Multiple siRNA delivery against cell cycle and anti-apoptosis proteins using lipid-substituted polyethylenimine in triple-negative breast cancer and nonmalignant cells

Manoj B. Parmar,[1] Bárbara E. Arteaga Ballesteros,[2] Timothy Fu,[2] Remant Bahadur K.C.,[2] Hamidreza Montazeri Aliabadi,[3] Judith C. Hugh,[4] Raimar Löbenberg,[1] Hasan Uludağ[1,2,5]

[1]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada
[2]Department of Chemical and Materials Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada
[3]School of Pharmacy, Chapman University, Irvine, California, USA
[4]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada
[5]Department of Biomedical Engineering, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada

Abstract: Conventional breast cancer therapies have significant limitations that warrant a search for alternative therapies. Short-interfering RNA (siRNA), delivered by polymeric biomaterials and capable of silencing specific genes critical for growth of cancer cells, holds great promise as an effective, and more specific therapy. Here, we employed amphiphilic polymers and silenced the expression of two cell cycle proteins, TTK and CDC20, and the anti-apoptosis protein survivin to determine the efficacy of polymer-mediated siRNA treatment in breast cancer cells as well as side effects in nonmalignant cells *in vitro*. We first identified effective siRNA carriers by screening a library of lipid-substituted polyethylenimines (PEI), and PEI substituted with linoleic acid (LA) emerged as the most effective carrier for selected siRNAs. Combinations of TTK/CDC20 and CDC20/Survivin siRNAs decreased the growth of MDA-MB-231 cells significantly, while only TTK/CDC20 combination inhibited MCF7 cell growth. The effects of combinational siRNA therapy was higher when complexes were formulated at lower siRNA:polymer ratio (1:2) compared to higher ratio (1:8) in nonmalignant cells. The lead polymer (1.2PEI-LA6) showed differential transfection efficiency based on the cell-type transfected. We conclude that the lipid-substituted polymers could serve as a viable platform for delivery of multiple siRNAs against critical targets in breast cancer therapy. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 3031–3044, 2016.

Key Words: amphiphilic polymers, siRNA delivery, breast cancer therapy, cell cycle protein, anti-apoptosis protein

INTRODUCTION

Conventional breast cancer therapies, based on broadly acting chemotherapeutic agents, have significant limitations and side effects due to their nonspecificity. More recently developed therapies mainly target estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER2) of breast cancer cells to decrease the tumor growth.[1–3] However, the common subtype of breast cancer, triple-negative breast cancer, occurs in 12–17% of patients, which has no expression of these receptors and will not be responsive to such therapies.[4] Triple-negative breast cancer is highly metastatic, display low response to chemotherapy, has a very high recurrence rates and poor prognoses for patient survival.[5,6] Targeted therapies for triple-negative breast cancer currently do not exist, so that search for novel avenues to fight against this deadly disease is warranted. A promising therapy in this regard is based on RNA interference (RNAi), where small interfering RNA (siRNA) is introduced into cells to silence the expression of aberrant genes. The latter may include proteins important for prolonged cell survival and/or causing un-checked tumor growth.[7,8] Upon introduction of siRNA to a cell, siRNA is incorporated into an RNA inducing silencing complex (RISC). RISC hydrolyses the double-stranded synthetic siRNA and degrades passenger strand while having the guide strand into the RISC assembly.[8,9] The guide strand of

siRNA directs the whole complex specifically toward its targeted mRNA. The siRNA–RISC complex binds to the targeted mRNA, and either cleave the mRNA or block the translation process, which results into silencing of specific target.[9] The major issue associated with siRNA therapy is the delivery. Since the siRNA is highly labile due to endogenous nucleases and its negative charge, its entry into the cell on its own is almost impossible.[10,11] The siRNA, therefore, needs a carrier that can deliver it into the cells by neutralizing its anionic charge, and protect it from extracellular degradation. We have synthesized a library of cationic polymeric carriers for such purpose, which is based on low molecular weight polyethylenimines (PEI).[12,13] PEIs were substituted with different lipidic moieties that help siRNA/polymer complexes to penetrate the plasma membrane and improve the uptake of complexes.[13,14] Cationic lipid-substituted PEIs provide protection to siRNA as well as facilitate its delivery into the cell so that siRNA can be assembled with RISC.

Controlled cell division and multiplication involve many proteins in a series of events, commonly known as the cell cycle. The deregulation of the cell cycle is one of the hallmarks of cancer, where cell cycle proteins are mostly upregulated.[15,16] Due to aberrant expression of cell cycle proteins, control over proliferation and multiplication of the cells is lost, resulting in a cancerous growth. If the expression of these up-regulated cell cycle proteins is silenced by siRNA therapy, the proliferation of malignant cells may be decreased and tumor growth can be halted. Here, we have targeted two cell cycle proteins, monopolar spindle 1 (MPS1) and cell division cycle protein 20 (CDC20) to silence their expression by siRNA. MPS1 is more commonly known as TTK protein kinase since it is able to phosphorylate tyrosine, serine and threonine residues of a substrate. It is exclusively associated with the cell proliferation.[17] It aligns chromosomes at the centromere and is required for the duplication of centrosome during mitosis.[18] TTK is a critical mitotic checkpoint protein for accurate segregation of chromosomes during mitosis, and it is upregulated in tumorigenesis.[19–22] A related cell cycle protein, CDC20, activates the anaphase-promoting complex (APC) in the cell cycle during mitosis, which initiates chromatid separation and entrance of the cell division into anaphase.[23] The reports have shown that inhibition of TTK protein disrupts the CDC20-MAD2 (mitotic arrest deficient 2; inhibitor of CDC20) complexation, causing premature activation of APC that accelerates anaphase during mitosis.[24,25] Therefore, silencing TTK along with CDC20 could be a promising strategy to achieve a synergistic reduction in malignant cell growth by combinational siRNA therapy.

The cell cycle process is often coordinated with the apoptosis machinery to maintain tissue homeostasis.[26] Apoptosis is a tightly regulated process of programmed cell death that is executed by activation of caspases.[27] The delicate balance between pro-apoptotic and anti-apoptotic proteins maintains the integrity of normal cell, and this balance is often shifted in favor of anti-apoptotic proteins in transformed cells to allow cells to resist drug therapy and to maintain an overproliferative state.[28–30] The member of inhibitor of apoptosis proteins (IAP), survivin is known to inhibit caspase activation and block apoptosis. Survivin is up-regulated in many cancer types, but is completely absent in terminally differentiated cells.[31] Transcriptional silencing of survivin may provide an excellent therapeutic opportunity as the discrimination between malignant and normal cells can be achieved. The evidences suggest a role for survivin in spindle checkpoint[32,33] and, therefore, targeting survivin along with cell cycle proteins by siRNA may lead to additive effect(s) to retard tumor growth.

In this study, we first determined the most effective polymeric carrier for siRNA delivery, and evaluated the efficacy of various TTK siRNAs targeting different locations of TTK transcripts using that effective polymeric carrier. We then evaluated the combinational siRNA therapy with TTK, CDC20 and survivin siRNAs in breast cancer cells. We hypothesize that siRNA combinations targeting mediators of abnormal cell cycle progression such as TTK, CDC20, and survivin may provide synergistic therapeutic effect on breast cancer cells. The effect of this combinational siRNA therapy was also evaluated in normal cells, including breast, endothelial, and bone marrow stromal cells.

MATERIALS AND METHODS
Materials
The amines of low molecular weight PEIs (0.6, 1.2, and 2.0 kDa) were substituted with linoleic acid via *N*-acylation (PEI-LA) and the degree of substitution was determined by NMR spectroscopy as previously described.[34,35] MTT [3-(4 5-dimethylthiazol-2-yl)−2 5-diphenyltetrazolium] and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Hank's balanced salt solution (HBSS) was prepared in-house.

TTK-1 (Cat. No. SI02223207), TTK-2 (Cat. No. SI02223214), TTK-3 (Cat. No. SI03062745), and TTK-4 (Cat. No. SI04898747) siRNAs were ordered from Qiagen (Valencia, CA), and TTK-5 (Cat. No. HSC.RNAI.N001166691.12.1) and TTK-6 siRNAs (Cat. No. HSC.RNAI.N001166691.12.2) were purchased from IDT (Coralville, IA). Negative control scrambled siRNA (Cat. No. DS NC1), CDC20 siRNA (Cat. No. HSC.RNAi.N001255.12.1) and survivin siRNA (Cat. No. HSC.RNAI.N001012271.12.1) were ordered from IDT. The 6-carboxyfluorescein (FAM) labeled scrambled siRNA was purchased from IDT. Primers for TTK (forward: GCCCGAAAAGTTAATACAGAGCAGA; reverse: GATGTTGATATTGGTGGTGACTGT), CDC20 (forward: CGCTATATCCCCCATCGCAG; reverse: GATGTTCCTTCTTGGTGGGC), survivin (forward: TGAGAACGAGCCAGACTTGG; reverse: ATGTTCCTCTATGGGGTCGT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: TCACTGTTCTCTCCCTCCGC; reverse: TACGACCAAATCCGTTGACTCC) were supplied by IDT.

