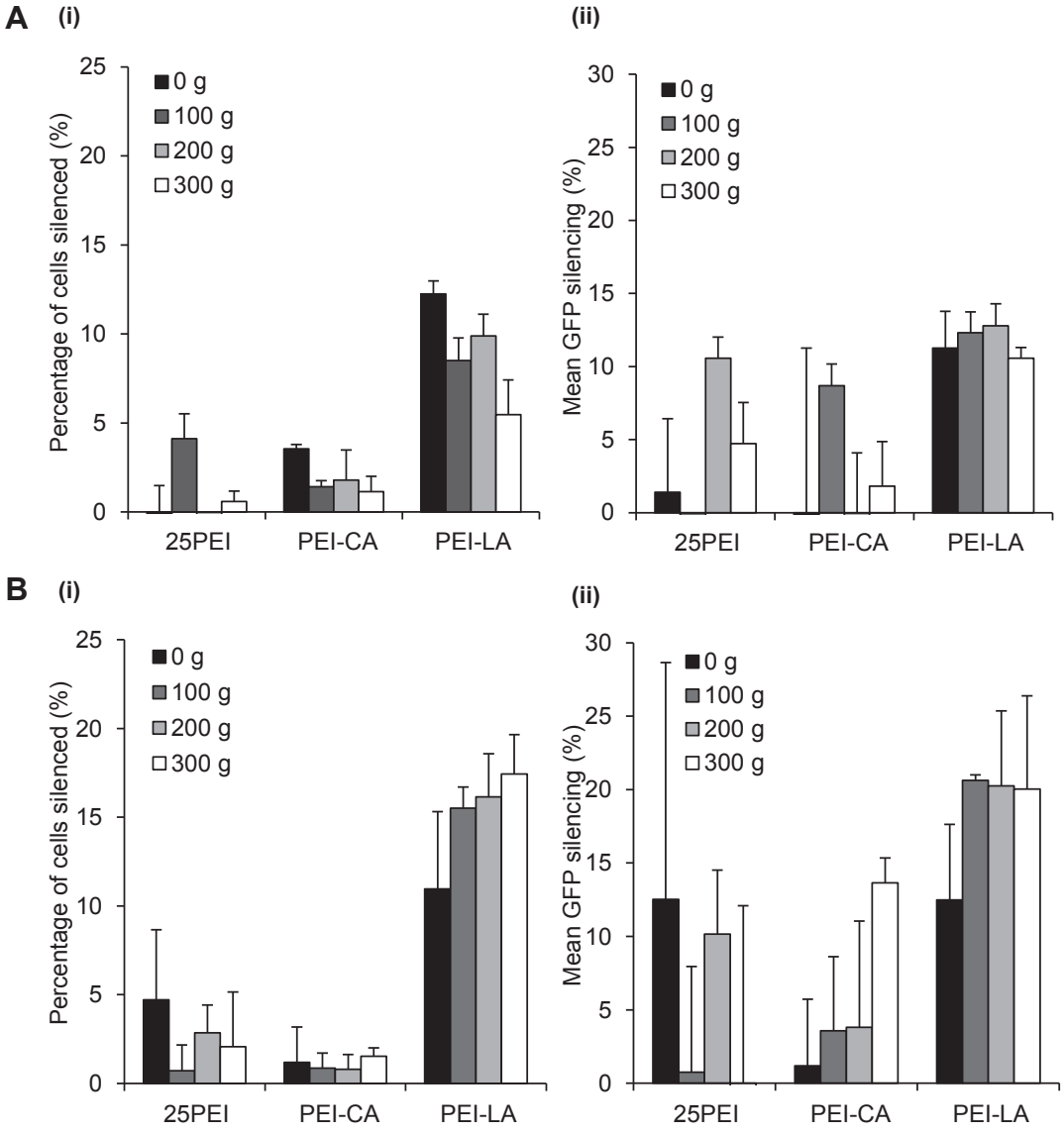

Fig. 3. Effect of centrifugation on GFP silencing. The cells were centrifuged for 5 min before (A; without complexes) and after (B; with complexes) addition of siRNA complexes of 25PEI, PEI-CA and PEI-LA. The results (mean ± SD) are summarized in terms of percentage of cells displaying reduced GFP expression (i) and percent decrease in mean GFP fluorescence (ii). The results were normalized against control (scrambled siRNA) treatment groups. The cells were analyzed after 72 h of treatment with anti-GFP siRNA complexes at siRNA: carrier ratio 1:4. The siRNA and polymer concentrations were 25 nM and 1.40 μg/mL, respectively.

