molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

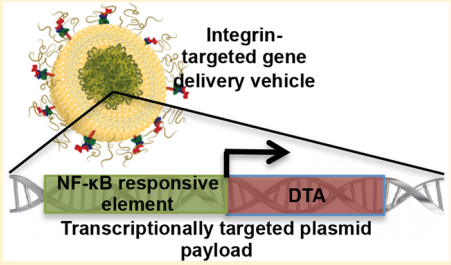
Increasing Cancer-Specific Gene Expression by Targeting Overexpressed $\alpha_5\beta_1$ Integrin and Upregulated Transcriptional Activity of NF-κB

Maroof M. Adil, Rachel M. Levine, and Efrosini Kokkoli*

Department of Chemical Engineering and Materials Science, University of Minnesota, 421 Washington Ave SE, Minneapolis, Minnesota 55455

Supporting Information

ABSTRACT: We developed a modular multifunctional nonviral gene delivery system by targeting the overexpressed cancer surface receptor $\alpha_5\beta_1$ integrin and the upregulated transcriptional activity of the cancer resistance mediating transcription factor NF-κB, thereby introducing a new form of transcriptional targeting. NF-κB regulated therapy can improve specificity of gene expression in cancer tissue and also may offset NF-κB mediated cancer resistance. We delivered a luciferase gene under the control of an NF-κB responsive element (pNF-κB-Luc) encapsulated in a PR_b peptide functionalized stealth liposome that specifically targets the $\alpha_5\beta_1$ integrin and achieved increased gene expression in DLD-1 colorectal cancer cells compared to BJ-fibroblast healthy cells *in vitro*. The multitargeted system was also able to differentiate between cancer cells and healthy cells better than either of the individually targeted systems. In addition, we constructed a novel cancer therapeutic plasmid by cloning a highly potent diphtheria toxin fragment A (DTA) expressing gene under the control of an NF-κB responsive element (pNF-κB-DTA). A dose-dependent reduction of cellular protein expression and increased cytotoxicity in cancer cells was seen when transfected with PR_b functionalized stealth liposomes encapsulating the condensed pNF-κB-DTA plasmid. Our therapeutic delivery system specifically eradicated close to 70% of a variety of cancer cells while minimally affecting healthy cells *in vitro*. Furthermore, the modular nature of the nonviral design allows targeting novel pairs of extracellular receptors and upregulated transcription factors for applications beyond cancer gene therapy.



KEYWORDS: *alpha(5)beta(1) integrin, NF-κB, transcriptional targeting, gene therapy, targeted delivery, nanoparticles*

INTRODUCTION

A major problem facing cancer therapy is nonspecific delivery of administered therapeutics. The nonspecific accumulation in healthy sites can often result in undesirable toxic side effects, necessitating the development of delivery vectors that can limit off-target cytotoxicity. A significant portion of the delivered therapeutic dose is taken up by the macrophagocytic system (MPS) leading to subsequent accumulation in the liver and the spleen. One strategy to address this issue is the encapsulation of therapeutics in nanoparticles functionalized with a polyethylene glycol (PEG) brush layer, which can help tumor accumulation by increasing circulation lifetime and the enhanced permeation and retention effect.[1,2] In addition, targeting ligands can be incorporated on the nanoparticle surface to bind to extracellular receptors overexpressed in tumor tissue and vasculature[3−6] thereby facilitating internalization into target cells.[7] While PEGylation and targeting can improve delivery efficiency compared to traditional delivery of free therapeutics, these systems continue to experience significant MPS uptake.[8] Also, many widely investigated cancer cell or cancer vasculature receptor targets such as integrins,[9] the endothelial growth factor receptor[10] and fibroblast growth factor receptor[11] are appreciably expressed in healthy tissues and vasculature.[12−14] These issues can lead to the same nonspecific side effects that targeted therapy is striving to avoid, and call for further levels of control to minimize nonspecific delivery.[15]

Gene therapy is becoming an increasingly popular form of cancer treatment. One way gene therapy can solve the problem of nonspecific delivery is through the addition of transcriptional targeting to the extracellular receptor targeting that is already in place, generating a multitargeted system for better control of gene expression. Transcriptional targeting has been widely investigated in the past, and has been shown to improve specific delivery to the target tissues.[16,17] Generally, transcriptional targeting involves delivering a gene under the control of a tissue specific promoter: allowing gene expression in target tissues while avoiding healthy areas. Multitargeting by combining transductional and transcriptional targeting has been shown before to effectively increase the specificity of a viral gene delivery system,[18−20] however it has not been sufficiently investigated in a nonviral delivery system.[21] Nonviral systems

Received: September 5, 2013
Revised: January 24, 2014
Accepted: February 3, 2014
Published: February 3, 2014

offer several advantages over viral gene delivery vectors: they are tunable, safer, cheaper and easier to build.[22,23] The tunable nature of these systems can facilitate the construction of a modular multitargeted system.

A second major problem in cancer therapy is the development of resistance to chemotherapy and radiation therapy in cancer cells, rendering these traditional forms of treatment largely ineffective. NF-κB is a well-characterized group of transcription factors that are upregulated in a variety of diseases including cancer[24] and is known to mediate resistance to chemotherapy and radiation therapy.[25] Inhibition of NF-κB activity has been shown to improve cancer therapy by reducing NF-κB mediated chemotherapy resistance.[26] However, its transcriptional activity has never been used as a therapeutic target before. Given the central role played by NF-κB as a transcription factor controlling a variety of cancer progression pathways, we postulated that its upregulated activity would be sufficient to mediate a therapeutic response specifically in cancer cells. In addition, targeting the transcriptional activity of a single upregulated transcription factor may offer better control and outcome predictability compared to conventional transcriptional targeting using entire promoter sequences of upregulated genes.

In this paper, we took advantage of the versatility of gene therapy and developed a multifunctional therapeutic system. As a delivery vehicle we chose PR_b functionalized stealth liposomes encapsulating branched polyethyleneimine (bPEI) condensed plasmid DNA (pDNA), a nonviral gene delivery vehicle recently developed in our group to specifically transfect $\alpha_5\beta_1$ integrin bearing cells.[27] This gene delivery system was extensively characterized in our previous work both *in vitro* and *in vivo*, in a mouse model of metastatic colon cancer, for stealth properties, targeting ability and specificity of delivery.[27] In addition, cryogenic transmission electron microscopy (cryo-TEM) was used to visualize the encapsulated condensed DNA nanoparticles in the aqueous core of the stealth liposomes.[27]

The PR_b targeting ligand used in this system is a fibronectin mimetic peptide developed by our group to specifically bind $\alpha_5\beta_1$ integrin with an affinity of 76.3 ± 6.3 nM,[28,29] and has since been used to improve nanoparticle delivery to $\alpha_5\beta_1$ integrin bearing cells for a variety of applications.[30−35] PR_b functionalized nanoparticles can outperform other RGD based peptide functionalized nanoparticles, and can even distinguish between cells with differential expression levels of $\alpha_5\beta_1$ integrin.[36,37] Additionally, the stealth and $\alpha_5\beta_1$ integrin targeting properties of the PR_b functionalized gene delivery system have been demonstrated *in vivo*.[27] Few, if any, previous studies have shown the same level of *in vivo* gene expression specificity as mediated by our delivery system, since the PR_b functionalized stealth liposomes differentiated between healthy and cancerous tissue within the same organ, the liver.[27]

$\alpha_5\beta_1$ integrin is a widely accepted and well-investigated cancer surface marker.[38] It is highly expressed on embryonic cell types but it is downregulated during development.[39] It is also highly upregulated in tumor vasculature and in tumor cells of breast and prostate cancer and, central to this work, colorectal cancer.[38,40] However, $\alpha_5\beta_1$ integrin is also present in normal tissues such as hepatic sinusoids, endothelial venules of lymph nodes, pancreatic ducts and intestinal smooth muscle, though most of these healthy sites are not accessible from the bloodstream.[12] For example, intravenously injected anti-$\alpha_5\beta_1$ antibody examined the amount and distribution of the integrin present *in vivo*, and this study revealed that the injected antibody strongly labeled tumor vessels but did not label most normal blood vessels and did not access pancreatic ducts or intestinal smooth muscle.[12] However, hepatic sinusoids and lymph nodes remain vulnerable to nonspecific transfection.[12]