Cell models
The triple-negative breast cancer MDA-MB-231 cells and MCF7 (estrogen- and progesterone-positive) cells were cultured in DMEM medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. MDA-MB-231 and MCF7 cells were generous gifts from Dr.

Michael Weinfeld, Department of Oncology, University of Alberta, and Dr. Afsaneh Lavasanifar, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, respectively. The normal breast cells MCF10A were cultured in DMEM/F12 medium with 500 ng/mL hydrocortisone, 20 ng/mL human epidermal growth factor (hEGF), 0.01 mg/mL human insulin, 100 ng/mL cholera toxin, 5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. MCF10A cells were gift from Dr. Judith Hugh, Department of Laboratory Medicine and Pathology, University of Alberta. Human umbilical vein endothelial cells (HUVEC), generous gift from Dr. Janet A. W. Elliott, Department of Chemical and Materials Engineering, University of Alberta, were cultured on rat tail type I collagen coated culture flask with EGM-2 medium that was supplied with manufacturer's growth factor bullet-kit, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human bone marrow stromal cells (hBMSC) were isolated from 29 years old female patient as previously described[36] with informed consent and approval from the Research Ethics Board, University of Alberta. hBMSC cells were cultured in DMEM/F12 medium with 10% FBS, 20 ng/mL basic fibroblast growth factor (bFGF), 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained at 37°C and 95/5% air/CO_2.

Screening of polymers and preparation of siRNA/polymer complexes

To determine most effective polymeric carrier for siRNA delivery, a library of synthesized polymers (Supporting Information Table I) was screened with TTK-1 and CDC20 siRNAs, and scrambled siRNA as a negative control. The inhibition of cell growth was assessed with the MTT assay after 72 h of siRNA transfection. The siRNAs were typically used at 40 nM concentration with 1:6 siRNA:polymer ratio. The lipid-based commercial carrier, Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA) was also used in the screening with 1:1 siRNA:Lipofectamine ratio. The siRNA/polymer complexes were prepared in 150 mM NaCl by allowing 30 min of incubation time after adding polymer into siRNA for their interaction with each other to form complexes. The complexes were added to cells after 30 min of incubation at room temperature. After 72 h of treatment, MTT was added to the cells at 1 mg/mL final concentration in HBSS. The cells were incubated for 1 h at 37°C and 5% CO_2. During this incubation, soluble MTT is transformed into insoluble formazan crystals due to the activity of mitochondrial dehydrogenase enzymes, giving a measure of cellular activity.[37] DMSO was added to the well to dissolve the formazan crystals formed because of cellular activity of live cells. The optical density (OD) was measured at 570 nm and the ODs were summarized as a percentage of cell growth based on nontreated cells (taken as 100% cell growth).

Size and ζ-potential of siRNA/polymer complexes

To characterize siRNA/polymer complexes based on different degree of lipid-substitutions in 1.2 kDa PEI, hydrodynamic diameter (Z-average) and surface charge (ζ-potential) of these complexes were determined in ddH_2O through dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using Zetasizer Nano ZS (Malvern, UK). The complexes were prepared as described above with 0.6 μg of scrambled siRNA at 1:6 and 1:1 siRNA:polymer and siRNA:Lipofectamine ratio, respectively, and were diluted to 1 mL ddH_2O before each measurement. The size and ζ-potential of siRNA/polymer complexes were characterized based on different siRNA:polymer ratios as well. Native 1.2 kDa PEI and 1.2PEI-LA6 were employed at 1:2, 1:4, and 1:8 siRNA:polymer ratios to determine the size and ζ-potential of complexes as described above.

siRNA uptake by flow cytometry

To determine the delivery efficiency of lipid-substituted PEIs based on different degree of LA substitutions, MDA-MB-231 cells were transfected with FAM-labeled siRNA at 40 nM with 1:6 siRNA:polymer ratio. Lipofectamine® 2000 (Thermo Fisher Scientific) was used at 1:1 siRNA:Lipofectamine ratio. Nonlabeled scrambled siRNA was used as a negative control. Cells were trypsinized after 24 h of siRNA transfection and fixed with 3.7% formaldehyde. The uptake of siRNA was quantified using BD LSRFortessa™ cell analyzer (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence of the recovered cell population and the percentage of cells showing FAM-fluorescence were determined after gating of the cell population as such that autofluorescence of untreated cells represented ∼1% of the total cell population.

To investigate the potential of the lead polymer 1.2PEI-LA6 to deliver siRNA, MDA-MB-231 and MCF7 cells were transfected with FAM-labeled siRNA at 30 nM with 1:2, 1:4, and 1:8 siRNA:1.2PEI-LA6 ratios. The uptake study by flow cytometry was also performed in normal cells, MCF10A, HUVEC, and hBMSC as described above to determine how effectively siRNA was delivered by the polymer to normal cells.

Targeting TTK with various siRNAs

TTK siRNAs targeting different locations of the gene were used to determine their efficacy by MTT assay in MDA-MB-231 and MCF7 cells using 20, 40, and 60 nM siRNA concentrations with 1:4 siRNA:polymer ratio. After 72 h of siRNA transfections, MTT assay was performed as described above to measure the inhibition of MDA-MB-231 and MCF7 cells growth by various TTK siRNAs.

Reverse transcription-quantitative PCR (RT-qPCR)

The MDA-MB-231 and MCF7 cells were transfected by TTK-1 and CDC20 siRNAs at 60 nM with 1:4 siRNA:1.2PEI-LA6 ratio. Total RNA was isolated from cells after 24 and 48 h of treatment using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was converted into cDNA using M-MLV reverse transcriptase (Invitrogen) according to manufacturer's instruction. The quantitative PCR (qPCR) was performed by taking 15 ng of cDNA from each sample using SYBR Green qPCR Mastermix (Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta, Edmonton, AB) and StepOne Real-Time PCR System

(Applied Biosystems, Foster City, CA) based on the recommendations of manufacturer. Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) in such a way that at least one primer (forward or reverse) spans the exon–exon junction of a gene so that the amplification of contaminating genomic DNA or heteronuclear RNA by PCR can be avoided. GAPDH was used as a reference gene in RT-qPCR and template cDNA was omitted from qPCR reaction as a negative control. The qPCR results were analyzed using $2^{-\Delta\Delta C_T}$ method and presented as relative quantity of transcripts. The qPCR conditions comprised an initial denaturation step for 10 min at 95.0°C, followed by 40 cycles at 95.0°C for 15 s (denaturation), and annealing and elongation at 60°C for 1 min.

Combinational siRNA therapy

Combinational siRNA delivery was performed in MDA-MB-231 and MCF7 using TTK-1, CDC20 and survivin siRNAs at 30 nM (15 nM each) total siRNA concentration with 1:2, 1:4, and 1:8 siRNA:1.2PEI-LA6 ratio. After 72 h of siRNA transfection, MTT assay was performed as described above to determine the efficacy of combinational siRNA therapy. Induction of apoptosis in MDA-MD-231 cells by combinational siRNA therapy was determined by caspase activity assay. To investigate caspase activity, total protein from MDA-MB-231 cells was isolated by applying freeze–thaw cycle three times after 72 h of combinational siRNA transfection. Cell debris was removed from isolated protein by centrifugation at 10,000g for 5 min. Total protein was incubated for 2 h with the fluorogenic substrate (Ac-DEVD-AFC; Cat. No. ALX-260-032, Enzo Life Sciences, Farmingdale, NY) at 37°C and the fluorescence of the substrate was measured at 390/510 nm excitation/emission. The fluorescence was normalized per μg of protein after determining the concentration of total protein for each sample by Pierce™ BCA Protein Assay Kit (Cat. No. 23227, Thermo Fisher Scientific) according to manufacturer's instructions. The caspase activity was presented as a percentage of activity based on nontreated cells (taken as 100% activity).

The validity of combinational siRNA therapy at mRNA transcript levels was determined by RT-qPCR. MDA-MB-231 and MCF7 cells were transfected with 30 and 60 nM total combinational siRNA concentration at 1:4 siRNA:1.2PEI-LA6 ratio. After 24 h of transfection, RNA was isolated and converted into cDNA as described above, which was used to perform qPCR.

siRNA delivery to nonmalignant cells

Combinational siRNAs against TTK-1, CDC20 and survivin were also delivered to normal cells, MCF10A, HUVEC and hBMSC to determine the side effects of siRNA therapy. MTT assay was performed to determine the inhibition of normal cell growth by siRNA transfection at 30 nM (15 nM each) total siRNA concentration with 1:2, 1:4, and 1:8 siRNA:1.2-PEI-LA6 ratio. Specific cell growth inhibition by combinational siRNA therapy compared to scrambled siRNA in all cell lines was calculated by removing cell growth inhibition

of scrambled siRNA from specific siRNA treated cells and the values were presented as a heat map.