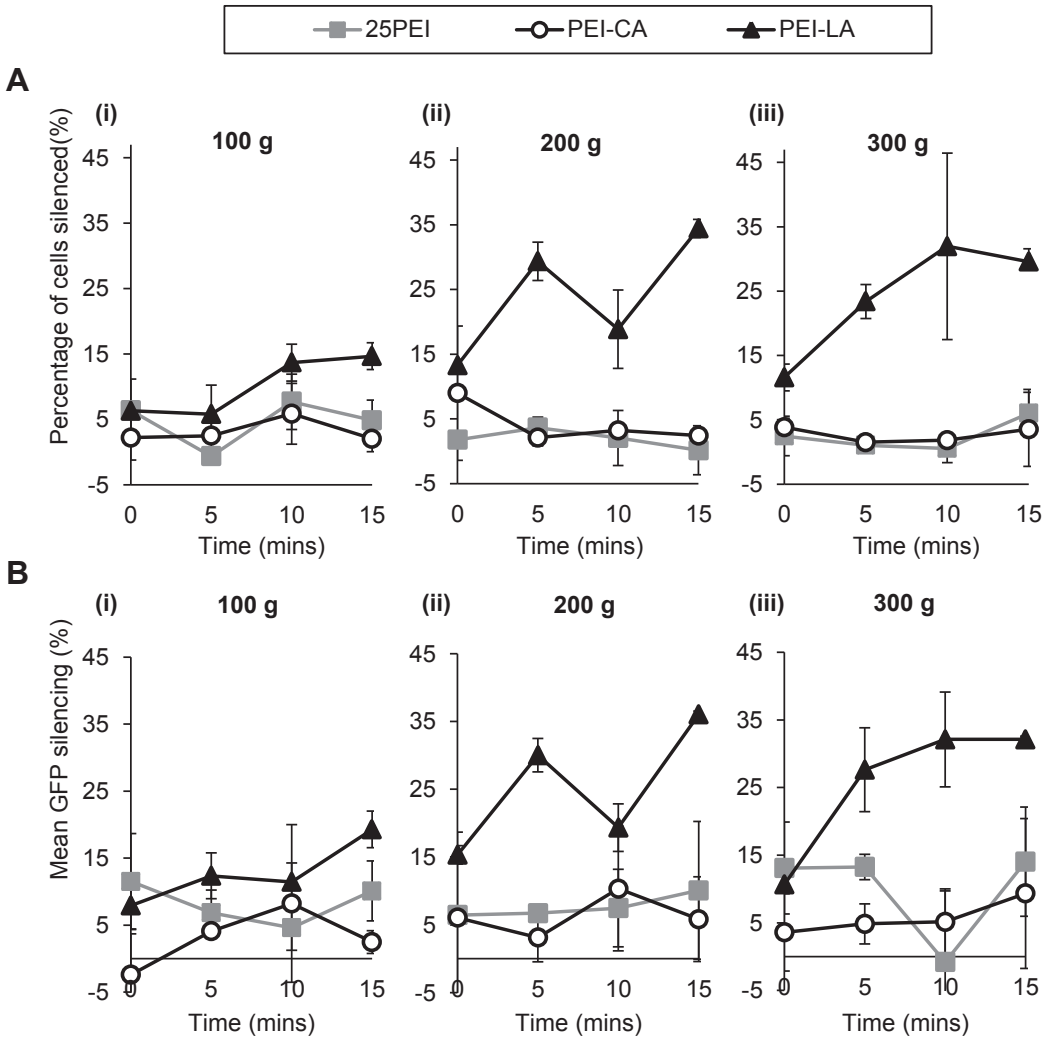

Fig. 4. Effect of centrifugation time on GFP silencing. The cells were centrifuged at 100 *g*, 200 *g* and 300 *g* for 0 (no centrifugation), 5, 10 and 15 min after addition of siRNA complexes of 25PEI, PEI-CA and PEI-LA. The results (mean ± SD) are summarized in terms of percentage of cells displaying reduced GFP expression (A) and percent decrease in mean GFP fluorescence (B). The results were normalized against control (scrambled siRNA) treatment groups. The cells were analyzed after 72 h of treatment with anti-GFP siRNA complexes at siRNA: carrier ratio 1:4. The siRNA and polymer concentrations were 25 nM and 1.40 μg/mL, respectively.

vortex to mix the reagents thoroughly. The siRNA volume added was adjusted to obtain the desired concentration of siRNA in contact with cells. In addition, the siRNA: carrier ratio was systematically varied between 1:2 and 1:8 (w/w). From a typical 35 μL complex volume, the complexes were added to the cells at 10 μL/well (in triplicate) at the end of the 30-min incubation time. Where indicated, Lipofectamine® RNAiMAX was used to prepare siRNA complexes by following the manufacturer's protocol. For investigation of siRNA uptake, FAM-siRNA was used to prepare the complexes at indicated siRNA: carrier ratios and added to the cells as above. To investigate the effect of centrifugation on silencing, complexes were added to cells and the plates were immediately centrifuged at 100–300 g for 5–15 min.

2.5. siRNAs against endogenous targets

Several targets were chosen to explore the possibility of reducing Hut78 cell growth by delivery of specific siRNAs. The siRNA used were specific against kinase spindle protein (KSP (KIF11); Cat. No AM16708/14672 from Ambion), cyclin-dependent kinase 18 (CDK18 (PCTK3); Cat. No AM16708/202296 from Ambion), mitogen activated protein kinase (MAP; Cat. No AM16708/143171 from Ambion), ribosomal protein S (RPS (RPS6KA5); Cat No AM16708/580 from Ambion), Fms-related Protein Kinase (STK (FLT3); Cat No AM16708/425 from Ambion), signal transducer and activator of transcription 3 (STAT3; Cat. No S100048363 (STAT3-1), S100048370 (STAT3-2), S102662338 (STAT3-7), and S102662898 (STAT3-8) from Qiagen), phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3 (PIK3CB); Cat. No AM16708/144255 from Ambion), and B-cell CLL/lymphoma 11B (Bcl11b; Cat. No AM16708/s224631 from Ambion). The complexes of these siRNAs were prepared as described above (siRNA: carrier ratios of 1:4) and added to cells at 25, 50 and 100 nM (in 48-well plates) after 30 min of incubation at the room temperature. After 72 h incubation, AlamarBlue® assay (see below) was used to determine cell viabilities after treatment with specific siRNA complexes, which were expressed as a percentage of the untreated cells (i.e., taken as 100% viability).

2.6. Flow cytometry for GFP silencing

After treatment of cells with complexes for a desired period of time, the cells were washed twice with HBSS and fixed with 3.7% formalin. The samples were analyzed for GFP expression using a Beckman Coulter Cell Lab Quanta™ SC flow cytometer. The threshold for GFP expression was determined using wild-type Hut78 and Jurkat cells and set for 1% of the population as the background. The mean GFP fluorescence and percentage of GFP-positive cell population was determined. The extent of GFP silencing with different polymers was expressed relative to the cells treated with the scrambled siRNA complexes of the corresponding polymers. For clarity, both reduction in mean GFP fluorescence and percentage of cells outside the main GFP peak (see Fig. 1S) were analyzed for evaluating the extent of silencing.

2.7. Assessment of polymer toxicities

The Hut78 cells in 24-well plates (200 μL/well) were exposed to scrambled siRNA complexes (between 0 and 10 μg/mL complexes based on polymer concentration) and centrifuged at 300 *g* for 5 min. After 72 h of incubation, the cell viabilities were determined by the AlamarBlue® and Trypan Blue exclusion assays. For the AlamarBlue® assay, which was designed to measure the metabolic (mitochondrial) activity of suspension growing cells, the cells were incubated with 5% (v/v) AlamarBlue® solution (10 μL) at 37 °C for 2 h. The fluorescence was read at 536 nm