We hypothesized that, by adding a second level of control to PR_b functionalized stealth liposomes via transcriptional targeting, we can improve the specificity of cancer cell transfection while minimizing off-target effects. Multitargeting, by combining extracellular targeting and transcriptional targeting, has not been extensively investigated using nonviral vectors before. We therefore targeted the upregulated transcriptional activity of NF-κB as a new form of transcriptional targeting, and showed increased cancer specificity. In addition, to address the problem of low gene expression mediated by most transcriptional targeting, we chose the highly potent diphtheria toxin fragment-A (DTA) as the protein product of gene expression. DTA consists of only the catalytic fragment of diphtheria toxin and lacks a cell-penetrating domain, which can minimize any bystander cell killing effect at off-target sites. A few molecules of DTA can be enough to result in cell death through inhibition of protein expression,[41] and DTA-encoding plasmids have been previously used to successfully kill cancer cells.[16,42] Moreover, some healthy cell lines have been shown to be resistant to DTA activity,[41] which further augments the potential cytotoxic specificity of our design. Thus, by delivering a DTA gene under the regulation of an NF-κB responsive element, we showed that the high transcriptional activity of NF-κB in cancer cells can mediate specific cytotoxicity in a variety of cancer cells compared to healthy cells *in vitro*.

By combining the targeting of two well-recognized cancer markers, $\alpha_5\beta_1$ integrin and upregulated NF-κB transcriptional activity, we created a general, modular platform for cancer-specific transfection agents. This multifunctional design may also offer a solution to NF-κB mediated cancer resistance to chemotherapy and radiation therapy, and can be a useful adjuvant to increase the efficiencies of these traditional forms of cancer treatment.

EXPERIMENTAL SECTION

Plasmid Construction and Cloning. pNF-κB-Luc (Agilent, Santa Clara, CA) was sequenced at the University of Minnesota Genomics Center (UMGC) with a Luc-B1 primer according to the manufacturer's instructions to verify the NF-κB binding region. The NF-κB binding domains were five tandem repeats of the 10 bp consensus sequence GGGGACTTTC. A gene sequence coding for diphtheria toxin fragment A (DTA)[43] was designed and manufactured in GeneArt (Life technologies, Grand Island, NY). The DTA gene was inserted into the pNF-κB-Luc plasmid in place of the luciferase gene using an In-Fusion HD Cloning kit (Clontech, Mountain View, CA) according to the manufacturer's protocol to yield pNF-κB-DTA. Further details and validation of the plasmid construction are presented in the Supporting Information. The plasmid construct was validated using gel electrophoresis and sequencing at UMGC.

Liposome Preparation. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG2000), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). PR_b peptide was purchased from UMGC, and PR_b peptide-amphiphiles were synthesized as previously described.[28] PR_b

peptide-amphiphiles were used to functionalize the stealth liposomes. PR_b functionalized or nontargeted stealth liposomes were made using a thin film lipid hydration method.[27,44] Briefly, a mixture of lipids containing x mol % PR_b peptide-amphiphiles, 5 mol % DPPE-PEG2000, $(60 - x)$ mol % DPPC and 35 mol % cholesterol were deposited in a round-bottom flask and dried under a stream of argon (x = 0 for nontargeted stealth liposomes and x = 5 for PR_b functionalized stealth liposomes). PR_b concentration on the liposomes was determined by the BCA assay (Thermo Scientific, Waltham, MA) following the manufacturer's protocol (actual concentrations are shown in the figure captions). Plasmid DNA (pDNA) was condensed with bPEI as previously described.[27] Condensed pDNA was then used to hydrate the lipid film for 2 h in a water bath held at 45 °C. The hydrated liposomes were then extruded through 400 nm polycarbonate membranes in a hand-held extruder (Avestin, Ontario, CA). Liposomes were dialyzed against water for 24 h in a 1000 kDa MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA) with frequent water changes. The ZetaPALS Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY) was used to determine both the zeta potential and diameter (by dynamic light scattering; DLS) of the stealth liposomes. Liposome sizes varied between 204 ± 6 nm for the nontargeted stealth liposomes and 279 ± 51 nm for the PR_b functionalized stealth liposomes, and these sizes were also verified by cryo-TEM images.[27] Zeta potentials were measured to be −15.3 ± 0.6 mV for stealth liposomes and −3.1 ± 1.8 mV for the targeted stealth liposomes.[27] The pDNA concentration in the liposomes was measured using a Cy5 DNA quantification assay (Mirus Biosciences, Madison, WI) as described previously, and was on average 5 μg/mL corresponding to a 40% encapsulation yield.[27]

Luciferase Transfection and Luminescence Measurement. DLD-1 colorectal cancer cells and BJ-fibroblast skin cells were plated at 5,000 cells per well in a white 96 well plate in 200 μL of Dulbecco's modified Eagle medium (DMEM). Medium was replenished the next day, and cells were transfected with 100 ng of pDNA encapsulated in different liposome formulations: PR_b functionalized stealth liposomes encapsulating pT2/Cal (a firefly luciferase expression plasmid, gift from Prof. McIvor, University of Minnesota), pNF-κB-Luc (Agilent, Santa Clara, CA) or pLuc-MCS (Agilent, Santa Clara, CA) and nontargeted stealth liposomes encapsulating pT2/Cal or pNF-κB-Luc (all plasmids were condensed with bPEI). Cells were incubated with the liposomes for 48 h at 37 °C and 5% CO_2 followed by luminescence measurement on a Synergy H1 plate reader (Biotek, Winooski, VT) using a luciferase assay kit according to the manufacturer's guidelines (Promega, Madison, WI). For experiments demonstrating the effect of pDTA on protein production, DLD-1 cells were cotransfected with 100 ng of bPEI condensed pT2/Cal encapsulated in PR_b functionalized stealth liposomes and different amounts of either bPEI condensed pNF-κB-DTA or bPEI condensed pmaxGFP (gift from Prof. Hu, University of Minnesota) in PR_b functionalized stealth liposomes.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) for DTA mRNA. DLD-1 cells were seeded in a 6 well plate at 1×10^5 cells/well in DMEM and cultured in an incubator at 37 °C and 5% CO_2. 24 h later, medium was replenished and bPEI condensed pNF-κB-DTA encapsulated in PR_b functionalized or nontargeted stealth liposomes was used to transfect the cells at 1 μg/well. Following incubation for 24 more hours, manufacturer's protocol was followed for mRNA harvest using an RNeasy extraction kit (Qiagen, Valencia, CA), for cDNA generation using RNA to cDNA EcoDry premix (Clontech, Mountain View, CA), and for running PCR on a Stratagene MX3000P using SYBR advantage PCR kit (Clontech, Mountain View, CA). The following PCR primer pairs were manufactured at UMGC and used for the detection of DTA mRNA: NF-κB-DTA-F GGCAGCAGCAGCGTGGAGTA and NF-κB-DTA-R CAGGCCTGGGCCATATATCGT. Human β-actin was used as a housekeeping gene control with primers h-Actin-F TGAGACCTTCAACACCCCAG and h-Actin-R AGATGGGCACAGTGTGGGT. The relative amount of DTA mRNA was calculated relative to the geometric mean of the cycle thresholds of the housekeeping gene β-actin using the $2^{-\Delta\Delta Ct}$ method.[45]

NF-κB-DTA Transfection and Cell Viability Measurement. DLD-1 colorectal cancer cells and BJ-fibroblast skin cells were plated at 5,000 cells per well in a clear 96 well plate in 200 μL of DMEM. Medium was replenished the next day, and cells were transfected with 100−250 ng of bPEI condensed pDNA encapsulated in different liposomes. The liposome formulations tested were PR_b functionalized stealth liposomes encapsulating either bPEI condensed pNF-κB-DTA plasmid or the bPEI condensed pT2/Cal plasmid, and nontargeted stealth liposomes encapsulating bPEI condensed pNF-κB-DTA. Liposomes were incubated with the cells for a period of 72 h in an incubator at 37 °C and 5% CO_2. Cells were then washed in PBS buffer, medium was replenished and the WST-1 assay (Promega, Madison, WI) was used to measure cell viability according to the manufacturer's protocol. Cell viability is reported as a percentage of untransfected cells. For experiments demonstrating the versatility of the pNF-κB-DTA plasmid, MCA38 murine colon cancer cells, MCF7 human breast cancer cells and TNF-α induced HEK293T human embryonic kidney cells were similarly transfected with 100 ng of bPEI condensed pNF-κB-DTA in PR_b functionalized stealth liposomes with pmaxGFP as the control. For experiments investigating the effect of DTA dosage on cell viability, different concentrations of bPEI condensed pNF-κB-DTA in PR_b functionalized stealth liposomes were delivered to DLD-1 cells, with pMaxGFP as the control.