Statistical analysis

All results were presented as mean + standard deviation. Results were analyzed by unpaired Student's t test, where an asterisk (*) indicated significantly different groups in figures. The significance ($p < 0.05$) was typically determined by comparing specific siRNA-treated groups to that of scrambled siRNA-treated groups.

RESULTS

Screening for effective carriers

Several LA-substituted PEIs were used to screen for the best polymeric carrier by determining inhibition of MDA-MB-231 cell growth with the TTK-1 and CDC20 siRNAs (Fig. 1). The synthesized polymers were designated based on the molecular weight of PEI (0.6, 1.2, and 2.0 kDa) backbone used for modification, followed by the substituted lipid moiety and the feed ratio of lipid/PEI during the synthesis of the polymers (see Supporting Information Table I for more detailed info). Lipofectamine® 2000, a widely used commercial carrier, was employed as a reference carrier that was unable to inhibit cell growth with TTK-1 siRNA while it inhibited cell growth ∼50% with the CDC20 siRNA. Many synthesized polymeric carriers were not effective to inhibit cell growth with TTK-1 siRNA, except 1.2PEI-LA6 that inhibited cell growth significantly [Fig. 1(A)]. With CDC20 siRNA delivery, 1.2PEI-LA6, 2.0PEI-LA6, and 2.0PEI-LA9 polymers were able to decrease cell growth significantly [Fig. 1(B)]. The 1.2PEI-LA6 was the most effective polymer as it inhibited ∼80% of cell growth with CDC20 siRNA and, since it was also effective with TTK-1 siRNA, it was used to carry out the rest of the studies. The 1.2PEI-LA8 was quite toxic in MDA-MB-231 cells as the growth of scrambled siRNA treated cells was inhibited drastically.

The siRNA/polymer complexes were characterized based on different degree of lipid-substitutions in 1.2 kDa PEI by measuring size and surface charge [Fig. 2(A)]. The size of complexes with Lipofectamine® 2000 was ∼100 nm, while it was less with native 1.2 kDa PEI without any modification (∼70 nm). Substitution of PEI with linoleic acid (1.2PEI-LA0.5) increased the complex size (∼127 nm), and complex size gradually decreased as the LA substitution in PEI was increased from 1.2PEI-LA0.5 to 1.2PEI-LA8 (∼70 nm). On the other hand, ζ-potential of complexes with Lipofectamine® 2000 was −21 mV, and initial (minimal) substitution of LA in PEI decreased surface charge from ∼17 mV (1.2PEI-LA0) to 10 mV (1.2PEI-LA0.5). The ζ-potential of complexes gradually increased as the substitution of LA has increased in PEI from 1.2PEI-LA0.5 (∼10 mV) to 1.2PEI-LA6 (28.8 mV), after which 1.2PEI-LA8 showed a significant drop in the surface charge (∼15 mV).

The uptake of siRNA/polymer complexes was determined for different LA substituted PEIs [Fig. 2(B)]. Lipofectamine® 2000 successfully delivered siRNA to MDA-MB-231 cells and transfected ∼80% cell population. As expected, nonmodified 1.2 kDa PEI was not able to deliver
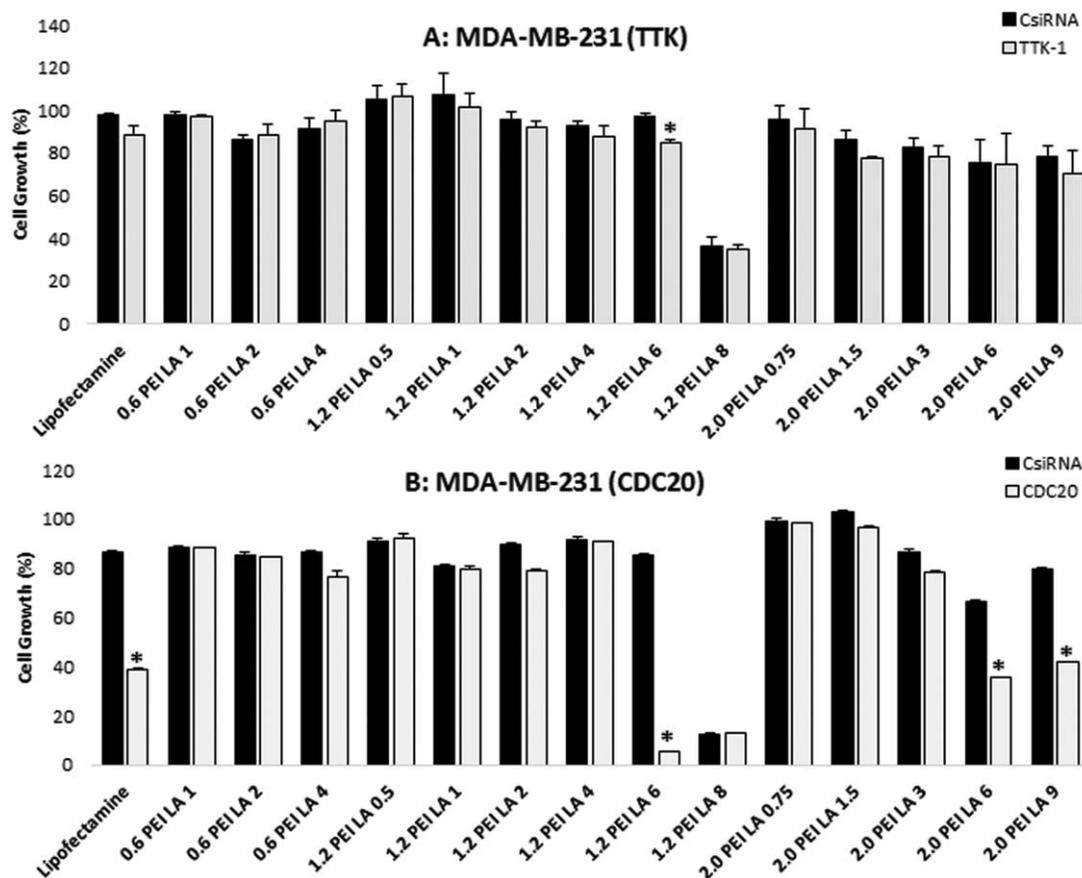
FIGURE 1. Screening of polymeric carriers using lipid-substituted PEIs in MDA-MB-231 cells. Modified PEIs were synthesized using linoleic acid (LA) with different feed ratios. The synthesized polymers were designated based on the molecular weight of PEI (0.6, 1.2, and 2.0 kDa) backbone used for modification, followed by the substituted lipid moiety and the feed ratio of lipid/PEI during the synthesis of polymers. The inhibition of cell growth by 40 nM siRNA against TTK-1 (**A**) and CDC20 (**B**) at 1:6 siRNA:polymer ratio was assessed with MTT assay. Lipofectamine® 2000 (Thermo Fisher Scientific) was used as a positive control with 1:1 siRNA:lipofectamine ratio. Scrambled siRNA (CsiRNA) was used as a negative control. The results were presented by taking nontreated cells as 100% cell growth. Asterisks represent the significant cell growth inhibition by TTK-1 or CDC20 siRNA compared to CsiRNA ($p < 0.05$).

siRNA at all to MDA-MB-231 cells. Similarly, siRNA uptake was not observed when it was delivered with 1.2PEI-LA0.5, 1.2PEI-LA2, and 1.2PEI-LA4. Polymers 1.2PEI-LA1 and 1.2PEI-LA8 delivered siRNA at some extent as higher mean fluorescence compared to 1.2PEI-LA0.5, 1.2PEI-LA2, and 1.2PEI-LA4 was observed in these cells. However, the transfection efficiency was lower as only 53 and 38% of cells were transfected with 1.2PEI-LA1 and 1.2PEI-LA8, respectively. The 1.2PEI-LA6 was the most effective polymer as the highest mean fluorescence was detected and 97% cell population were transfected with this polymer.

Delivery of TTK siRNAs to breast cancer cells
The efficacy of various TTK siRNAs targeting different locations of the mRNA was determined by inhibition of MDA-MB-231 and MCF7 cell growth (Fig. 3). TTK-1, TTK-2, TTK-3, and TTK-4 siRNAs at 20 nM decreased the MDA-MB-231 cell growth by 10–40%, while 40 nM of siRNA inhibited cell growth by 70–90%. Similarly, 60 nM of TTK siRNAs decreased MDA-MB-231 cell growth significantly compared to scrambled siRNA. CDC20 siRNA was able to inhibit the

MDA-MB-231 cell growth drastically at all the concentrations used. Furthermore, MCF7 cells were not responsive to TTK-1, TTK-2, TTK-3, TTK-4, and CDC20 siRNAs at all concentrations.