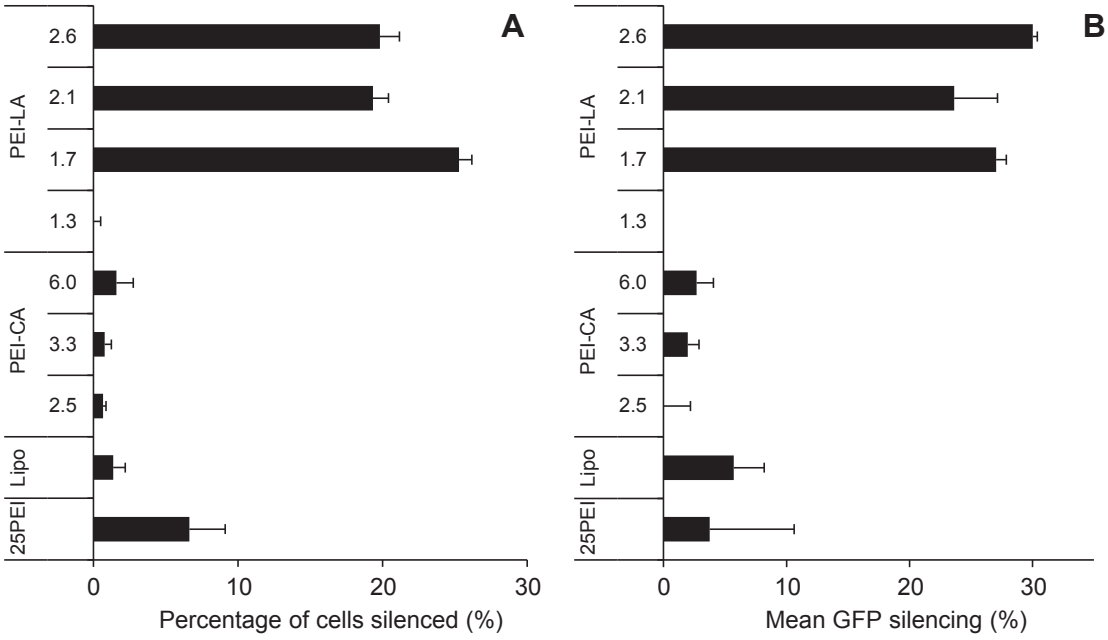

Fig. 5. Effect of lipid substitution on GFP silencing. The cells were centrifuged at 300 *g* for 5 min after addition of siRNA complexes of 25PEI, Lipofectamine® RNAiMAX, PEI-CA and PEI-LA. The levels of lipid substitution for the latter 2 polymers are indicated in the vertical axis. The results (mean ± SD) are summarized in terms of percentage of cells displaying reduced GFP expression (A) and percent decrease in mean GFP fluorescence (B). The results were normalized against control (scrambled siRNA) treatment groups. The cells were analyzed after 72 h of treatment with anti-GFP siRNA complexes at siRNA: carrier ratio 1:4. The siRNA and polymer concentrations were 25 nM and 1.40 μg/mL, respectively.

excitation and 604 nm emission using a plate reader (Fluoroskan Ascent; Thermo Labsystems), and relative cell viability (%) was determined by normalizing the fluorescence of complex-treated cells to that of untreated cells. For Trypan Blue assay, 10 μL of 0.4% Trypan Blue dye was mixed with 10 μL of cell samples in a 0.5 mL eppendorf tube, and allowed to equilibrate for ~2 min. The cell suspension was loaded into a hemocytometer and counted for the numbers of Trypan blue positive (dead) and negative (viable) cells. The percentage of viable cells was calculated by dividing the number of Trypan blue-negative cells with the total number of cells.

2.8. *FlowTACS™ apoptosis detection*

Trevigen's FlowTACS™ Apoptosis Detection Kit utilizes terminal deoxynucleotidyl transferase (TdT) to incorporate biotinylated nucleotides to fragmented DNA, which is detected as a measure of apoptotic cell population with streptavidin-FITC using a flow cytometer. The cells were seeded in 24-well plates at 300 μL (6 wells per group) and 72 h after the complex addition, 3 wells were pooled and analysis was performed in duplicate. The assay was performed as instructed by the

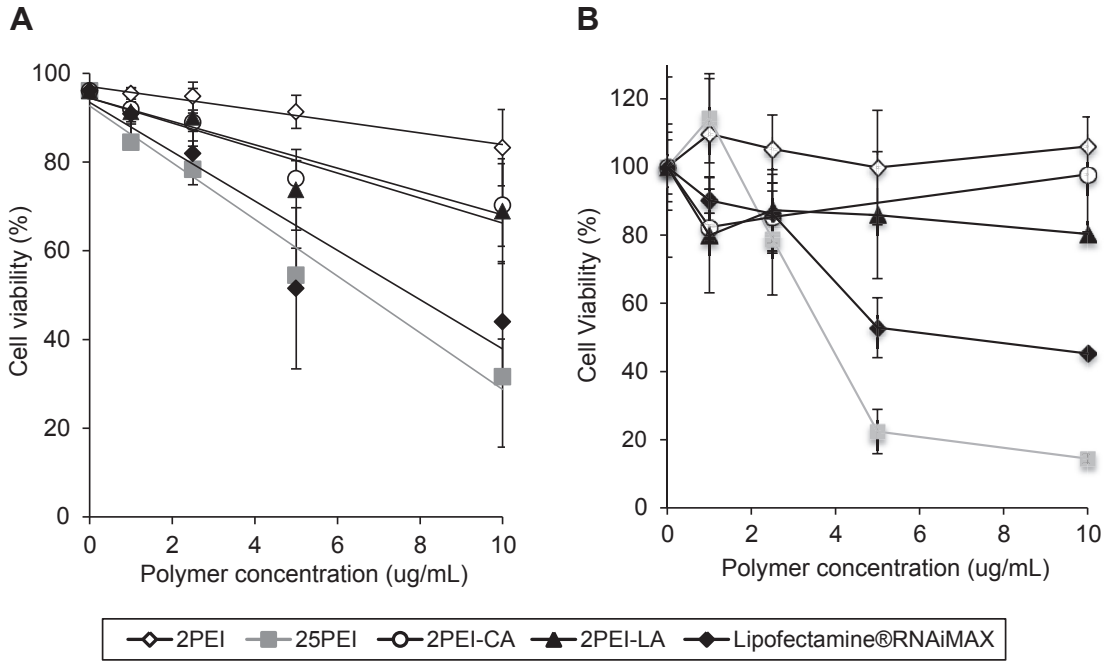

Fig. 6. Effect of siRNA carriers on cell viability. The results (mean ± SD) are summarized in terms of percentage of viable cells based on Trypan Blue staining (A) or AlamarBlue® viability assay (B). The cell viabilities were determined after 72 h of treatment with the indicated scrambled siRNA complexes and normalized with no-treatment groups (taken as 100%). The complexes were prepared at siRNA: carrier ratio of 1:4, and the cells were centrifuged at 300 *g* for 5 min after complex addition. Linear trendlines were fitted to guide the eye in A but not in B, given the saturation observed in some of the study groups in the latter case.