RESULTS

Multitargeting Increases Specificity of Luciferase Gene Expression in Cancer Cells Compared to Healthy Cells. We hypothesized that, in specific situations where a cancer-related extracellular target receptor is also overexpressed in healthy cell lines, further levels of control would be needed for cancer specific gene delivery. For that we used DLD-1 colorectal cancer cells and BJ-fibroblasts that express the $\alpha_5\beta_1$ integrin at a higher level than DLD-1 cancer cells (Figure S1 and experimental details are included in the Supporting Information). BJ-fibroblasts are primary fibroblasts from the foreskin of a newborn, and $\alpha_5\beta_1$ is highly expressed in embryonic cell types while it is downregulated during development in adult tissues. While this primary cell line is not likely to be encountered by gene delivery vehicles administered *in vivo* in an adult, it serves as a useful model for healthy cells expressing the receptor of choice. Thus this relative expression level demonstrates a scenario where the first level of extracellular receptor targeting by itself would be unable to differentiate between healthy cells and cancer cells, leading to undesirable side effects in off-target cells. To identify a model

for the second level of intracellular targeting that would increase specificity of gene delivery to cancer cells, we chose to investigate the transcription factor NF-κB that is strongly implicated in the development and progression of cancer. NF-κB can exist in several different forms in cells: as homo- or heterodimers of RelA, RelB, cRel, NF-κB1 or NF-κB2.[24] mRNA levels of NF-κB in DLD-1 cells and BJ-fibroblasts were measured using RT-PCR, and four out of the five NF-κB proteins tested were seen to be upregulated in DLD-1 colorectal cancer cells compared to the healthy BJ-fibroblasts (Figure S2A and experimental details are included in the Supporting Information). To further validate the differential NF-κB expression in healthy versus cancer or NF-κB-induced cells, NF-κB expression levels were investigated in normal or TNF-α induced human embryonic kidney HEK293T cells, as TNF-α inflames HEK293T cells and induces NF-κB activation. NF-κB levels were also seen to be upregulated in induced HEK293T cells compared to uninduced cells (Figure S2B and experimental details are included in the Supporting Information). Thus NF-κB is an intracellular target that could be utilized successfully to differentiate between healthy and diseased cells and in particular the DLD-1 cells and BJ-fibroblasts that are used here as a cancer and healthy cell model for therapeutic delivery.

To demonstrate increased specificity with the multitargeting approach DLD-1 cells and BJ-fibroblasts were transfected with two different plasmids: pT2/Cal, which constitutes the firefly luciferase gene under the control of a CAGS constitutive promoter,[46] or pNF-κB-Luc, where the firefly luciferase gene is controlled by an NF-κB responsive element. The plasmids were condensed with bPEI and encapsulated in either PR_b functionalized or nontargeted stealth liposomes. In both cases, nontargeted stealth liposomes resulted in low transfection levels, demonstrating the effectiveness of the first level of extracellular targeting (Figure 1). When pT2/Cal was delivered to these cells using PR_b functionalized stealth liposomes, both DLD-1 and BJ-fibroblasts were seen to express the luciferase gene at very similar levels. This demonstrates a crucial part of our hypothesis, namely, the inability to differentiate between cancer cells and healthy cells with a single level of extracellular targeting in situations where the extracellular receptor is upregulated in off-target tissues. These results also show similar gene expression levels between these two cell lines, thereby justifying a fair comparison. The presence of the targeting molecule PR_b increased transfection efficiency of the pmaxGFP plasmid delivered with stealth liposomes by 2-fold and 8-fold in DLD-1 (32.6 ± 1.2% transfected cells) and BJ-fibroblasts (51.6 ± 0.7% transfected cells), respectively, as quantified by flow cytometry (Figure S3 and experimental details are included in the Supporting Information). This reiterates both the effectiveness of the first level of extracellular targeting for improving transfection efficiency in cells that express the extracellular target and the need for a second level of transcriptional targeting in the presence of healthy cells expressing this extracellular target. The transfection efficiency of pmaxGFP delivered with PR_b functionalized stealth liposomes was higher in BJ-fibroblasts than in DLD-1 cells (Figure S3 in the Supporting Information), while the luciferase expression levels measured after delivery of pT2/Cal with PR_b functionalized liposomes was comparable for both cells (Figure 1). This is likely because the gene expression levels measured from luciferase expression (Figure 1) is a result of the percent of cells transfected and cell growth and metabolism, while the transfection efficiency (Figure S3 in the Supporting Information) measures only the percent of cells transfected. BJ-fibroblasts express higher levels of $\alpha_5\beta_1$ (Figure S1 in the Supporting Information), while DLD-1 cells exhibit higher cell growth and metabolism (data not shown), resulting in comparable luciferase expression between the two cell lines with different transfection efficiencies. When pNF-κB-Luc was delivered to these cells with PR_b functionalized stealth liposomes (Figure 1), DLD-1 cells maintained similar levels of luciferase expression compared to pT2/Cal transfection, while expression in healthy BJ-fibroblasts dropped to control levels as seen in the case where plasmids were delivered using nontargeted stealth liposomes. Therefore, these results show that introducing a second level of intracellular targeting significantly increased transfection specificity in cancer cells. And to further prove that the luciferase expression seen after transfection with pNF-κB-Luc was actually due to the activity of NF-κB, the pLuc-MCS plasmid, which is the same plasmid lacking the NF-κB responsive element, was used as a control. Luciferase expression was not detectable in either cell line, relating any measurable expression to NF-κB transcriptional activity. With almost 2 orders of magnitude difference between the levels of gene expression in DLD-1 cells versus BJ-fibroblasts, we concluded that using a multitargeted approach holds promise for cancer-specific gene therapy. In addition, this experiment was repeated using HEK293T cells in their uninduced and induced form to demonstrate that multitargeting can increase specificity in other diseases where NF-κB levels are upregulated, and trends were similar (Figure S4 in the Supporting Information).

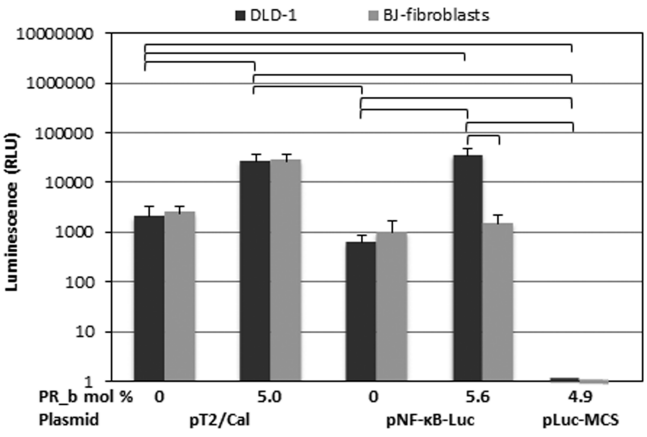


Figure 1. Luciferase expression in DLD-1 cells and BJ-fibroblasts. PR_b functionalized or nontargeted stealth liposomes were used to deliver 100 ng of bPEI condensed pT2/Cal plasmid (luciferase under a constitutive CAGS promoter), pNF-κB-Luc (luciferase under the NF-κB responsive element) or pLuc-MCS (promoterless luciferase plasmid) into DLD-1 cells or BJ-fibroblasts. Luminescence from luciferase expression was measured after 48 h incubation at 37 °C. Data presented as average ± standard error from three independent experiments (n = 3), done in triplicate. Student's t test statistical analysis was performed for DLD-1 cells and comparisons between DLD-1 and BJ-fibroblasts for the same sample: brackets indicate $p < 0.05$, absence of brackets for $p > 0.05$.