The TTK-5 and TTK-6 siRNAs decreased the MDA-MB-231 cell growth ~90% at 40 nM siRNA, while no significant difference between TTK-5 and TTK-6 siRNA treated cells and scrambled siRNA treated cells was found at 60 nM siRNA (Fig. 3). TTK-5 siRNA decreased MCF7 cell growth 20–40% at 40 and 60 nM, while TTK-6 siRNA was not able to inhibit the cell growth significantly compared to scrambled siRNA.

To determine the functionality of TTK-1 and CDC20 siRNAs at the transcripts level, RT-qPCR was performed for MDA-MB-231 and MCF7 cells. Significant reduction in the levels of TTK and CDC20 mRNA transcripts was found using TTK-1 and CDC20 siRNAs after 24 and 48 h of siRNA transfection (Fig. 4). RT-qPCR confirmed that the siRNAs against TTK and CDC20 proteins were effective in obtaining the desired silencing and the polymeric carrier was able to deliver siRNAs successfully into the cells.
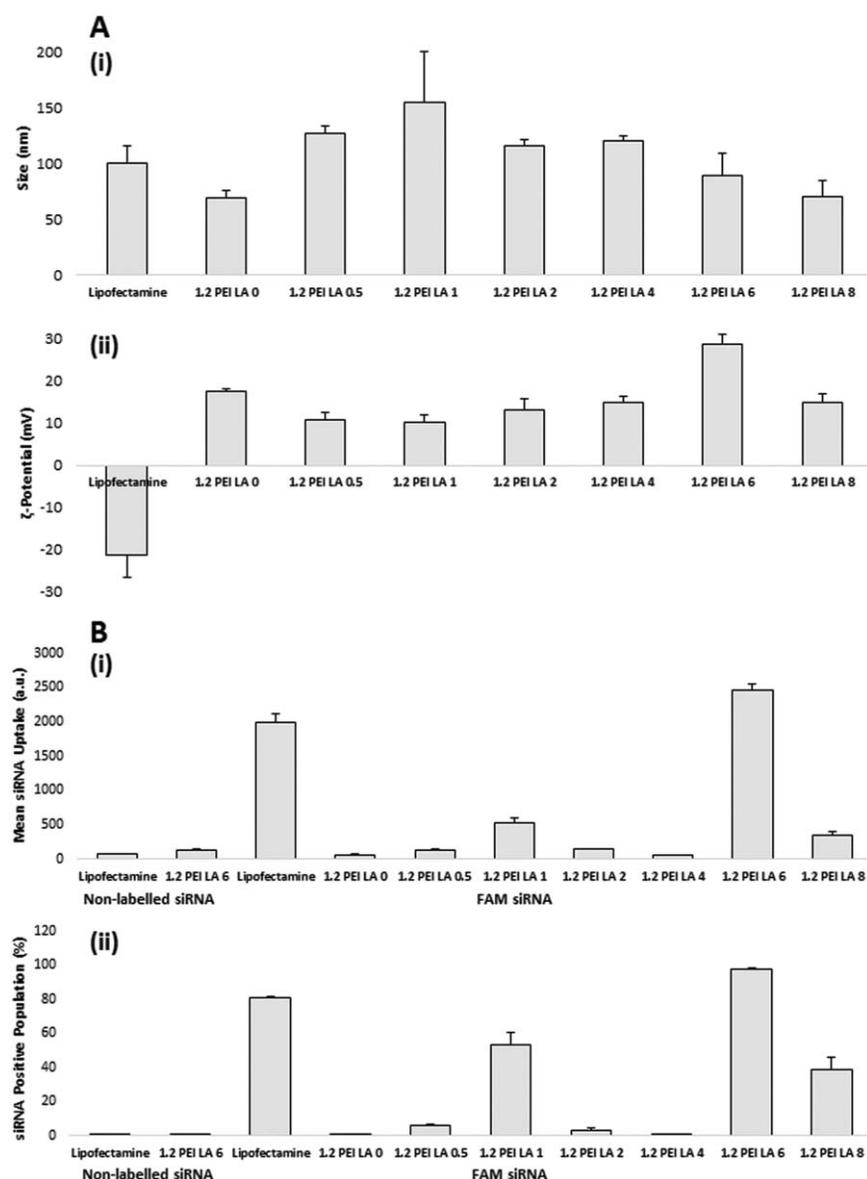
FIGURE 2. Physicochemical characterization and cellular uptake of siRNA/polymer complexes. (A) Size (i) and surface charge (ii; ζ-potential) of siRNA/polymer complexes were determined using polymers with different degree of lipid-substitutions in 1.2 kDa PEI. The complexes were prepared with 0.6 μg of scrambled siRNA at 1:6 and 1:1 siRNA:polymer and siRNA:Lipofectamine ratios, respectively. (B) FAM-labeled scrambled siRNA was used to determine cellular uptake of siRNA/polymer complexes by flow cytometry at 40 n*M* siRNA with 1:6 and 1:1 siRNA:polymer and siRNA:lipofectamine ratios, respectively. Nonlabeled scrambled siRNA was used as a control to investigate autofluorescence of complexes. The mean fluorescence (i) and FAM-positive cell population (ii) was determined for all siRNA carriers.

Combinational siRNA delivery against TTK, CDC20, and survivin

Combinational siRNA therapy was performed in MDA-MB-231 and MCF7 cells to determine if a synergistic effect can be achieved with carefully selected combination of siRNAs. A relatively low concentration of siRNA (total 30 n*M*; 15 n*M* siRNA each) was chosen for this purpose. The siRNA delivery against single targets at these concentrations (15 n*M* specific siRNA + 15 n*M* scrambled siRNA) served as a reference treatment. The siRNA against TTK (using TTK-1 siRNA; referred as TTK siRNA hereafter) and CDC20 alone was not effective in MDA-MB-231 as no inhibition of cell growth was found due to very low concentration of siRNA. However,

survivin siRNA alone decreased cell growth by ∼25% at 1:4 siRNA:polymer ratio. The synergistic effect of dual therapy was detected with TTK-CDC20 and CDC20-survivin siRNA combination in MDA-MB-231 cells at 1:4 ratio as the cell growth was inhibited by ∼30 and ∼45%, respectively compared to scrambled siRNA as well as individual targeted siRNAs [Fig. 5(A)]. The synergism was not detected for dual therapy at 1:2 and 1:8 siRNA:polymer ratios. Caspase activity assay was performed using MDA-MB-231 cells to investigate the initiation of apoptosis in the cell by combinational siRNA therapy. No increase in the caspase activity was found with TTK siRNA alone, while CDC20 and survivin siRNAs showed drastic increase in the caspase activity
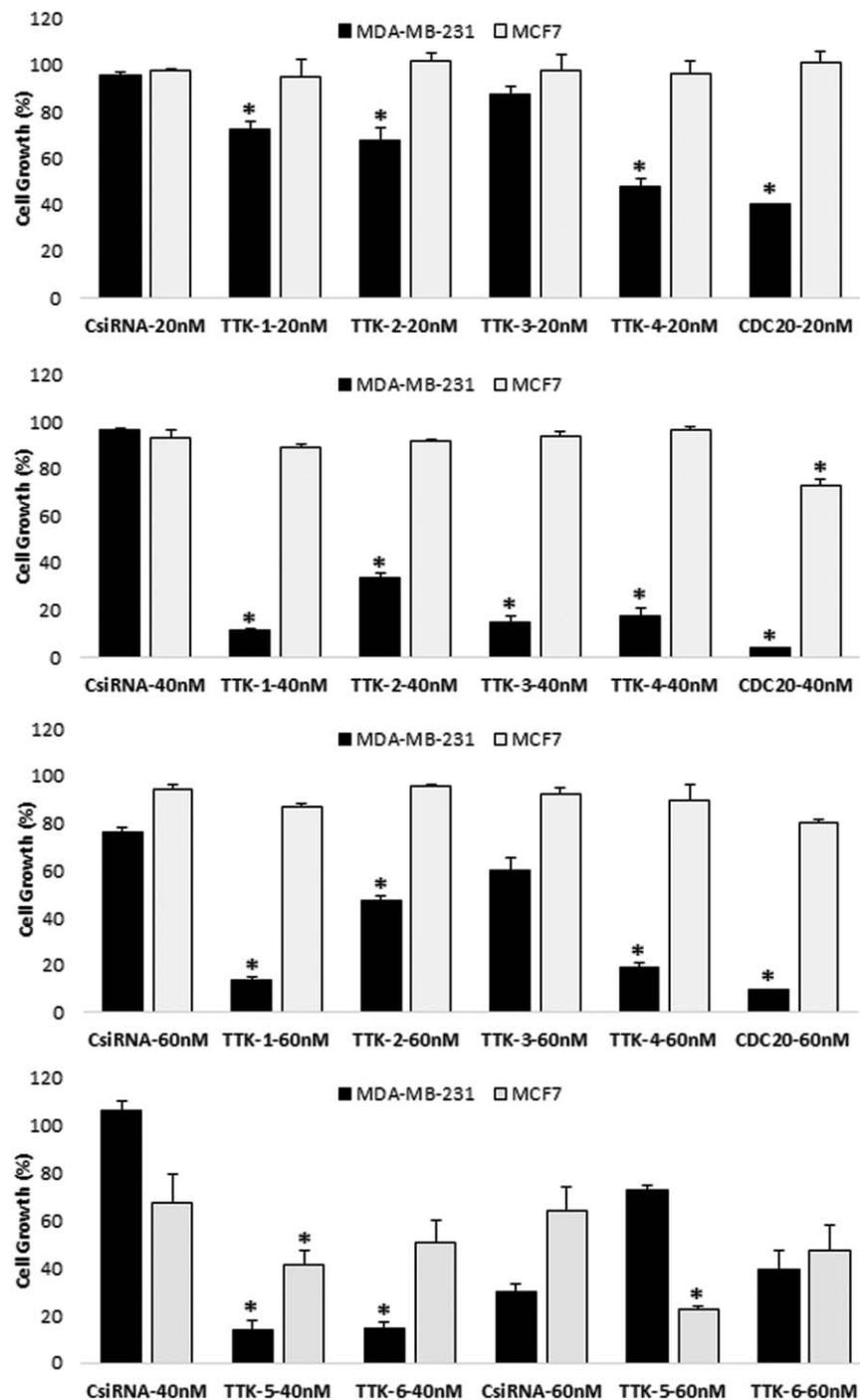
FIGURE 3. Targeting TTK with various siRNAs in MDA-MB-231 and MCF7 cells. TTK-1, TTK-2, TTK-3, and TTK-4 siRNAs were delivered with 20 (**A**), 40 (**B**), and 60 nM (**C**) siRNA, while TTK-5 and TTK-6 siRNAs (**D**) were delivered with 40 and 60 nM siRNA at 1:4 siRNA:1.2PEI-LA6 ratios. The inhibition of cell growth was assessed by MTT assay and scrambled siRNA (CsiRNA) was used as a negative control. Asterisks represent the significant cell growth inhibition by specific siRNA compared to CsiRNA ($p < 0.05$).