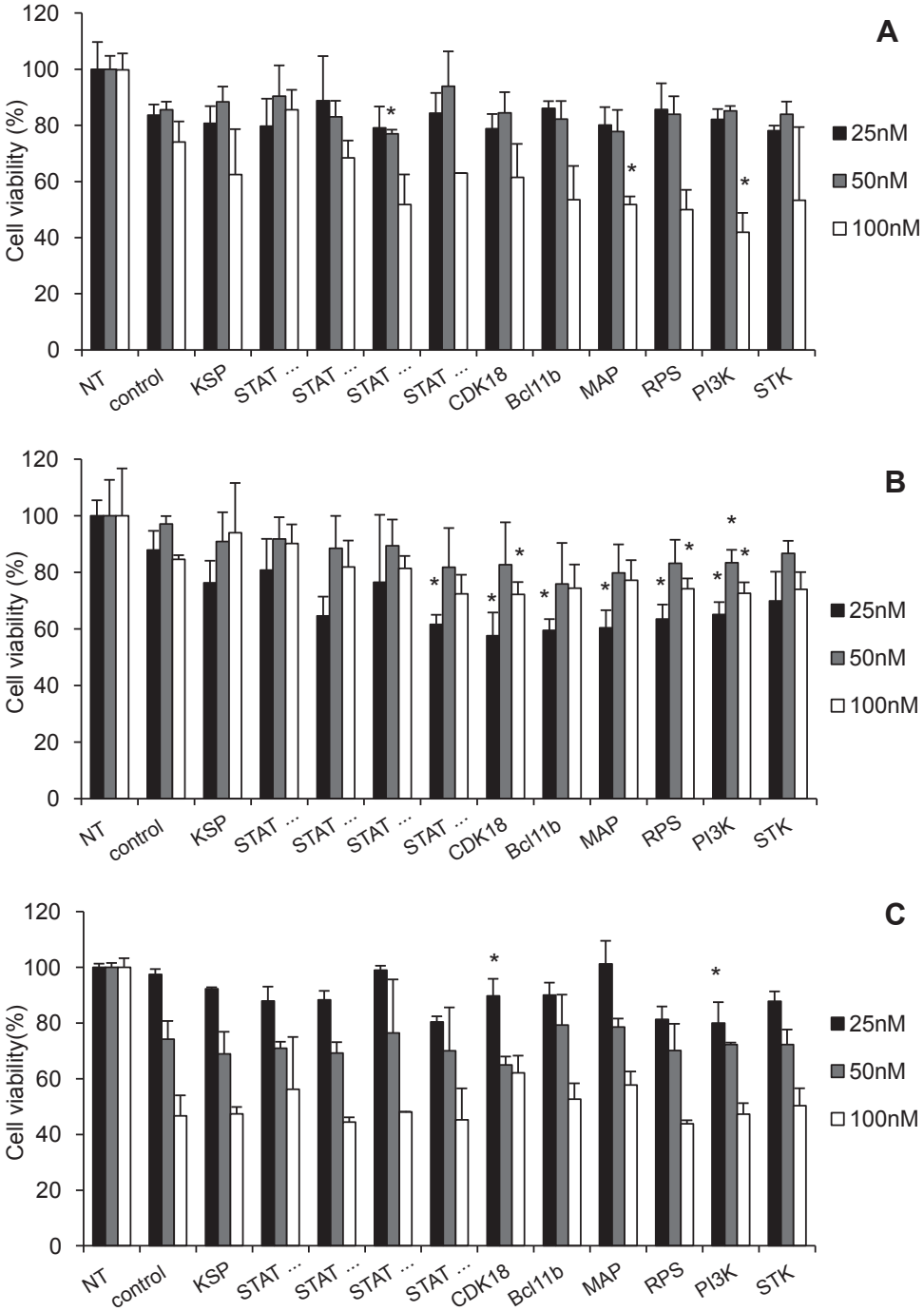

Fig. 7. Effect of specific siRNA silencing on Hut78 viabilities. Percentage of cell viability after treatment with siRNAs complexes of PEI-LA (A), PEI-CA (B) and Lipofectamine® RNAiMAX (C). The complexes were prepared at the siRNA: carrier ratio of 1:4 and added to cells at 25, 50 and 100 nM siRNA (polymer concentration of 1.4, 2.8 and 5.6 μg/mL, respectively). The relative viabilities compared to untreated cells were analyzed with AlamarBlue® assay after 72 h of incubation with complexes. Groups significantly different from the corresponding control siRNA treated groups (at respective doses) were indicated with an *.

manufacturer (without the use of propidium iodide) and FITC-positive cells was quantified using Beckman Coulter Cell Lab Quanta™ SC flow cytometer in the FL1 channel. Relative extent of apoptosis (%) was determined by normalizing against a positive control as instructed by the manufacturer.

2.9. Droplet digital polymerase chain reaction (ddPCR)

Hut78 cells were seeded in 24-well plate at 200,000 cells/mL (0.5 mL/well). Cells were transfected with PIK3CB (referred as PI3K from hereon) siRNA (50 nM) at 1:4 siRNA: carrier ratio. Total RNA was isolated after 48 h of treatment using TRIzol. Total RNA (400 ng) was converted into cDNA using oligo-dT, random primers and M-MLV reverse transcriptase. The silencing of PIK3CB mRNA copy number was detected by ddPCR (QX100, Bio-Rad) using 2 ng of each sample and ddPCR supermix for the specific probes (Bio-Rad). The PrimeTime qPCR assays for PIK3CB (forward primer 5′-CGC TTG ATG GAT TTA CTC TGG A-3′, reverse primer 5′-GAG GTG CTC ACA ACT TCA ATG-3′ and probe 5′-/FAM/TGG TTT GGA/ZEN/TCT TCG GAT GTT GCC T/IABkFQ/-3′) and a reference gene, β-actin (forward primer 5′-GCG AGA AGA TGA CCC AGA T-3′, reverse primer 5′-CCA GTG GTA CGG CCA GA-3′ and probe 5′-/HEX/CCA TGT ACG/ZEN/TTG CTA TCC AGG CTG T/IABkFQ/-3′) were obtained from Integrated DNA Technologies (IDT; Coralville, Iowa). 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