Multitargeting Can Specifically Eradicate Cancer Cells While Sparing Healthy Cells. To investigate the therapeutic potential of the multitargeted gene delivery system, we designed a cancer specific suicide gene therapy regime. As a cytotoxic gene of interest we chose the DTA encoding gene,

which has been previously shown to effectively kill cancer cells.[47,48] pNF-κB-DTA (Figure S5 in the Supporting Information) was created by cloning in the DTA gene in the place of the luciferase gene in pNF-κB-Luc (details of plasmid construction are presented in the Supporting Information). To show cancer specific therapy, pNF-κB-DTA was delivered into DLD-1 cells and BJ-fibroblasts with PR_b functionalized or nontargeted stealth liposomes (Figure 2). As a negative control,

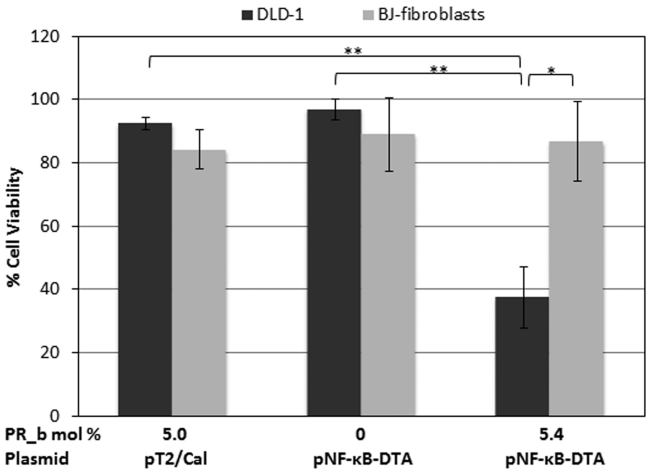


Figure 2. DTA mediated cytotoxicity in DLD-1 cells and BJ-fibroblasts. 250 ng of bPEI condensed pNF-κB-DTA or pT2/Cal encapsulated in PR_b functionalized or nontargeted stealth liposomes were used to transfect cells for 72 h at 37 °C, followed by measuring cell viability using a WST-1 metabolic assay and shown as a percentage of untreated samples. Data presented as average ± standard error of three independent experiments (n = 3), done in triplicate. Student's t test statistical analysis was performed for DLD-1 cells and comparisons between DLD-1 and BJ-fibroblasts for the same sample: * $p < 0.05$, ** $p < 0.005$, absence of brackets for $p > 0.05$.

we chose a generic DNA delivery vector not linked with NF-κB. Given the similar transfection behavior between pT2/Cal and pNF-κB-Luc shown in Figure 1, we deemed it appropriate to use pT2/Cal in PR_b functionalized stealth liposomes as a negative control. Successful cytotoxic therapy was observed only when PR_b functionalized stealth liposomes were used to transfect pNF-κB-DTA in DLD-1 colorectal cancer cells, that is when both levels of targeting were used and both targets were upregulated in the cells. While more than 60% of cancer cells were killed, on average 15% of the healthy cells were affected. This low level of nonspecific cell cytoxicity likely came from a combination of the bPEI used to condense the pDNA and the stealth liposomes themselves. Figure S6 in the Supporting Information shows cell cytotoxicity mediated by different concentrations (100−500 ng) of bPEI condensed DNA (pmaxGFP and pT2/Cal) and empty PR_b functionalized stealth liposomes (1−5 μmol/mL of lipid concentrations) in both DLD-1 and BJ-fibroblast cells at 48 and 72 h (the experimental details are discussed in the Supporting Information). bPEI condensed DNA affected the healthy cells more than the cancer cells, as at 72 h bPEI condensed DNA caused on average 54% cell death in BJ-fibroblasts. Empty PR_b functionalized stealth liposomes appeared to result in low levels of cytotoxicity at most concentrations and both time points. Lipid concentrations necessary for the delivery of up to 250 ng of encapsulated condensed DNA caused much less cytotoxicity in BJ-fibroblasts at 72 h (17% on average cell death) compared to the much higher lipid concentrations required for the delivery of 500 ng of encapsulated condensed DNA, that gave rise to an average of 45% BJ-fibroblast cell death at 72 h. The toxicity from the DNA encapsulated liposomes, however, is less than the sum of the cytotoxic effects from the bPEI condensed DNA and the empty stealth liposomes individually, and in some cases much less than the cytotoxicty from bPEI condensed DNA alone. One major mechanism of bPEI toxicity comes from its binding to plasma membrane proteoglycans, resulting in membrane destabilization.[49] When bPEI is encapsulated within the liposomes, this toxicity mechanism may be avoided, thus decreasing the overall cytotoxicity from the delivery vehicle.

Further Characterization of DTA Activity. To relate the cytotoxic effect seen in the cancer cells to DTA production, we further investigated the effect of delivering pNF-κB-DTA to DLD-1 cancer cells using several different techniques. First, RT-PCR (amplification curves are shown in Figure S7 in the Supporting Information) was used to measure the amount of DTA mRNA produced in DLD-1 cells after transfection with pNF-κB-DTA using PR_b functionalized or nontargeted stealth liposomes. DTA mRNA was detected in transfected cells, and the levels were seen to be 4.5 ± 0.6 (average ± standard error, n = 3) times higher when PR_b functionalized stealth liposomes were used versus nontargeted stealth liposomes. This explains the low level of cytotoxicity seen when DLD-1 cells were transfected with pNF-κB-DTA encapsulated in nontargeted stealth liposomes, and shows the control over gene expression exerted by the first level of our multitargeting approach: targeting the extracellular $\alpha_5\beta_1$ integrin.

Second, to investigate the mode of action of DTA in killing cells we measured protein expression after transfecting DLD-1 cells with pNF-κB-DTA encapsulated in PR_b functionalized stealth liposomes. DTA is thought to cause cell death by inhibiting protein expression.[50] DLD-1 cells were cotransfected with pT2/Cal and pNF-κB-DTA, and luciferase expression was measured relative to cells that received only pT2/Cal (Figure 3). Cotransfection with pmaxGFP, a GFP expressing plasmid, was used as a control. A dose dependent reduction in luciferase expression was seen with the delivery of increasing amounts of pNF-κB-DTA. A substantial level of protein inhibition was observed even at 25 ng of pNF-κB-DTA delivered, the lowest amount of pDNA delivery investigated in the current studies. When 250 ng of pNF-κB-DTA was delivered, we observed instances where the level of protein expression went down below detectable levels.

Third, to complement these protein expression inhibition studies we measured the effect of pDNA dosage on cytotoxicity in DLD-1 cells. With increasing amounts of pNF-κB-DTA delivered in PR_b functionalized stealth liposomes, increased cytotoxicity was seen (Figure 4). There was no corresponding trend observed when pmaxGFP was used as a control, showing on average less than 10% nonspecific cytoxicity at all pDNA concentrations delivered. Interestingly, cytotoxicity was not observed in DLD-1 cells at 100 ng of pNF-κB-DTA delivered, an amount which resulted in 82.5 ± 0.5% protein inhibition (Figure 3). Our results therefore suggest that protein production may need to be completely halted before cytotoxicity results in DLD-1 cancer cells.

pNF-κB-DTA Is a Versatile Plasmid Capable of Killing a Variety of Cancer types. We also wanted to investigate the versatility of the pNF-κB-DTA plasmid in killing different types of cancer and diseased cells. To this end, pNF-κB-DTA

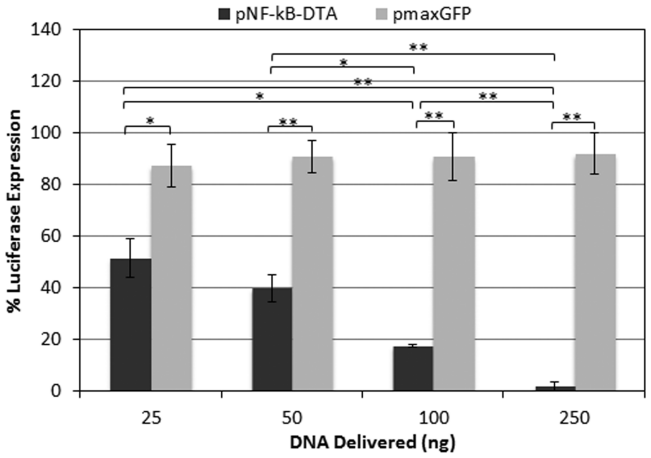


Figure 3. DTA mediated inhibition of protein expression in DLD-1 cells. 5 mol % PR_b functionalized stealth liposomes was used to transfect cells with 100 ng of bPEI condensed pT2/Cal plasmid and different amounts of pNF-κB-DTA or pmaxGFP for 48 h at 37 °C. Luminescence was measured with a luciferase expression assay kit and reported as a percentage of expression from control cells that received only the pT2/Cal plasmid. Data presented as average ± standard error of three independent experiments (n = 3), done in triplicate. Student's t test statistical analysis was performed for pNF-κB-DTA and comparisons between pNF-κB-DTA and pmaxGFP for the same sample: * $p < 0.05$, ** $p < 0.005$, absence of brackets for $p > 0.05$.