(Supporting Information Fig. 1). Similarly, all siRNA combinations confirmed the initiation of apoptosis in MDA-MB-231 cells as caspase activity was found significantly higher.

No significant decrease in MCF7 cell growth was found by individual siRNA against TTK, CDC20 and survivin at 30 nM total concentration. TTK-CDC20 combination has shown synergistic effect as ~25% of cell growth was inhibited by

these siRNAs at 1:4 ratio in MCF7 cells compared to scrambled siRNA as well as individual TTK and CDC20 siRNAs [Fig. 5(B)].

The efficacy of the combinational siRNA therapy was also determined by RT-qPCR in MDA-MB-231 and MCF7 cells at 30 and 60 nM total siRNAs (Fig. 6). The amount of mRNA transcripts for each targeted protein was significantly
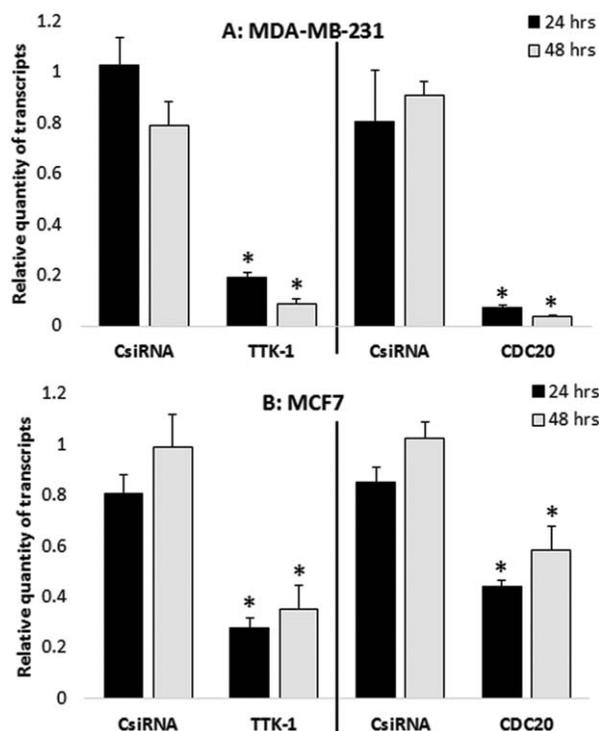
FIGURE 4. RT-qPCR for MDA-MB-231 (**A**) and MCF7 (**B**) cells treated with TTK-1 and CDC20 siRNAs. TTK-1 and CDC20 were targeted with 60 nM siRNA at 1:4 siRNA:1.2PEI-LA6 ratio. RT-qPCR was performed after 24 and 48 h of transfection. Scrambled siRNA (CsiRNA) was used as a negative control. Asterisks represent the significant reduction in the quantity of transcripts compared to CsiRNA ($p < 0.05$).

decreased after individual and dual siRNA delivery, which confirmed that TTK, CDC20, and survivin siRNAs were able to silence its target upon successfully delivered by polymeric carrier.

Cellular uptake of siRNA in breast cancer and nonmalignant cells

To investigate how effectively 1.2PEI-LA6 delivers siRNA into the cells, the uptake of FAM-labeled siRNA was performed in breast cancer MDA-MB-231 and MCF7 cells and normal cells, MCF10A, HUVEC, hBMSC (Fig. 7). No autofluorescence of the complexes was detected as the mean fluorescence and FAM-positive population of nontreated and nonlabeled scrambled siRNA treated cells were similar (data not shown). Based on the mean fluorescence of MDA-MB-231 cells, the cellular uptake of siRNA increased with higher siRNA:polymer ratio (1:4 and 1:8) compared to lower ratio (1:2). However, no major difference was found in MDA-MB-231 cellular uptake between 1:4 and 1:8 ratio. Similarly, an equivalent uptake of siRNA was found at all ratios in MCF7 cells, which was quite low compared to MDA-MB-231 cells.

The cellular uptake of siRNA at 1:2 and 1:4 siRNA:polymer ratios was similar in normal breast MCF10A cells, while the uptake was significantly increased at 1:8 ratio (Fig. 7). No difference was evident among all siRNA:polymer ratios in the cellular uptake of HUVEC and hBMSC cells. Considering the cellular uptake by breast cancer and normal

cells, 1:4 siRNA:polymer ratio could be better formulation of siRNA and 1.2PEI-LA6 due to quite low siRNA delivery efficiency of polymer at this ratio in normal cells compared to MDA-MB-231 cells.

Targeting TTK, CDC20, and survivin in nonmalignant cells

The TTK, CDC20 and survivin siRNAs were delivered to normal cells to determine possible effects of siRNA therapy on nonmalignant cells. Normal breast cells MCF10A were quite responsive to siRNAs at 1:2 and 1:4 ratios as 20–40% of cell growth was inhibited with individual or combinational siRNAs [Fig. 8(A)]. However, 1:8 ratio of siRNA was not at all effective to MCF10A cells. No inhibition of HUVEC cell growth was found with complexes formed at higher ratios (1:4 and 1:8). However, complexes at 1:2 ratio inhibited HUVEC cell growth by 20–50% with individual/combined CDC20 and survivin siRNAs [Fig. 8(B)]. The hBMSC cell growth was significantly inhibited by the CDC20 siRNA at 1:2 and 1:4 ratios whether it was delivered individually or in combination [Fig. 8(C)]. However, 1:8 ratio was unable to decrease the cell growth by all siRNAs. Specific cell growth inhibition by combinational siRNA therapy is presented for breast cancer and normal cells in Figure 8(D). Overall, complexes formulated at higher siRNA:polymer ratio showed the least siRNA effect on nonmalignant cells and, therefore, the ratio of siRNA:polymer will need to be chosen carefully to decrease the side effects of siRNAs on normal cells.