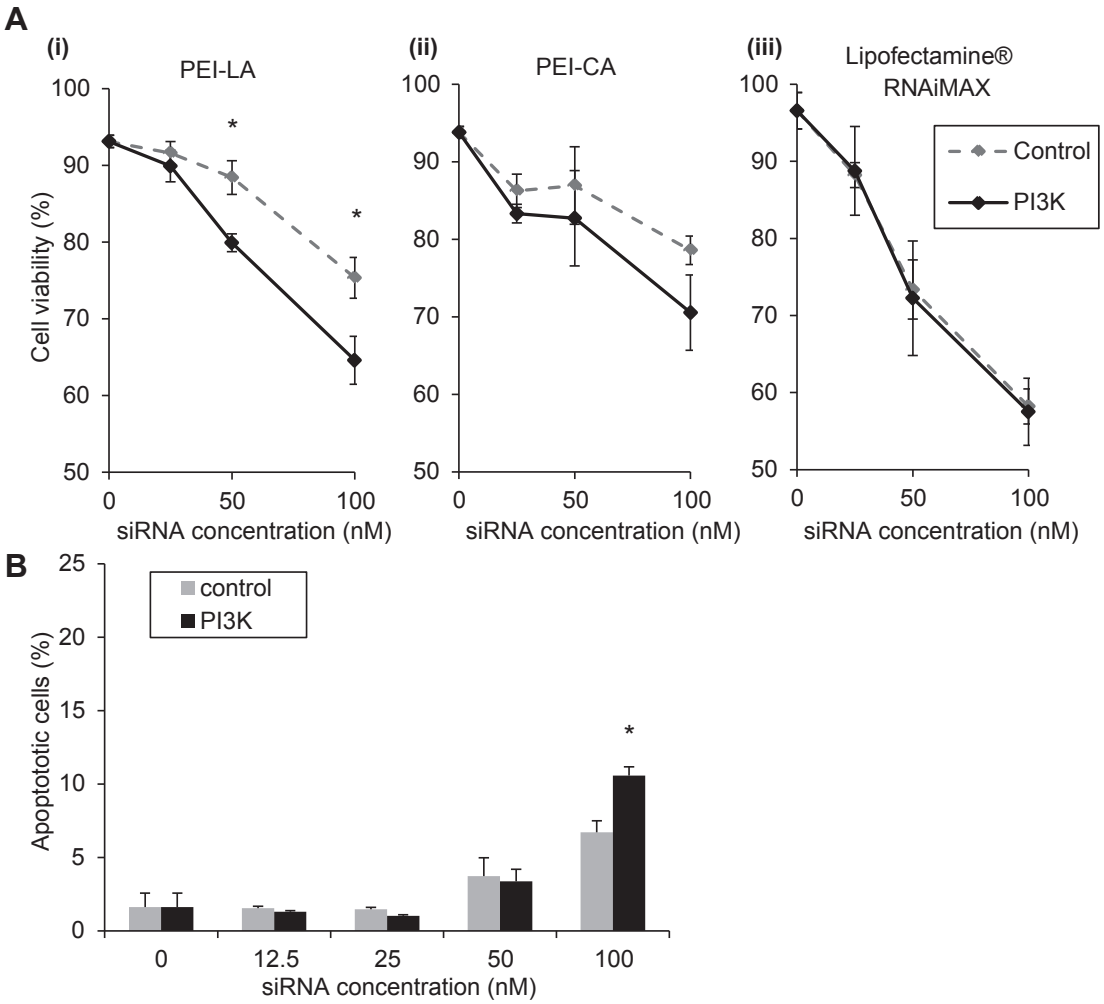

Fig. 8. Effect of anti-PI3K siRNA on Hut78 viabilities. (A) The cells were treated with the complexes of PEI-LA (i), PEI-CA (ii) and Lipofectamine® RNAiMAX (iii) prepared at siRNA: carrier ratio 1:4. The siRNA concentrations were 25, 50 and 100 nM (polymer concentration of 1.4, 2.8 and 5.6 μg/mL, respectively). The relative viabilities compared to untreated cells were analyzed with Trypan Blue staining after 72 h of incubation with complexes. Groups significantly different from the corresponding control siRNA treated groups (at respective doses) were indicated with an *. (B) The percentage of apoptotic cells after treatment with the complexes of PEI-LA and anti-PI3K siRNA (siRNA: carrier ratio 1:4). The cell apoptosis was analyzed with FlowTACS™ Apoptosis Detection Kit after 72 h of incubation with the complexes. *: $p < 0.05$ vs. control siRNA treatment group.

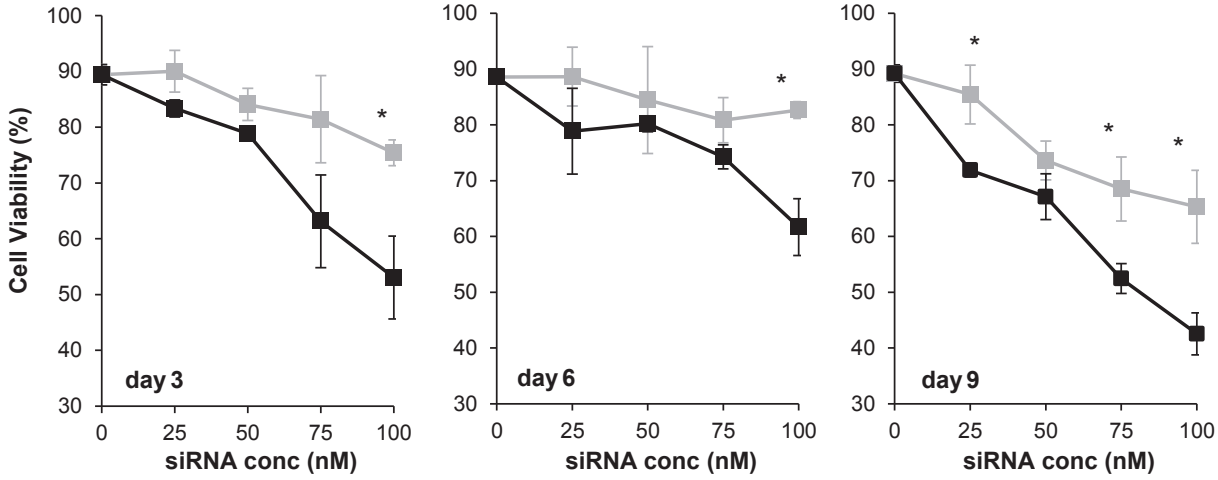

Fig. 9. Effect of anti-CDK18 siRNA on Hut78 viabilities. The cells were treated with CDK18 siRNA (black squares) and control siRNA (gray squares) complexes of PEI-LA prepared at siRNA: carrier ratio 1:4. The relative cell viabilities were determined by the Trypan Blue staining after 3, 6 and 9 days of treatment. Groups significantly different from the corresponding control siRNA treated groups (at respective doses) were indicated with an *.

10 min at 98 °C. Template DNA was omitted from the ddPCR reaction as a no template control and the results of ddPCR were analyzed using the QuantaSoft Software (Bio-Rad).

2.10. Statistical analysis

All data are summarized as mean SD and variations between groups means was analyzed by the 2 sample Student's *t*-test ($p < 0.05$) [29].

3. Results

3.1. siRNA delivery to Hut78 and Jurkat cells

The PEI-CA and PEI-LA, which were previously shown to be effective in anchorage-independent cells [18], were initially employed to investigate siRNA delivery to Hut78 and Jurkat cells. The siRNA delivery was quantitated using FAM-labeled siRNA at siRNA: carrier ratios of 1:2, 1:4 and 1:8 and using 25PEI as a reference reagent (Fig. 1). In Hut78 cells, 25PEI showed superior delivery at all siRNA: carrier ratios based on both mean siRNA delivery (Fig. 1Ai) and percentage of siRNA-positive cells (Fig. 1Aii), approaching 100% siRNA-positive cells at the 1:8 ratio. PEI-CA and PEI-LA significantly enhanced siRNA uptake at 1:4 and 1:8 ratios and were generally equivalent in siRNA delivery efficiency. In Jurkat cells (Fig. 1B), none of the polymers gave significant delivery at siRNA: carrier ratio of 1:2. At a ratio of 1:4, 25PEI gave the best result in terms of cell percentage displaying uptake (54.1 ± 3.5%; Fig. 1Bii) but not in terms of mean siRNA uptake (82.9 ± 14.4 a.u.), where PEI-CA was the most effective (215.1 ± 72.7 a.u.; Fig. 1Bi). The siRNA delivery by the PEI-LA was not significant at this ratio (39.8 ± 8.7 a.u. for mean and 13.2 ± 5.0% for percentage of siRNA-positive cells), but it increased significantly at the ratio 1:8. The 25PEI was again the best carrier in terms of both mean fluorescence (288.1 ± 76.9 a.u.) and cell percentage (80.0 ± 3.4%) at the ratio of 1:8.