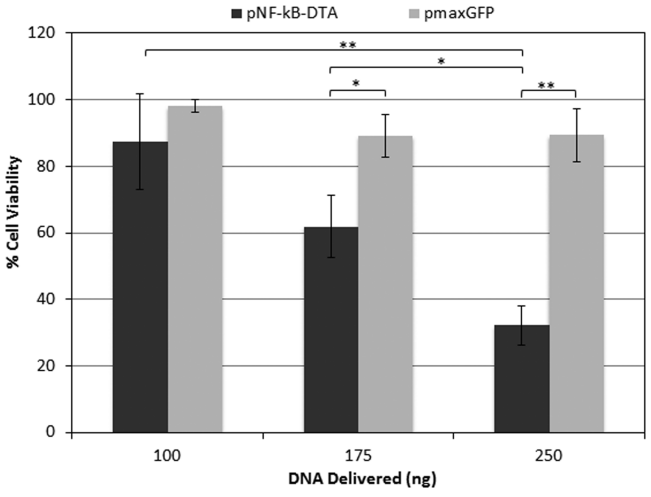


Figure 4. Cytotoxic dose response to increasing pNFκB-DTA delivery in DLD-1 cells. Cells were transfected with different amounts of bPEI condensed pNF-κB-DTA or pmaxGFP encapsulated in 5 mol % PR_b functionalized stealth liposomes for 72 h at 37 °C. Cell viability was measured using WST-1 metabolic assay and reported as a percentage of the viability of untreated cells. Data presented is average ± standard error of four independent experiments (n = 4), done in triplicate. Student's t test statistical analysis was performed for pNF-κB-DTA and comparisons between pNF-κB-DTA and pmaxGFP for the same sample: * $p < 0.05$, ** $p < 0.005$, absence of brackets for $p > 0.05$.

encapsulated in PR_b functionalized stealth liposomes was delivered to MCA38 murine colon cancer cells, MCF7 human breast cancer cells and TNF-α induced HEK293T human embryonic kidney cells while using pmaxGFP as a control. In all these cell lines, a high level of cytotoxicity was observed after transfection with pNF-κB-DTA compared to the control (Figure 5A). Furthermore, for each of the cell lines tested DTA was shown to inhibit protein production (Figure 5B). Taken together, these results demonstrate the versatility of the pNF-κB-DTA plasmid in killing cancer cells and TNF-α induced HEK293T cells. Also, pNF-κB-DTA was able to kill MCA38, MCF7 and HEK293T induced cells effectively at only 100 ng delivery, in contrast to the higher amounts required for DLD-1 cells. This is likely due to differences in transfection efficiency and differential effectiveness of DTA in different cell lines.[41]

DISCUSSION

In this paper we focused on the problem of specific delivery of nonviral gene delivery vehicles to cancer cells versus healthy cells *in vitro*. However, the same problem of off target extracellular receptor expression is prevalent in other therapeutic strategies. For example, an exciting new development in gene therapy is reengineered T cells that can be specifically activated upon interaction with the CD19 receptor found on B cell lymphomas.[51] Unfortunately, CD19 is also expressed on normal B cells.[52] Similarly, myelodysplastic syndrome associated stem cells that progress into amyeloid leukemia offer no distinguishing extracellular receptors compared to normal cells.[53] In situations like these, a multitargeted therapeutic system could aid recognition of and therapeutic delivery into target cells and subsequently transcriptionally control the expression of a desired gene therapy product.

The regulation of gene expression in mammalian cells however is a complex process. Tumor specific promoters may be regulated by many different transcriptional components. For example, tumor specific promoters previously used in transcriptional targeting such as survivin and H19[54,55] are regulated by the SP1, E2F and C/EBP families of transcription factors and their cofactors.[56,57] The involvement of multiple transcription factors and regulatory components may substantially increase the noise in gene expression.[58] While noise in gene expression can be useful in regulatory events in the cell, it can also lead to unpredictable heterogeneous outcomes following uniform inputs.[59] Therefore, the simplicity of a design where gene expression is mediated by a single upregulated transcription factor may be attractive for building more controllable and predictable gene delivery vectors. A similar effect can be obtained by trimming full promoters of upregulated genes to the bare minimum essential for transcription, as was done for HIF-1α.[60] In our design, expression of DTA is regulated by five tandem repeats of a 10 bp NF-κB responsive element (Figure S5 in the Supporting Information).

We developed a multitargeted gene delivery system combining extracellular receptor targeting and transcriptional targeting and showed improved specificity of gene expression in cancer cells compared to healthy cells *in vitro*. The PR_b peptide targets the $\alpha_5\beta_1$ integrin receptor and allows for subsequent internalization of the nanoparticles and release of the payload by cells that overexpress the integrin compared to nontargeted stealth liposomes (Figures 1 and 2). Previous work in our group showed that PR_b functionalized nanoparticles bind with high specificity to $\alpha_5\beta_1$ and internalization of PR_b functionalized vesicles was absent on cells where the integrin was minimally expressed.[31,36] We further showed that the strategy of using a second level of control for gene expression can successfully relieve the problems of nonspecificity faced by current extracellular receptor targeted gene delivery vectors. The increased transcriptional activity of NF-κB in cancer cells versus healthy cells (Figure S2A in the Supporting Information)

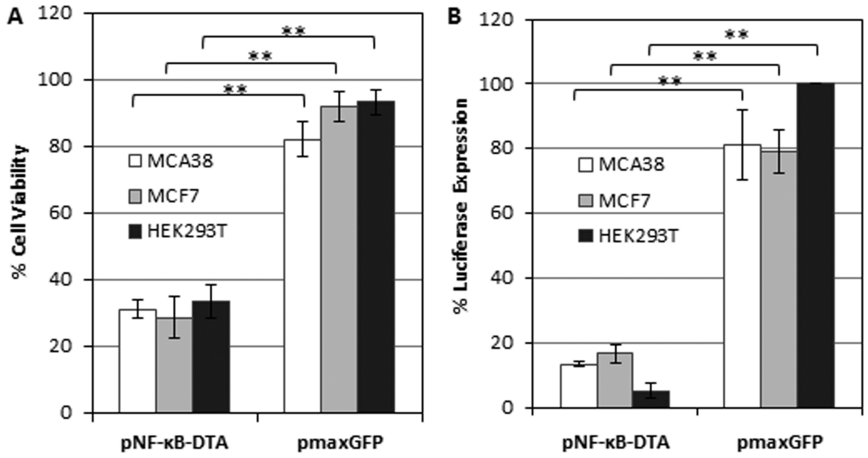


Figure 5. (A) DTA mediated cytotoxicity in MCA38 murine colon cancer cells, MCF7 human breast cancer cells and TNF-α induced HEK293T human embryonic kidney cells. 100 ng of bPEI condensed pNF-κB-DTA or pmaxGFP encapsulated in 5 mol % PR_b functionalized stealth liposomes was used to transfect cells for 72 h at 37 °C, followed by measuring cell viability using a WST-1 metabolic assay as a percentage of untreated samples. (B) DTA mediated inhibition of protein expression in MCA38 cells, MCF7 cells and TNF-α induced HEK293T cells. 5 mol % PR_b functionalized stealth liposomes was used to transfect cells with 100 ng of bPEI condensed pT2/Cal plasmid and 100 ng of pNF-κB-DTA or pmaxGFP for 48 h at 37 °C. Luminescence was measured with a luciferase expression assay kit and reported as a percentage of expression from control cells that received only the pT2/Cal plasmid. Data presented as average ± standard error of three independent experiments (n = 3), done in triplicate. Student's t test statistical analysis was performed: ** $p < 0.005$.

resulted in a 20-fold increase in luciferase expression (Figure 1) and substantial cytotoxicity in DLD-1 colorectal cancer cells compared to healthy BJ-fibroblast cells (Figures 2 and 4). Furthermore, the lack of detectable gene expression in the absence of this NF-κB binding element demonstrates the strict control over gene expression exerted by a single transcription factor (Figure 1). The versatility of the constructed pNF-κB-DTA plasmid was demonstrated by successfully inhibiting protein expression and inducing apoptosis in DLD-1 human colorectal cancer cells, MCA38 murine colon cancer cells, MCF7 human breast cancer cells and TNF-α induced HEK293T human embryonic kidney cells (Figure 5). While the inhibition of protein expression seemed to be similarly effective in all cell lines tested (Figures 3 and 5B), DLD-1 cells seemed to require a higher dose of pNF-κB-DTA for a cytotoxic effect (Figure 4). This may be an indication of differential activity of DTA in different cancer or diseased cell lines.[41] We also noticed a low background level of cytotoxicity (Figure 2) that could be generated by a combination of DTA expression from background NF-κB activity and from the delivery vehicle itself.