DISCUSSION

The RNAi has become a viable approach in the last decade to develop a targeted cancer therapy, where a specific protein can be silenced to achieve a reduction in tumor growth.[7,8] The siRNA therapy has its limitations, and one of them is the development of a nontoxic carrier that can successfully deliver siRNA into the cell. We evaluated a library of polymeric carriers based on low molecular weight PEIs (0.6, 1.2, and 2.0 kDa) to deliver siRNA as high molecular weight branched (>25 kDa) and linear (>750 kDa) PEIs possess unacceptable cytotoxicity.[38] PEI can escape the endosomal compartment due to its inherent 'proton-sponge' effect so that siRNA can be effectively delivered into cytoplasm after the cellular uptake of siRNA/polymer complexes.[38] The cellular uptake of siRNA using low molecular weight PEIs is quite low[34,35] and no cellular uptake of siRNA was observed with nonmodified 1.2 kDa PEI [Fig. 2(B)]. Therefore, we substituted the amines of PEIs with lipidic moieties to improve the delivery of siRNA. The lipidic moieties presumably improves chemical compatibility of siRNA/polymer complexes with cell membrane so that siRNA uptake can be increased significantly.[14] In addition, the degree of substitutions in PEI plays a major role for uptake of complexes. No siRNA uptake was observed with low LA substitutions [Fig. 2(B)], and high LA substituted PEI showed higher toxicity (Fig. 1). The relationship between the uptake and LA substitution was not monotonous, since a critical degree of substitution (>2 LA/1.2 PEI)
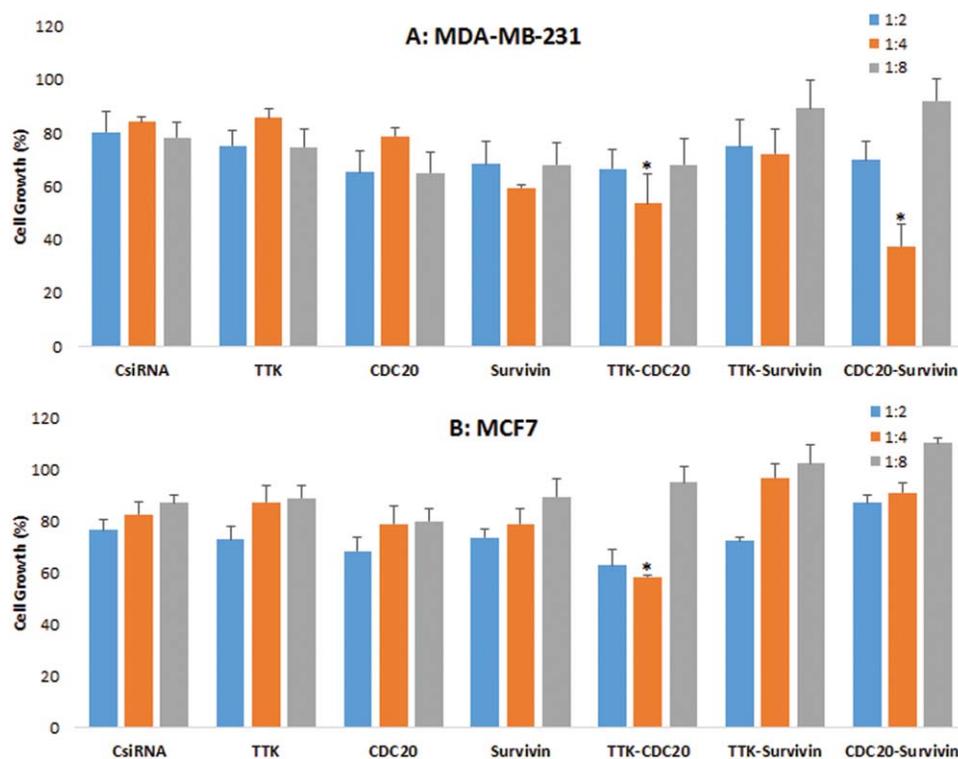
FIGURE 5. Combinational siRNA delivery against TTK-1 (referred as TTK), CDC20 and survivin in MDA-MB-231 (**A**) and MCF7 (**B**) cells. Combination was performed with total 30 nM (15 + 15 nM each) siRNA at 1:2, 1:4, and 1:8 siRNA:1.2PEI-LA6 ratio. Scrambled siRNA (CsiRNA) was used as a negative control. Asterisks represent the significant cell growth inhibition with combinational siRNA therapy compared to CsiRNA and specific siRNA alone ($p < 0.05$).

was required to enable uptake of siRNA complexes. An optimal LA substitution in PEI is necessary to have higher uptake and less cytotoxicity, and 1.2PEI-LA6 seemed to have both characteristics based on cellular uptake and inhibition of cell growth. Moreover, physicochemical characteristics of the complexes play an important role in the uptake of siRNA as well. Hydrodynamic size of complexes with 1.2PEI-LA6 has decreased significantly [Fig. 2(A)], while ζ-potential has dramatically increased compared to other LA substituted polymers, which could have contributed to better uptake of siRNA with 1.2PEI-LA6 compared to other polymers. Among many synthesized polymer, 1.2PEI-LA6 was the most effective polymer that inhibited cell growth drastically when it was used to deliver CDC20 siRNA (Fig. 1), which was consistent with uptake results (Fig. 2). Several other polymers inhibited MDA-MB-231 cell growth as well with CDC20 siRNA. However, only 1.2PEI-LA6 showed significant decrease in the cell growth with TTK siRNA. The efficacy of growth inhibition by TTK delivery, however, was quite low compared to CDC20 siRNA, which suggests that CDC20 may be more potent target for silencing compared to TTK. This is not surprising since CDC20 was previously identified from a siRNA library screen, where we selected the most efficacious siRNA from a library of 169 siRNAs.[39] The polymers derived from the 0.6 kDa PEI were generally not effective, presumably due to smaller size and relatively less efficient binding to siRNA, and higher LA substitutions on

1.2 and 2.0 kDa PEIs generally led to more effective growth inhibition.

We targeted TTK protein in this study with various siRNAs since the up-regulation of this gene in many cancer types was reported leading to uncontrolled proliferation of those cancer cells.[21,22] As TTK is associated with cell proliferation, it has been silenced in pancreatic, prostate and liver cancers by siRNA using commercially available carriers to decrease cell proliferation.[22,40–42] In this study, we showed that TTK is also a viable target in breast cancer and delivering the TTK siRNA using polymeric carrier would lead to decrease in the breast cancer cell growth (Fig. 3). The results were confirmed at transcripts level as well by RT-qPCR (Fig. 4). We used six different siRNAs against TTK to determine the efficacy of each siRNA. The outcome was expected to depend on how thermodynamically asymmetric siRNA is and where siRNA is targeting in the gene.[43] Based on the thermodynamic asymmetry of siRNA, the components of RISC bind to siRNA and the selection of guide strand is made.[44] The various siRNAs for the same gene, therefore, should have different efficiency of silencing its target. TTK-5 and TTK-6 siRNAs that were supplied by IDT were 27 base pair dicer–substrate siRNA, which has a longer nucleotide sequence compared to regular 21 base pair siRNAs. Dicer–substrate siRNA interacts with the dicer enzyme before its incorporation into the RISC assembly, which may lead to increased potency by engaging to natural siRNA processing pathway.[45] However, no difference in the
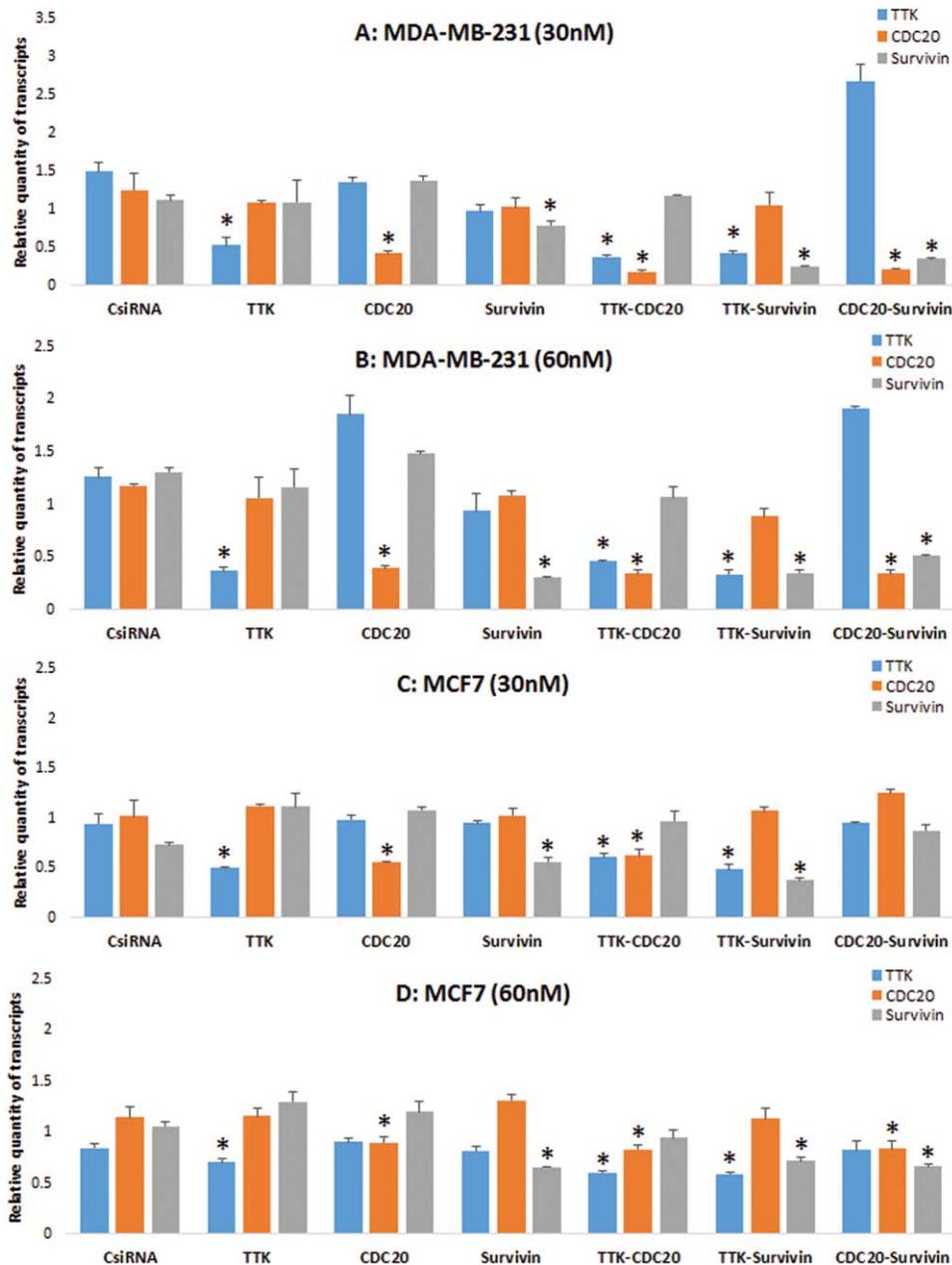
FIGURE 6. RT-qPCR for MDA-MB-231 and MCF7 cells treated with combinational siRNAs. Cells were transfected with total 30 nM (15 + 15 nM each) and 60 nM (30 + 30 nM each) siRNA at 1:4 siRNA:1.2PEI-LA6 ratio. RT-qPCR was performed after 24 h of transfection. Scrambled siRNA (CsiRNA) was used as a negative control. Asterisks represent the significant reduction in the quantity of transcripts compared to CsiRNA ($p < 0.05$).