3.2. GFP silencing in Hut78 and Jurkat cells

The GFP-positive Hut78 and Jurkat cells were treated with anti-GFP siRNA complexes of 25PEI, PEI-CA and PEI-LA, and GFP levels were assessed after 24, 48 and 72 h (Fig. 2). The siRNA: carrier weight ratios were 1:4 and 1:8 for Hut78 cells and 1:2, 1:4 and 1:8 for Jurkat cells. The GFP levels obtained with anti-GFP siRNA treatments were normalized against the corresponding control (scrambled) siRNA treatments. Based on the percentage of silenced cells (Fig. 2Ai), 25PEI and PEI-CA were not efficient in Hut78 cells at any of the time points evaluated, while PEI-LA gave significant ($p < 0.05$) silencing at 1:4 ratio at 24 h (10.7 ± 3.0%), at both 1:4 and 1:8 ratios at 48 h (18.7 ± 2.0% and 22.3 ± 2.2%, respectively) and 72 h (18.4 ± 2.2% and 20.2 ± 0.3%). Based on mean GFP levels (Fig. 2Aii), only PEI-LA at a ratio of 1:4 was efficient at 24 h (23.6 ± 7.1%). At 48 h, 25PEI at the ratio of 1:8 (13.7 ± 1.8%) and PEI-LA at the ratios of 1:4 and 1:8 (18.8 ± 1.8% and 25.4 ± 3.7%, respectively) gave significant silencing. At 72 h, PEI-CA and 25PEI at the ratio of 1:8, and PEI-LA at ratios of 1:4 and 1:8 (19.5 ± 1.3% and 31.4 ± 1.8%, respectively) showed significant silencing efficiency.

The extent of GFP silencing was not significant ($p > 0.05$) in Jurkat cells, either based on the percentage of cells silenced (Fig. 2Bi) or the mean GFP silencing (Fig. 2Bii). Some silencing observed based on mean GFP silencing was associated with large variations in these groups (e.g., 25PEI and PEI-CA at the ratio 1:8). Since repeat experiments did not indicate any silencing in Jurkat cells, the polymers were deemed ineffective in Jurkat cells and these cells were not pursued any further in this study.

3.3. Optimizing GFP silencing in Hut78 cells

We next attempted to optimize silencing efficiency in Hut78 cells by several measures. The effect of cell agitation during siRNA treatments was first explored with the goal of increasing the physical encounter between the cells and the siRNA complexes. Using siRNA: carrier ratios of 1:2, 1:4 and 1:8 for 25PEI, PEI-CA and PEI-LA, no effect of agitation was evident, since the study groups either displayed the same level of silencing, or even reduced silencing after shaking (not shown).

The effect of centrifugation on GFP silencing efficiency was next explored. Centrifugations at 0 *g* (normal incubation without centrifugation), 100 *g*, 200 *g* and 300 *g* were performed before and after complex addition; i.e., centrifugation of cells alone before complexes addition vs. centrifugation of cells together with complexes. Using siRNA: carrier weight ratio of 1:4 and 5-min centrifugation time, no effect of the centrifugation was observed with any of the polymers after 72 h when the cells were centrifuged before the addition of complexes (Fig. 3A). The delivery with PEI-LA was effective, but centrifugation did not increase silencing efficiency even with this polymer. However, when the centrifugation was performed after the complex addition (Fig. 3B), the efficiency of PEI-LA slightly increased with increasing centrifugation while 25PEI and PEI-CA remained ineffective. However, no significant difference was reached among the applied g-forces based on the percentage of cells silenced (Fig. 3Bi). Based on mean GFP silencing, 25PEI remained ineffective, PEI-CA gave a significant silencing at 300 *g* (0 *g*: 1.2 ± 4.5% vs. 300 *g*: 13.7 ± 1.7%), and PEI-LA showed increased efficiency with centrifugation (0 *g*: 12.5 ± 5.2%, 100 *g*: 20.6 ± 0.4%, 200 *g*: 20.2 ± 5.1%, 300 *g*: 20.0 ± 6.3%; Fig. 3Bii; $p > 0.05$ between 0 *g* and 300 *g*).

Effect of centrifugation time between 0 and 15 min was next investigated. Based on the percentage of cells silenced (Fig. 4A), 25PEI and PEI-CA showed no silencing that was dependent of either the magnitude or the duration of centrifugation. PEI-LA, on the other hand, showed an increase with the duration of centrifugation at 100 *g* (0 min: 6.3 ± 0.5% vs. 15 min: 14.7 ± 2.1%), 200 *g* (0 min: 15.3 ± 1.3% vs. 15 min: 34.4 ± 1.4%) and 300 *g* (0 min: 11.6 ± 2.1% vs. 15 min: 30.0 ± 2.0%). The results based on the mean GFP silencing showed a similar trend (Fig. 4B).

Finally, the effect of lipid substitution on 2PEI was investigated by preparing complexes with PEI-CA and PEI-LA polymers at different lipid substitution ratios (substitution ratios on 2PEI were controlled with the feed ratio during the synthesis). The complexes were prepared at siRNA: carrier weight ratio of 1:4, centrifugation was performed at 300g/15 min, and GFP silencing efficiency was assessed after 72 h of complex addition. Based on both the percentage of cells silenced (Fig. 5A), and mean GFP silencing (Fig. 5B), 25PEI and PEI-CA at different lipid substitutions were again inefficient, while PEI-LA was inefficient at the lowest substitution ratio of 1.29 LA/PEI, but effective at the higher substitution ratios between 1.7 and 2.6 LA/PEI.

3.4. Polymer toxicity

To assess the relative toxicities of polymeric siRNA carriers, Hut78 cells were treated with varying concentrations of control siRNA complexes prepared at siRNA: carrier ratio of 1:4. Complexes prepared with the commercial reagent Lipofectamine® RNAiMAX and relatively non-toxic 2PEI [15] were also used as reference reagents. The toxicity was evaluated by Trypan Blue staining (Fig. 6A) and AlamarBlue® assay (Fig. 6B). Based on Trypan Blue staining, 2PEI was non-toxic to Hut78 cells up to 10 μg/mL, while 25PEI was the most toxic with only 31.6 ± 8.6% of the cells viable at 10 μg/mL. The PEI-CA and PEI-LA had similar toxicities (~30% loss of viability)

at the highest concentration tested, which were more toxic than the unsubstituted 2PEI complexes but significantly less toxic than the 25PEI complexes. Lipofectamine® RNAiMAX complexes showed a similar effect to that of 25PEI complexes, significant toxicity starting at 5 μg/mL reaching up to ~55% loss of viability at 10 μg/mL. Based on the AlamarBlue® assay, 2PEI was not toxic, while 25PEI complexes were highly toxic again. The PEI-CA and PEI-LA were minimally toxic showing a similar trend. Lipofectamine® RNAiMAX complexes were not as toxic as the 25PEI complexes in this assay, but were still more toxic than the other carriers.