Additionally, we noticed cell killing was limited to about 70% and even increasing the amount of pNF-κB-DTA delivered to DLD-1 and MCF-7 cancer cells to 500 ng did not increase cell killing (Figure S8 in the Supporting Information). This is likely due to a combination of factors including transfection efficiency, NF-κB activity and expression of DTA that play a role in determining the cytotoxicity in target cells. The transfection efficiency of pmaxGFP delivered with PR_b functionalized liposomes in DLD-1 cells was found to be 32.6% (Figure S3 in the Supporting Information), while uptake efficiency, reported as the percentage of cells with internalized DNA compared to untransfected cells, was 98.7%,[61] suggesting that there are cellular barriers to effective transfection independent of internalization. The inherent variation in NF-kB expression within a population has been shown in the literature.[62] However, these fluctuations occurring on the time scale of a couple of hours may likely be evened out over the duration of the 72 h experiment presented here, and do not fully explain the levels of cell killing efficiency seen in our study. DTA mediated cytotoxicity was investigated in a variety of different cell types, and the demonstrated cytotoxicity, ranging from 40 to 80%, was related to the transfection efficiencies in the different cell types.[16] However, DTA cytotoxicity may not scale linearly with transfection efficiency, as variability in DTA activity among different cell types has also been shown.[41] Thus, a variety of factors can influence the level of DTA mediated cytotoxicity in a particular cell type.

In addition to differential activity of cytotoxic gene products, several other factors including variability in cell transfectability, transfection agents, and promoter strengths of genes administered can make it difficult to directly compare the reported cytotoxicities mediated by different nonviral therapeutic gene delivery vectors across different studies. Keeping this in mind, some of the common nonviral gene delivery vectors previously used to deliver cytotoxic therapeutic genes *in vitro* include Fugene 6, jetPEI and Lipofectamine. Cell cytotoxicity was investigated in a variety of different cell lines following transfection using a DTA expression plasmid under a Rad51 promoter delivered using Fugene 6 (a commercial transfection agent), and observed cytotoxities ranging from 40 to 80% depending on the cell line at 100 ng of plasmid delivered.[16] In comparison, our results show that we were able to achieve 70% cell killing in a variety of different cell types. Using strong promoters, such as H19 and P3 to drive DTA expression, colon cancer cells were transfected using jetPEI and obtained 70−95% reduction in protein expression.[63] In a different study, Lipofectamine-complexed DTA (regulated by full promoter sequences) was able to almost completely inhibit protein production in human ovarian tumor cells.[42] In comparison, using an NF-κB binding region as a minimal promoter we can inhibit more than 80% of protein production in a variety of different cell lines. Taken together, current results presented here demonstrate the promise of our system as a therapeutic gene delivery vector.

Upregulated NF-κB can be responsible for chemoresistance in cancer.[25] Some successful cancer therapy strategies involve administering NF-κB inhibitors in conjunction to chemotherapy,[26,64] where the reduction in cancer chemoresistance leads to better treatment outcomes. In contrast, our approach uses NF-κB itself to drive therapy in cancer cells and turns cancer cell strength into a weakness. For example, in cases where inhibiting NF-κB activity is ineffective in promoting cancer therapy,[65] targeting NF-κB activity itself to drive therapy can be a potential solution. MCF7 breast cancer cells, one of the cell lines that failed to respond to chemotherapy after NF-κB inhibition,[65] were eradicated with pNF-κB-DTA. This form of NF-κB mediated cancer therapy may also have the potential to be an adjuvant to current chemotherapy and radiation therapy. In fact, by identifying and killing NF-κB overexpressing, potentially chemoresistant cancer cells, NF-κB mediated therapy may reduce chemotherapy and radiation therapy dosage, thereby alleviating undesirable side effects of these traditional forms of therapy.

Our results demonstrate the potential of driving therapeutic gene expression where both the $\alpha_5\beta_1$ integrin and a single transcription factor, NF-κB, are upregulated and warrant further investigation of our design as a new form of cancer multitargeting. One drawback of the current target pair though is that they may both be expressed in noncancerous inflamed tissues, and can therefore limit the application of our current delivery system when inflammatory diseases (such as rheumatoid arthritis or asthma) and cancer are both present.[66,67] However, with recent advances in high throughput screening and the ability to determine cell phenotypes quickly, other cancer specific pairs of extracellular receptors and transcriptional targets can be identified.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details and figures as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 612-626-1185. Fax: 612-626-7246. E-mail: kokkoli@umn.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by the CAREER award NSF/CBET-0846274, by NSF/CBET-1159967 and by the Shell Land Grant Chair at the University of Minnesota. We thank Prof. Kaznessis's group members, Dr. Katherine Volzing and Dr. Juan Borrero for helpful discussions during the design and synthesis of the pNF-κB-DTA plasmid.

■ REFERENCES

(1) Liu, D.; Mori, A.; Huang, L. Role of Liposome Size and RES Blockade in Controlling Biodistribution and Tumor Uptake of GM1-Containing Liposomes. *Biochim. Biophys. Acta* **1992**, *1104*, 95−101.

(2) Drummond, D. C.; Meyer, O.; Hong, K.; Kirpotin, D. B.; Papahadjopoulos, D. Optimizing Liposomes for Delivery of Chemotherapeutic Agents to Solid Tumors. *Pharmacol. Rev.* **1999**, *51*, 691−743.

(3) Pearce, T. R.; Shroff, K.; Kokkoli, E. Peptide Targeted Lipid Nanoparticles for Anticancer Drug Delivery. *Adv. Mater.* **2012**, *24*, 3803−3822.

(4) Ogris, M.; Wagner, E. To Be Targeted: Is the Magic Bullet Concept a Viable Option for Synthetic Nucleic Acid Therapeutics? *Hum. Gene Ther.* **2011**, *22*, 799−807.

(5) Levine, R. M.; Scott, C. M.; Kokkoli, E. Peptide Functionalized Nanoparticles for Nonviral Gene Delivery. *Soft Matter* **2013**, *9*, 985−1004.

(6) Pangburn, T. O.; Petersen, M. A.; Waybrant, B.; Adil, M. M.; Kokkoli, E. Peptide- and Aptamer-Functionalized Nanovectors for Targeted Delivery of Therapeutics. *J. Biomech. Eng.* **2009**, *131*, 074005.

(7) Guo, X.; Huang, L. Recent Advances in Nonviral Vectors for Gene Delivery. *Acc. Chem. Res.* **2011**, *45*, 971−979.

(8) Moghimi, S. M.; Szebeni, J. Stealth Liposomes and Long Circulating Nanoparticles: Critical Issues in Pharmacokinetics, Opsonization and Protein-Binding Properties. *Prog. Lipid Res.* **2003**, *42*, 463−478.

(9) Juliano, R. L.; Ming, X.; Nakagawa, O.; Xu, R.; Yoo, H. Integrin Targeted Delivery of Gene Therapeutics. *Theranostics* **2011**, *1*, 211−219.

(10) Ciardiello, F. EGFR Antagonists in Cancer Treatment. *N. Engl. J. Med.* **2008**, *358*, 1160−1174.

(11) Wesche, J.; Haglund, K.; Haugsten, E. M. Fibroblast Growth Factors and Their Receptors in Cancer. *Biochem. J.* **2011**, *437*, 199−213.