potency of 21 and 27 base pairs siRNAs was observed for TTK siRNAs, and inhibition of MDA-MB-231 cell growth was found at some extent with all six siRNAs (Fig. 3). Previously, we have observed high efficacy with dicer–substrate siRNA against CDC20 compared to regular 21 base pair siRNA,[39] but it seems that the relative efficiency might depend on the target and/or exact sequence (siRNA binding site in a gene) of the siRNA. MCF7 cells were not as responsive as MDA-MB-231 to TTK siRNAs despite displaying effective silencing (Fig. 4), which suggests that MCF7 cells may not critically rely on

TTK protein for its survival and may have found an alternative protein to undertake cell division.

CDC20 has a key role during mitosis to activate APC that leads cells from metaphase to anaphase. We have validated the efficacy of various siRNAs against CDC20 previously in two breast cancer models (MDA-MB-435 and MDA-MB-231 cells).[39] CDC20 silencing showed significant cell death at 40 nM of siRNA in MDA-MB-231 cells [Fig. 1(B)], while it displayed poor efficacy at 15 nM of siRNA in both MDA-MB-231 and MCF7 cells (Fig. 5). Survivin siRNAs
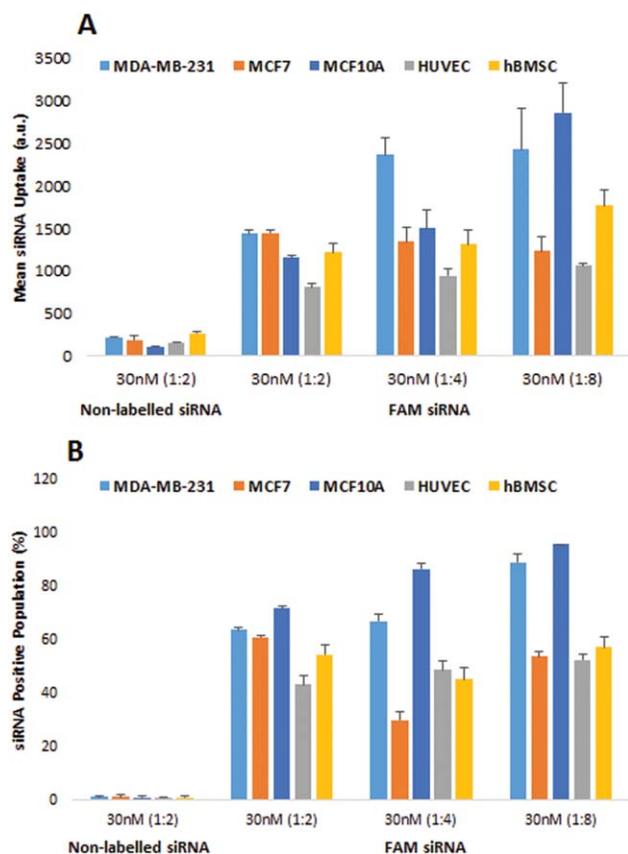
FIGURE 7. Cellular uptake of siRNA by flow cytometry. FAM-labeled scrambled siRNA was used to determine cellular uptake of siRNA/polymer complexes at 30 nM siRNA with 1:2, 1:4, and 1:8 siRNA:polymer ratios. Nonlabeled scrambled siRNA was used as a control to investigate autofluorescence of complexes at 30 nM siRNA with 1:2 ratio. The mean fluorescence **(A)** and FAM-positive cell population **(B)** was determined for breast cancer and normal cells.

employed here was dicer–substrate siRNAs and has shown increased potency in MDA-MB-231 cells at 15 nM concentration [Fig. 5(A)]. Since survivin has been shown to be a critical target for cancer therapy as well, the siRNA against survivin was applied to breast cancer cells and the efficacy of targeting survivin was validated previously.[46]

Silencing the expression of two essential proteins simultaneously is a promising strategy as the chances of survival for malignant cells could drastically be decreased. Not only the synergistic effect of the combinational therapy can be achieved, but also the doses of siRNAs can be lowered to have less side effects on nonmalignant cells. We have targeted the cell cycle proteins, TTK and CDC20 with siRNAs and observed a synergism in the therapy at low doses of siRNAs. RT-qPCR confirmed the functionality of combinational TTK-CDC20 siRNA therapy at transcripts level (Fig. 6) so that we do not seem to be overloading the RISC-mediated silencing machinery with dual siRNA delivery. Elevation in TTK transcripts was consistently observed in MDA-MB-231 cells upon silencing CDC20 individually or with combination, which suggests that cells may have upregulated TTK expression upon detecting loss of CDC20 protein. Upon depletion of cell cycle

proteins during cell proliferation, cell cycle is arrested until the error in the cell cycle is corrected.[47] If the essential proteins for cell cycles are missing, especially TTK and CDC20 that participate in mitosis, the error cannot be corrected and pathways for cell death is expected to be initiated. The mitotic catastrophe[48] or initiation of apoptotic pathway by caspase activation (cell death mechanisms) may have played role upon depletion of TTK and CDC20 in breast cancer cells as the cell death (Fig. 5) and the elevation in caspase activity (Supporting Information Fig. 1) were evident with combinational siRNA therapy against these cell cycle proteins. Several lines of evidence suggest that cell cycle and anti-apoptosis proteins have conjunctional roles during cell division.[49,50] Moreover, arrested cell cycle due to inhibition of a specific cell cycle protein could increase the production of anti-apoptosis protein to block the apoptotic pathway that may have been initiated in order to overcome cell cycle arrest. Therefore, targeting anti-apoptosis protein such as survivin with TTK and CDC20 seems appropriate to have a synergism in combinational siRNA delivery. The synergistic effect of the combinational CDC20/survivin siRNA therapy was evident in MDA-MB-231 cells [Fig. 5(A)].

The siRNA:polymer ratio plays a major role for the efficacy of siRNA treatment. The synergistic effect of dual siRNA therapy was not observed at lower 1:2 siRNA:polymer ratio in MDA-MB-231 cells [Fig. 5(A)] due to poor uptake of complexes (Fig. 7). On the other hand, the synergistic effect was only observed at 1:4 siRNA:polymer ratio even though the uptake of siRNA was similar at 1:4 and 1:8 siRNA:polymer ratios. A possible reason for ineffective siRNA treatment at 1:8 ratio could be the dissociation of complexes in cytoplasm; siRNA may have dissociated poorly from polymer at the higher 1:8 ratio because of strong binding between anionic siRNA and cationic polymer, resulting in poor efficacy of siRNA (Supporting Information Fig. 2). In addition, the size and ζ-potential of complexes at different siRNA:polymer ratios may have contributed for the efficacy of siRNA treatment as well. The size of siRNA/1.2PEI-LA6 complexes decreased from ~130 to ~63 nm as the polymer amount was increased from 1:2 to 1:8 ratios (Supporting Information Fig. 3). Similarly, the ζ-potential has increased as the siRNA:polymer ratio increased. No significant difference was observed in size and ζ-potential of complexes at 1:4 and 1:8 ratios, which may support the uptake of siRNA results as similar uptake was found at both of these ratios in MDA-MB-231 cells.