3.5. Silencing endogenous targets for inhibition of cell growth

The feasibility of silencing endogenous mediators in Hut78 cells was explored by targeting several mediators whose silencing (i) decreased cell viability in other cell lines (KSP, CDK18, MAP, RPS and STK), or (ii) induced apoptosis in Hut78 (STAT3, PI3K and Bcl11b) based on the literature [30–33]. The carriers used were PEI-LA, PEI-CA and Lipofectamine® RNAiMAX with the overall goal of assessing the effect of target silencing on the viability of Hut78 cells. A single siRNA formulation was used against the selected targets, except for STAT3 for which 4 different siRNAs were used against different sequences within the target mRNA. With PEI-LA mediated delivery, none of the siRNAs decreased the viability significantly at 25 nM siRNA as compared to control siRNA (Fig. 7A). At 50 nM, only STAT3-7 siRNA had a statistically significant effect on the viability of Hut78 cells while MAP and PI3K siRNAs had significant effects on cell viability at 100 nM. With PEI-CA, CDK18, Bcl11b, MAP, RPS, PI3K and STK siRNAs had significant effects at 25 nM siRNA dose, while only PI3K siRNA was effective at the 50 and 100 nM concentration (Fig. 7B). The CDK18 and RPS siRNAs had also significant effects on the viability of the cells at 100 nM concentration. With Lipofectamine® RNAiMAX, CDK18 and PI3K siRNA had significant effects at 25 nM, while none of these siRNAs had any effect at higher doses (Fig. 7C).

3.6. Targeting PI3K and CDK18 for inhibition of cell growth

The initial screen of siRNAs indicated PI3K and CDK18 to be relatively more effective among the chosen targets in reducing cell growth. More detailed studies were performed with these siRNAs and Hut78 cells. The siRNA delivery with PEI-LA resulted in significant loss of cell viability with anti-PI3K siRNA (at 50 and 100 nM) as compared to control siRNA (Fig. 8i). The siRNA delivery with PEI-CA and Lipofectamine® RNAiMAX did not result in statistically significant loss of cell viability, where the more pronounced toxicity of the latter reagent was again evident. The extent of apoptosis after anti-PI3K siRNA delivery was determined by a nuclear fragmentation assay based on flow cytometry (Fig. 8B). There was no clear apoptosis in the cells treated at 0, 12.5 and 25 nM siRNA while, at 50 nM siRNA, there was limited apoptosis that was equal in both control and anti-PI3K siRNA groups. At 100 nM siRNA, a significant difference in apoptosis was evident between the control and anti-PI3K siRNA treated groups.

The CDK18 silencing was investigated using PEI-LA for siRNA delivery and cell viability was investigated after 3, 6 and 9 days of complex treatment (Fig. 9). A significant difference in cell viabilities between anti-CDK18 and control siRNA was evident with 100 nM siRNA on day 3 (Fig. 9i) and day 6 (Fig. 9ii). On day 9 (Fig. 9iii), all anti-CDK18 siRNA doses except for the 50 nM had a significant effect on the viability of Hut78 cells compared to the control siRNA treatment. As in PI3K silencing, the extent of apoptosis was investigated after 3 days, but there was no significant difference in apoptotic cell populations for the cells treated with control and anti-CDK18 siRNA (not shown).

Finally, to confirm the specific effect of siRNA-mediated silencing, the extent of PI3K mRNA levels was quantitated by ddPCR. The siRNA delivery was undertaken by the PEI-LA, PEI-CA and Lipofectamine® RNAiMAX, and mRNA levels were assessed 24 h after complex addition (Fig. 10). A significant reduction in PI3K mRNA levels were evident when PEI-LA was used to deliver the specific siRNA, while the difference between control and PI3K siRNA were not as significant with the PEI-CA and Lipofectamine® RNAiMAX mediated delivery.

4. Discussion

A polymer-based approach for *in vitro* siRNA delivery to cutaneous T-cell lymphoma cells was investigated in this study. Among the polymers explored, linoleic acid-substituted 2PEI was most effective in order to undertake siRNA delivery to Hut78 cells, but widely applicable carriers Lipofectamine® RNAiMAX and 25PEI were found ineffective in these cells. Of the two lipid-modified polymers evaluated (PEI-LA and PEI-CA), the former gave consistently better outcomes. Since both lipids are attached by the same chemical reaction to polymeric amines, the superior functionality of the PEI-LA was not due to modification of the polymeric amines *per se*, but rather the nature of the lipid substituent on the polymer. It was interesting to note that the PEI-LA that was successful in Hut78 cells was not effective in the phenotypically related T-cell line, Jurkat cells. The delivery efficiencies were relatively similar in the two cell types (based on uptake of FAM-siRNA), but silencing of a model gene (GFP) was observed only in the Hut78 cells. This was in contrast to a previous study where a functional response to Bcl11b silencing was similar in both cell types as a result of siRNA delivery by electroporation [34]. The Hut78 cells display more differentiated features than the Jurkat (adult lymphoid leukemia) cells and the RISC pathway may be more active in Hut78 as compared to Jurkat cells, making the former cells more responsive to siRNA treatment. Even for the Hut78 cells, siRNA delivery did not correlate with silencing efficiency, where the most effective carrier for siRNA delivery (25PEI) did not sustain GFP silencing. Differences in intracellular trafficking of siRNA and/or siRNA dissociation from complexes inside the cells, which were not explored in this study,

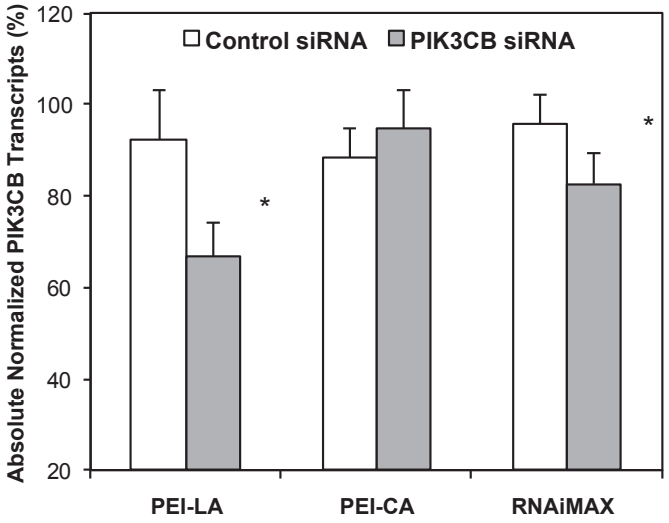

Fig. 10. PI3K silencing after specific siRNA delivery with PEI-LA, PEI-CA and Lipofectamine® RNAiMAX. The cells were treated with PI3K (gray bars) and control siRNA (white bars) complexes of the carriers (siRNA: carrier ratio of 1:4; siRNA concentration of 50 nM in medium). The relative PI3K mRNA levels (normalized with β-actin) were determined by ddPCR after 24 h of siRNA treatment. *: $p < 0.05$.

might have been responsible for this observation. We previously established that the PEI-LA polymers display increased propensity for dissociation due to the bulky and disordered LA substituents [18], possibly making the siRNA more available intracellularly for silencing.