(12) Parsons-Wingerter, P.; Kasman, I. M.; Norberg, S.; Magnussen, A.; Zanivan, S.; Rissone, A.; Baluk, P.; Favre, C. J.; Jeffry, U.; Murray, R.; McDonald, D. M. Uniform Overexpression and Rapid Accessibility of $\alpha_5\beta_1$ Integrin on Blood Vessels in Tumors. *Am. J. Pathol.* **2005**, *167*, 193−211.

(13) Koretz, K.; Schlag, P.; Möller, P. Expression of Epidermal Growth Factor Receptor in Normal Colorectal Mucosa, Adenoma, and Carcinoma. *Virchows Arch. A: Pathol. Anat. Histopathol.* **1990**, *416*, 343−349.

(14) Hughes, S. E. Differential Expression of the Fibroblast Growth Factor Receptor (FGFR) Multigene Family in Normal Human Adult Tissues. *J. Histochem. Cytochem.* **1997**, *45*, 1005−1019.

(15) Parodi, A.; Quattrocchi, N.; van de Ven, A. L.; Chiappini, C.; Evangelopoulos, M.; Martinez, J. O.; Brown, B. S.; Khaled, S. Z.; Yazdi, I. K.; Enzo, M. V.; Isenhart, L.; Ferrari, M.; Tasciotti, E. Synthetic Nanoparticles Functionalized with Biomimetic Leukocyte Membranes Possess Cell-Like Functions. *Nat. Nanotechnol.* **2013**, *8*, 61−68.

(16) Hine, C. M.; Seluanov, A.; Gorbunova, V. Use of the Rad51 Promoter for Targeted Anti-Cancer Therapy. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 20810−20815.

(17) Gu, J.; Kagawa, S.; Takakura, M.; Kyo, S.; Inoue, M.; Roth, J. A.; Fang, B. Tumor-Specific Transgene Expression from the Human Telomerase Reverse Transcriptase Promoter Enables Targeting of the Therapeutic Effects of the Bax Gene to Cancers. *Cancer Res.* **2000**, *60*, 5359−5364.

(18) Barnett, B. G.; Tillman, B. W.; Curiel, D. T.; Douglas, J. T. Dual Targeting of Adenoviral Vectors at the Levels of Transduction and Transcription Enhances the Specificity of Gene Expression in Cancer Cells. *Mol. Ther.* **2002**, *6*, 377−385.

(19) Barker, S. D.; Dmitriev, I. P.; Nettelbeck, D. M.; Liu, B.; Rivera, A. A.; Alvarez, R. D.; Curiel, D. T.; Hemminki, A. Combined Transcriptional and Transductional Targeting Improves the Specificity and Efficacy of Adenoviral Gene Delivery to Ovarian Carcinoma. *Gene Ther.* **2003**, *10*, 1198−1204.

(20) Müller, O. J.; Leuchs, B.; Pleger, S. T.; Grimm, D.; Franz, W.-M.; Katus, H. A.; Kleinschmidt, J. A. Improved Cardiac Gene Transfer by Transcriptional and Transductional Targeting of Adeno-Associated Viral Vectors. *Cardiovasc. Res.* **2006**, *70*, 70−78.

(21) Nahde, T.; Müller, K.; Fahr, A.; Müller, R.; Brüsselbach, S. Combined Transductional and Transcriptional Targeting of Melanoma Cells by Artificial Virus-Like Particles. *J. Gene Med.* **2001**, *3*, 353−361.