We have also determined how much damage the siRNAs are causing to normal cells since siRNA/polymer complexes will not be restricted to transfect only tumor cells when administered *in vivo*. As the results indicate, normal breast cells (MCF10A) seemed to be more sensitive to these complexes compared to endothelial (HUVEC) and bone marrow stromal cells (hBMSC; Fig. 8). Moreover, the higher siRNA:polymer ratio used to prepare complexes was not effective to decrease cell growth of normal cells compared to lower ratio that inhibited the cell growth drastically in normal cells, which clearly showed how important it is to determine the specific formulation ratio carefully for siRNA
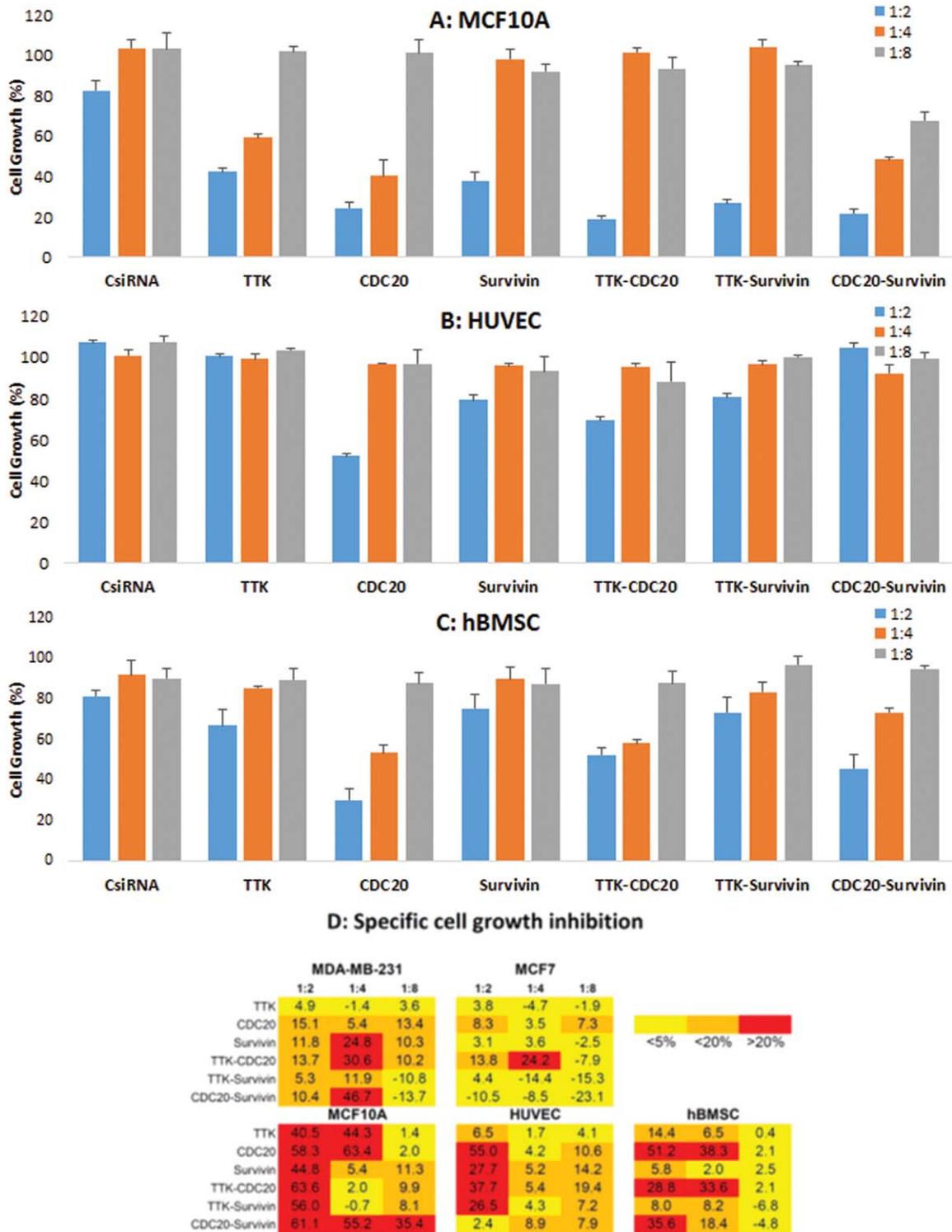
FIGURE 8. Combinational siRNA delivery against TTK, CDC20, and survivin in nonmalignant cells. Combination was performed with total 30 n*M* (15 + 15 n*M* each) siRNA at 1:2, 1:4, and 1:8 siRNA:1.2PEI-LA6 ratio in MCF10A **(A)**, HUVEC **(B)**, and hBMSC **(C)** cells. Scrambled siRNA (CsiRNA) was used as a negative control. A heat-map **(D)** represents specific cell growth inhibition by combinational siRNA therapy compared to CsiRNA in breast cancer and normal cells.

therapy. The employed polymer (1.2PEI-LA6) did not have any cell-targeted moieties so that it is not surprising to observe its functionality in all of these adherent cells. Since no growth inhibition was found for the scrambled siRNA treated normal cells with all ratios, it was obvious that the polymeric delivery system was not toxic and the observed

effect was due to specific siRNAs delivered. Furthermore, the cellular uptake of siRNA was found to be similar at all ratios in normal cells (Fig. 7), which suggests that the polymer had successfully delivered siRNA into the cells at all ratios. However, the dissociation of polymer from siRNA in cytoplasm may have been higher at lower 1:2 siRNA:polymer ratio so that the effect of siRNA was evident in normal cells, and siRNA may have dissociated poorly from polymer at higher 1:8 ratio, resulting in poor efficacy of siRNA (Supporting Information Fig. 2). The 1:4 siRNA:polymer ratio showed synergistic effect with TTK and CDC20 siRNA in breast cancer cells (Fig. 5), and the same ratio was not effective in normal cells except hBMSC that showed ∼30% cell death (Fig. 8). Therefore, 1:4 ratio at total 30 n*M* siRNA could be an effective dose to target TTK and CDC20 in breast cancer cells. The siRNA:polymer ratio could play a critical role under *in vivo* conditions at preclinical models, but this issue remains to be addressed at this stage; such an optimization with siRNA:polymer ratio might be necessary with the chosen molecular targets *in vivo*.

The synthesized polymer showed different efficacy in siRNA delivery to breast cancer and normal cells. Comparing the two breast cancer cells, we noted lower uptake of siRNA by MCF7 cells compared to MDA-MB-231 that may explain the lower silencing efficiencies (that is, mRNA reduction based on RT-qPCR) and reduced response (that is, cell growth inhibition) to specific siRNA delivery in MCF7 cells. The siRNA uptake was similar in HUVEC and hBMSC, suggesting no particular selectivity of polymer toward these normal cells. MCF10A, however, showed higher uptake of siRNA and were more responsive, leading to the selectivity of employed polymer to transfect breast cells efficiently compared to other cell types. We have observed in the past that certain polymers can only transfect particular cell lines, for example, the polymer used to deliver siRNA to breast cancer cells has displayed poor efficacy in leukemia cells.[51] This is expected to some extent since adherent cells probably possess different membrane structure than the nonadherent leukemic cells. For more effective clinical utility, it might be necessary to make the current polymers without a targeting moiety selective toward a particular cell type. Perhaps, coating of complexes with polyethylene glycol[52] or hyaluronic acid[53] could make the complexes selective for cancer cells due to reduced uptake by initially encountered normal cells. The selectivity may arise from the choice of siRNA as well i.e., selection of specific oncotargets, but with the three specific targets explored here, the selectivity was not universal and could only be partially achieved by fine-tuning of siRNA:polymer ratio used in formulating the nucleic acid complexes. Tailored polymers that can exclusively transfect malignant cells remain to be designed, synthesized and tested.

CONCLUSIONS
The proteins TTK, CDC20, and survivin could be promising targets for siRNA therapy to decrease the proliferation of triple-negative breast cancer cells, such as MDA-MB-231

cells employed here. This study has indicated improved therapeutic efficacy of siRNAs by employing combinational siRNAs against cell cycle and anti-apoptosis proteins in breast cancer cells. The lipid-substituted polymers could serve as a viable platform for delivery of multiple siRNAs against critical targets, and co-delivery of siRNAs did not impair the desired silencing efficiency observed with individual siRNAs. However, the siRNA therapy with the chosen targets showed significant effects on nonmalignant cells *in vitro*. While the side-effects could be minimized with the optimization of siRNA:polymer ratio, more selective therapies (either engineered polymers or siRNA against therapeutic targets) might be needed to target cancer cells solely.

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