As with both anchorage dependent [17] and independent cells [18], the siRNA delivery was related to the siRNA: carrier ratio used for complex formation; increased delivery at higher ratios was previously attributed to higher zeta-potential of the complexes [16]. A higher siRNA: carrier ratio, although optimal for delivery, might lead to higher cytotoxicity that might affect target silencing. It is possible that the toxicity of 25K PEI to be the underlying reason for the failure of this polymer to induce gene silencing upon siRNA delivery. Similarly for Lipofectamine® RNAiMAX, when used for anti-PI3K siRNA delivery, its toxicity might have prevented a clear manifestation of a functional outcome (see Fig. 8Aiii). Centrifugation at short duration (5 min) did not enhance the efficiency of PEI-LA, but a more prolonged (15 min) centrifugation was effective in further enhancing the efficacy of PEI-LA. The encounter of the siRNA complexes with cells might therefore be limiting efficient delivery of siRNA to Hut78 cells and better Hut78-associating complexes might further improve the silencing efficiency.

Assessing GFP silencing within target cells is a convenient and precise method to compare the relative efficiency of siRNA carriers. However, being an artificial gene expression system with a typically higher level of expression as compared to endogenous proteins, it may be difficult to directly relate the GFP silencing results to silencing endogenous genes. The forced GFP expression might also have undesired effects on cells, such as increasing the permeability of cellular membranes to solutes (and nanoparticles), changing the proliferation rate and/or enhancing cellular sensitivity to treatment effects (i.e., cytotoxicity) [35]. We are aware that maximal silencing efficiency obtained with GFP (30–35%) is not clearly transferrable to endogenous genes. We therefore attempted to silence several promising targets to obtain a functional response in the form of inhibition of cell growth. The chosen functional outcome is not a straightforward silencing response, since growth inhibition will not be directly related to extent of target silencing (presumably a certain level of silencing will need to be achieved before a functional response is seen) and that other mediators could substitute for the target proteins to continue supporting cell proliferation. Nevertheless, successful inhibition of growth was possible and PEI-LA again yielded the largest number of hits among the chosen targets, as compared to other carriers used. PEI-CA gave some hits even though it was not as effective as PEI-LA in silencing GFP expression. The lower level of endogenous gene expression, hence the need for less siRNA, might have been the reason for this. The Lipofectamine® RNAiMAX was not successful in this regard, and it is possible that the toxicity of this reagent might have again interfered with obtaining a functional response (note that control siRNA treated cells gave significant growth inhibition with this reagent, more so than the other reagents). Not all targets and delivery systems showed a robust dose-response effect, since toxicities at the highest doses might have prevented such a clear response.

Among the employed targets, PI3K and CDK18 were identified as more robust targets to inhibit cell proliferation. The possibility of PI3K as a therapeutic target in T-cell lymphomas has been recognized [36] and Hut78 cells were shown to express PI3K and prone to PI3K isoform-specific silencing with siRNA (siRNA delivery by electroporation [37]). The silencing was marginal in that study (only at 48 h between 24 and 96 h analysis) and no apoptosis was seen in that case. This was unlike our case where PIK3 silencing was shown at the mRNA level with PEI-LA and significant apoptosis was evident at the 100 nM siRNA concentration, which may possibly indicate more efficient siRNA delivery with the PEI-LA. The overall response, although significant, was not very strong in our hands and additional targets, such as MEK and PI3K combination [37], might be required for silencing to obtain a robust functional response. The CDK18 (also known as serine/threonine-protein kinase PCTAIRE-3), on the other hand, has not been explored in T-cell lymphomas and the reason for our use of this target was the availability of an effective siRNA that curbed cell growth in breast cancer cells (unpublished data). We were unable to find any information in the literature on the possible role of CDK18 in lymphomas, so this protein could be a novel target for CTCL therapy.

Finally, we must note that other groups have employed RNAi-mediated control of Hut78 cell growth and other CTCL cells (primary cells or cell lines). With the Hut78 cells, lentiviral-mediated shRNA expression was targeted against polo-like kinase 1 [38], but the safety concerns with lentiviruses impede their applicability in a clinical setting. Electroporation-mediated siRNA delivery has been tested against NRAS [39], the apoptosis-inducible substrate regulated by p53, Noxa [40], and the anti-apoptotic protein Bcl11b [34] in Hut78 cells. In the latter case, ~80% reduction in protein level was obtained that led to significant population of apoptotic cells (>50% over 3 days). Although we attempted to repeat this outcome, our delivery system in this study did not indicate a reproducible response to Bcl11b siRNA for reasons not clear at this stage. It is possible that the electroporation previously used for Bcl11b siRNA delivery was more potent than our polymer-mediated siRNA delivery or affected cells in a way that led to a stronger response to Bcl11b silencing. In other CTCL cells, electroporation was again the most common means of delivering siRNAs, for example against STAT5 [41], FRA2 and JUND [42], SHP-1 [43], STAT3 and JAK5 [7], and PDCD-10 [44]. While these studies provided a proof-of-principle for deployment of RNAi to control malignant cell growth, the electroporation used for siRNA is not translatable for therapy, being restricted to *in vitro* studies. A few non-viral carriers were employed in the CTCL cells; the proprietary cationic polymer Turbofect was used to deliver siRNA against forced expression of p53 [8], while Lipofectamine® RNAiMAX and an unspecified lipofection agent were used for siRNA against Notch1/2/3 [45] and JAK1/3 [46], respectively. The Lipofectamine® RNAiMAX was not effective in our hands, but the choice of our target protein and the functional response assessed here might have accounted for this difference. A full range of studies exploring the use of these non-viral carriers for siRNA therapy is lacking and no follow up studies were reported. In this regard, our extensive description of the performance of the polymeric carrier represents a valuable step, by providing a clinically-feasible delivery system for molecular therapy of CTCLs. However, we recognize the need to improve the delivery efficiency of the described polymers for a more robust effect as well as the animal studies to assess *in vivo* delivery. The PEI-LA was recently shown to be effective in subcutaneous breast cancer xenografts [47], so that the polymers are promising for *in vivo* delivery. Future studies are planned to further improve the delivery efficiency to CTCL cells and explore delivery to CTCL xenografts in animal models.

5. Conclusions

We found a polymeric carrier (PEI-LA) suitable for *in vitro* delivery of siRNA to cutaneous T-cell lymphoma cells. The polymeric carrier was superior to commonly used carriers, the Lipofectamine® RNAiMAX and 25PEI, and could serve as an alternative to electroporation, which is an effective (but damaging) approach for *in vitro*

delivery of molecular agents into cells, but cannot be used in pre-clinical animal models. A threshold value for LA substitution amount was noted for effective PEI-LA carriers, beyond which the extent of substitutions did not influence the silencing efficiency. The polymeric carriers were capable of delivering other siRNAs that may play a significant role in proliferation of the cutaneous T-cell lymphoma model, Hut78 cells. Beyond the employed model, the polymers could be further used for delivery of other nucleic acids as well as expression systems for proteins and short hairpin RNA (shRNA), so that they could be broadly applicable reagent for molecular therapy in oncological transformations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2014.07.029.

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