(22) Li, S.-D.; Huang, L. Non-Viral Is Superior to Viral Gene Delivery. *J. Controlled Release* **2007**, *123*, 181−183.
(23) Mintzer, M. A.; Simanek, E. E. Nonviral Vectors for Gene Delivery. *Chem. Rev.* **2009**, *109*, 259−302.
(24) Karin, M.; Cao, Y.; Greten, F. R.; Li, Z.-W. NF-kappaB in Cancer: From Innocent Bystander to Major Culprit. *Nat. Rev. Cancer* **2002**, *2*, 301−310.
(25) Baldwin, A. S. Control of Oncogenesis and Cancer Therapy Resistance by the Transcription Factor NF-κB. *J. Clin. Invest.* **2001**, *107*, 241−246.
(26) Bivona, T. G.; Hieronymus, H.; Parker, J.; Chang, K.; Taron, M.; Rosell, R.; Moonsamy, P.; Dahlman, K.; Miller, V. A.; Costa, C.; Hannon, G.; Sawyers, C. L. FAS and NF-κB Signalling Modulate Dependence of Lung Cancers on Mutant EGFR. *Nature* **2011**, *471*, 523−526.
(27) Adil, M.; Belur, L.; Pearce, T. R.; Levine, R. M.; Tisdale, A. W.; Sorenson, B. S.; McIvor, R. S.; Kokkoli, E. PR_b Functionalized Stealth Liposomes for Targeted Delivery to Metastatic Colon Cancer. *Biomater. Sci.* **2013**, *1*, 393−401.
(28) Mardilovich, A.; Craig, J. A.; McCammon, M. Q.; Garg, A.; Kokkoli, E. Design of a Novel Fibronectin-Mimetic Peptide-Amphiphile for Functionalized Biomaterials. *Langmuir* **2006**, *22*, 3259−3264.
(29) Shroff, K.; Pearce, T. R.; Kokkoli, E. Enhanced Integrin Mediated Signaling and Cell Cycle Progression on Fibronectin Mimetic Peptide Amphiphile Monolayers. *Langmuir* **2012**, *28*, 1858−1865.
(30) Pangburn, T. O.; Georgiou, K.; Bates, F. S.; Kokkoli, E. Targeted Polymersome Delivery of siRNA Induces Cell Death of Breast Cancer Cells Dependent Upon Orai3 Protein Expression. *Langmuir* **2012**, *28*, 12816−12830.
(31) Petersen, M. A.; Hillmyer, M. A.; Kokkoli, E. Bioresorbable Polymersomes for Targeted Delivery of Cisplatin. *Bioconjugate Chem.* **2013**, *24*, 533−543.
(32) Garg, A.; Kokkoli, E. pH-Sensitive PEGylated Liposomes Functionalized with a Fibronectin-Mimetic Peptide Show Enhanced Intracellular Delivery to Colon Cancer Cell. *Curr. Pharm. Biotechnol.* **2011**, *12*, 1135−1143.
(33) Atchison, N. A.; Fan, W.; Papas, K. K.; Hering, B. J.; Tsapatsis, M.; Kokkoli, E. Binding of the Fibronectin-Mimetic Peptide, PR_b, to $\alpha_5\beta_1$ on Pig Islet Cells Increases Fibronectin Production and Facilitates Internalization of PR_b Functionalized Liposomes. *Langmuir* **2010**, *26*, 14081−14088.
(34) Garg, A.; Tisdale, A. W.; Haidari, E.; Kokkoli, E. Targeting Colon Cancer Cells Using PEGylated Liposomes Modified with a Fibronectin-Mimetic Peptide. *Int. J. Pharm.* **2009**, *366*, 201−210.
(35) Demirgöz, D.; Pangburn, T. O.; Davis, K. P.; Lee, S.; Bates, F. S.; Kokkoli, E. PR_b-Targeted Delivery of Tumor Necrosis Factor-α by Polymersomes for the Treatment of Prostate Cancer. *Soft Matter* **2009**, *5*, 2011−2019.
(36) Pangburn, T. O.; Bates, F. S.; Kokkoli, E. Polymersomes Functionalized via "Click" Chemistry with the Fibronectin Mimetic Peptides PR_b and GRGDSP for Targeted Delivery to Cells with Different Levels of $\alpha_5\beta_1$ Expression. *Soft Matter* **2012**, *8*, 4449−4461.
(37) Demirgöz, D.; Garg, A.; Kokkoli, E. PR_b-Targeted PEGylated Liposomes for Prostate Cancer Therapy. *Langmuir* **2008**, *24*, 13518−13524.
(38) Gong, J.; Wang, D.; Sun, L.; Zborowska, E.; Willson, J. K. V; Brattain, M. G. Role of $\alpha_5\beta_1$ Integrin in Determining Malignant Properties of Colon Carcinoma Cells. *Cell Growth Differ.* **1997**, *8*, 83−90.
(39) Darribère, T.; Skalski, M.; Cousin, H. L.; Gaultier, A.; Montmory, C.; Alfandari, D. Integrins: Regulators of Embryogenesis. *Biol. Cell* **2000**, *92*, 5−25.
(40) Jayne, D. G.; Heath, R. M.; Dewhurst, O.; Scott, N.; Guillou, P. J. Extracellular Matrix Proteins and Chemoradiotherapy: Alpha5beta1 Integrin as a Predictive Marker in Rectal Cancer. *Eur. J. Surg. Oncol.* **2002**, *28*, 30−36.
(41) Zhang, Y.; Schulte, W.; Pink, D.; Phipps, K.; Zijlstra, A.; Lewis, J. D.; Waisman, D. M. Sensitivity of Cancer Cells to Truncated Diphtheria Toxin. *PLoS One* **2010**, *5*, e10498.
(42) Huang, Y.-H.; Zugates, G. T.; Peng, W.; Holtz, D.; Dunton, C.; Green, J. J.; Hossain, N.; Chernick, M. R.; Padera, R. F.; Langer, R.; Anderson, D. G.; Sawicki, J. A. Nanoparticle-Delivered Suicide Gene Therapy Effectively Reduces Ovarian Tumor Burden in Mice. *Cancer Res.* **2009**, *69*, 6184−6191.
(43) Greenfield, L.; Bjorn, M. J.; Horn, G.; Fong, D.; Buck, G. A.; Collier, R. J.; Kaplan, D. A. Nucleotide Sequence of the Structural Gene for Diphtheria Toxin Carried by Corynebacteriophage Beta. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6853−6857.
(44) Levine, R. M.; Pearce, T. R.; Adil, M.; Kokkoli, E. Preparation and Characterization of Liposome-Encapsulated Plasmid DNA for Gene Delivery. *Langmuir* **2013**, *29*, 9208−9215.
(45) Livak, K. J.; Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods* **2001**, *25*, 402−408.
(46) Belur, L. R.; McIvor, R. S.; Wilber, A. Liver-Directed Gene Therapy Using the Sleeping Beauty Transposon System. *Methods Mol. Biol.* **2008**, *434*, 267−276.
(47) Hine, C. M.; Seluanov, A.; Gorbunova, V. Rad51 Promoter-Targeted Gene Therapy Is Effective for In Vivo Visualization and Treatment of Cancer. *Mol. Ther.* **2011**, *20*, 347−355.
(48) Pang, S. Targeting and Eradicating Cancer Cells by a Prostate-Specific Vector Carrying the Diphtheria Toxin A Gene. *Cancer Gene Ther.* **2000**, *7*, 991−996.
(49) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. A Two-Stage Poly(ethylenimine)-Mediated Cytotoxicity: Implications for Gene Transfer/Therapy. *Mol. Ther.* **2005**, *11*, 990−995.
(50) Morimoto, H.; Bonavida, B. Diptheria Toxin - and Pseudomonas A Toxin-Mediated Apoptosis ADP Ribosylation of Elongation Factor-2 Is Required for DNA Fragmentation and Cell Lysis and Synergy with Tumor Necrosis Factor-a. *J. Immunol.* **1992**, *149*, 2089−2094.
(51) Porter, D. L.; Kalos, M.; Zheng, Z.; Levine, B.; June, C. Chimeric Antigen Receptor Therapy for B-Cell Malignancies. *J. Cancer* **2011**, *2*, 331−332.
(52) Wang, K.; Wei, G.; Liu, D. CD19: A Biomarker for B Cell Development, Lymphoma Diagnosis and Therapy. *Exp. Hematol. Oncol.* **2012**, *1*, 36−43.
(53) Xie, W.; Wang, X.; Du, W.; Liu, W.; Qin, X.; Huang, S. Detection of Molecular Targets on the Surface of CD34+CD38- Bone Marrow Cells in Myelodysplastic Syndromes. *Cytometry* **2010**, *77*, 840−848.
(54) Zhu, Z. B.; Makhija, S. K.; Lu, B.; Wang, M.; Kaliberova, L.; Liu, B.; Rivera, A. A.; Nettelbeck, D. M.; Mahasreshti, P. J.; Leath, C. A.; Barker, S.; Yamaoto, M.; Li, F.; Alvarez, R. D.; Curiel, D. T. Transcriptional Targeting of Tumors with a Novel Tumor-Specific Survivin Promoter. *Cancer Gene Ther.* **2004**, *11*, 256−262.
(55) Sidi, A. A.; Ohana, P.; Benjamin, S.; Shalev, M.; Ransom, J. H.; Lamm, D.; Hochberg, A.; Leibovitch, I. Phase I/II Marker Lesion Study of Intravesical BC-819 DNA Plasmid in H19 over Expressing Superficial Bladder Cancer Refractory to Bacillus Calmette-Guerin. *J. Urol.* **2008**, *180*, 2379−2383.
(56) Li, F.; Altieri, D. C. Transcriptional Analysis of Human Survivin Gene Expression. *Biochem. J.* **1999**, *344*, 305−311.
(57) Banet, G.; Bibi, O.; Matouk, I.; Ayesh, S.; Laster, M.; Kimber, K. M.; Tykocinski, M.; de Groot, N.; Hochberg, A.; Ohana, P. Characterization of Human and Mouse H19 Regulatory Sequences. *Mol. Biol. Rep.* **2000**, *27*, 157−165.
(58) Tkacik, G.; Gregor, T.; Bialek, W. The Role of Input Noise in Transcriptional Regulation. *PLoS One* **2008**, *3*, e2774.
(59) Chalancon, G.; Ravarani, C. N. J.; Balaji, S.; Martinez-Arias, A.; Aravind, L.; Jothi, R.; Babu, M. M. Interplay Between Gene Expression Noise and Regulatory Network Architecture. *Trends Genet.* **2012**, *28*, 221−232.

(60) Liu, Y.; Cox, S. R.; Morita, T.; Kourembanas, S. Hypoxia Regulates Vascular Endothelial Growth Factor Gene Expression in Endothelial Cells: Identification of a 5′ Enhancer. *Circ. Res.* **1995**, *77*, 638−643.
(61) Adil, M. M.; Erdman, Z. S.; Kokkoli, E. Transfection Mechanisms of Polyplexes, Lipoplexes, and Stealth Liposomes in $\alpha_5\beta_1$ Integrin Bearing DLD-1 Colorectal Cancer Cells. *Langmuir* **2014**, submitted.
(62) Paszek, P.; Ryan, S.; Ashall, L.; Sillitoe, K.; Harper, C. V; Spiller, D. G.; Rand, D. A.; White, M. R. H. Population Robustness Arising from Cellular Heterogeneity. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 11644−11649.
(63) Ohana, P.; Schachter, P.; Ayesh, B.; Mizrahi, A.; Birman, T.; Schneider, T.; Matouk, I.; Ayesh, S.; Kuppen, P. J. K.; De Groot, N.; Czerniak, A.; Hochberg, A. Regulatory Sequences of H19 and IGF2 Genes in DNA-Based Therapy of Colorectal Rat Liver Metastases. *J. Gene Med.* **2005**, *7*, 366−374.
(64) Wang, C. Y.; Cusack, J. C.; Liu, R.; Baldwin, A. S. Control of Inducible Chemoresistance: Enhanced Anti-Tumor Therapy through Increased Apoptosis by Inhibition of NF-κB. *Nat. Med.* **1999**, *5*, 412−417.
(65) Bentires-Alj, M.; Hellin, A. C.; Ameyar, M.; Chouaib, S.; Merville, M. P.; Bours, V. Stable Inhibition of NF-κB in Cancer Cells Does Not Increase Sensitivity to Cytotoxic Drugs. *Cancer Res.* **1999**, *59*, 811−815.
(66) Makarov, S. NF-kappaB in Rheumatoid Arthritis: a Pivotal Regulator of Inflammation, Hyperplasia, and Tissue Destruction. *Arthritis Res.* **2001**, *3*, 200−206.
(67) Gagliardo, R.; Chanez, P.; Mathieu, M.; Bruno, A.; Costanzo, G.; Gougat, C.; Vachier, I.; Bousquet, J.; Bonsignore, G.; Vignola, A. M. Persistent Activation of Nuclear Factor-kappaB Signaling Pathway in Severe Uncontrolled Asthma. *Am. J. Respir. Crit. Care Med.* **2003**, *168*, 1190−1198.