Image-Guided Breast Tumor Therapy Using a Small Interfering RNA Nanodrug

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Abstract

Iron oxide nanoparticles offer a feasible tool for combined imaging and delivery of small interfering RNA (siRNA) to tumors, stimulating active interest in exploring different imaging and delivery platforms suitable for detection by a variety of modalities. In this study, we describe the synthesis and testing of a tumor-targeted nanodrug (MN-EPPT-siBIRC5) that is designed to specifically shuttle siRNA to human breast tumors. The nanodrug binds the tumor-specific antigen uMUC-1, which is found in >90% of human breast adenocarcinomas. MN-EPPT-siBIRC5 consists of superparamagnetic iron oxide nanoparticles [for magnetic resonance imaging (MRI)], the dye Cy5.5 (for near-IR optical imaging), peptides (EPPT) that specifically target uMUC-1, and a synthetic siRNA that targets the tumor-specific antiapoptotic gene BIRC5. Nanodrug uptake by human breast adenocarcinoma cells resulted in a significant downregulation of BIRC5. Following i.v. delivery into subcutaneous mouse models of breast cancer, the nanodrug showed a preferential tumor uptake, which could be visualized by MRI and near-IR optical imaging. Furthermore, MRI could be used to quantitatively monitor nanodrug bioavailability in the tumor tissue throughout the course of treatment. Intravenous injection of the agent once a week over 2 weeks resulted in the induction of considerable levels of necrosis and apoptosis in the tumors, translating into a significant decrease in tumor growth rate. Our strategy permits the simultaneous tumor-specific delivery of siRNA to tumors and the imaging of the delivery process. More generally, it illustrates the potential to apply this approach to many human cancer studies, including for basic tumor biology and therapy. Cancer Res; 70(19); 7553–61. ©2010 AACR.

Introduction

RNA interference (RNAi) holds considerable potential as a molecular therapeutic tool due to its broad applicability and exquisite specificity (1–3). The main hurdle to the application of RNAi in vivo is the difficulty in achieving efficient delivery of the small interfering RNA (siRNA) to the target tissue due to phenomena such as RNase degradation, interaction with blood components, and insufficient translocation across the cell membrane. To address these obstacles, many approaches have been proposed for in vivo siRNA delivery. They include liposome-mediated delivery of siRNA in stable nucleic acid–lipid particles (4), polymer-based delivery using atelocollagen and chitosan (5, 6), conjugation to cholesterol (7, 8), and complexing with positively charged peptides or proteins (9–11), to name a few. Various nanoparticle carriers have been proposed for siRNA delivery in vitro (12–14) and in vivo (refs. 15, 16; also reviewed in ref. 17).

To evaluate the success of siRNA-mediated therapy, it is also very important to monitor its bioavailability following in vivo administration as well as the associated therapeutic effect because this will help to develop more successful delivery strategies. In this regard, noninvasive imaging plays an important role as a technology that permits the in vivo monitoring of siRNA delivery.

Magnetic resonance imaging (MRI) represents a suitable modality for this purpose because it is characterized by a high spatial resolution, tomographic capability, and the potential to provide quantitative information about contrast agent abundance in tissue (18). At present, few reports have described the application of MRI for image-guided siRNA delivery in vitro (19) and in vivo (20–22).

Previously, we have shown the feasibility of MRI-guided siRNA delivery to tumors using myristoylated polyarginine-conjugated magnetic nanoparticles (MN) that accumulate in tumor tissue through enhanced permeability and retention effect (20). Despite their capability to mediate very efficient silencing in vivo, these nanoparticles exhibited a high degree of nonspecific uptake, especially by the liver, leading to a more rapid degradation and a reduced effective dose of the agent at the tumor site. A more biologically relevant approach toward improving the bioavailability of the siRNA complex would involve the implementation of a tumor-targeted...
design. This is a necessary step to transform our original delivery agent into an effective, clinically relevant nanodrug.

In the present study, we address this issue by constructing a tumor-selective probe (MN-EPPT-siBIRC5), which consists of MN (for MRI), labeled with near-IR dye Cy5.5 (for optical imaging) and conjugated to a peptide (EPPT), which targets the tumor-specific antigen uMUC-1, as well as to siRNA against the antiapoptotic gene birc5, which encodes survivin. We have previously established the in vivo tumor-targeting properties of the MN-EPPT platform in a variety of adenocarcinoma models, including breast cancer (23–25). Our present results show the application of this platform for the efficient image-guided delivery of siRNA to breast tumors and the mediation of a robust therapeutic effect, illustrating the potential of this agent as a novel cancer nanodrug.

Materials and Methods

Nanodrug synthesis

The EPPT peptide was synthesized by general Fmoc chemistry using 2-6H-benzotriazole-1-yl-9,1,3,3-tetramethylammonium hexafluorophosphate and 1-hydroxybenzotriazole activating agents. Amino acids and resin were purchased from EMD Chemicals. All other reagents were purchased from Advanced Chemtech, Sigma-Aldrich, GE Life Sciences, and Fisher Scientific and used without further purifications. The sequence Cys-(PEG)2-Tyr-Cys(Acm)-Ala-Arg-Pro-Pro-Thr-Arg-Thr-Phe-Ala-Tyr-Trp-Gly-Lys(FITC)-CONH2 (EPPT) was synthesized in a 0.1 mmol scale of rink amide methylbenzyl hydroxamine resin. The Lys (Dde) side chain on the resin was selectively cleaved using 2% hydrazine in dimethylformamide and coupled with FITC. Finally, the resin was cleaved by 5 mL of cocktail mixture [81.5% trifluoroacetic acid (TFA), 5% thioanisole, 5% phenol, 5% water, 2.5% ethanedithiol, and 1% triisopropylsilane], precipitated in cold ether, purified by high-performance liquid chromatography using eluting solvents A and B (A = 0.1% TFA in 95% water + 5% acetonitrile and B = 0.1% TFA in 90% acetonitrile + 10% water), and characterized by matrix-assisted laser desorption/ionization mass spectrometry (2,827.17 calculated and 2,828.60 found).

The parental MNs (cross-linked dextran-coated superparamagnetic iron oxide nanoparticles) were synthesized as described (26).

The subsequent steps are outlined in Fig. 1A. The MN-Cy5.5 precursor was synthesized as described (23, 25). The targeting EPPT peptide was coupled to MN-Cy5.5 for the resultant MN-EPPT precursor probe using a protocol modified from previous reports (23, 25). Briefly, the nanoparticles were...
conjugated to the heterobifunctional cross-linker N-succinimidyl 3-(2-pyridyldithio) propionate (Pierce Biotechnology), and the product was purified using a Sephadex PD-10 column (GE Life Sciences) against a 100 mmol/L sodium phosphate solution (pH 8.1). The peptide EPPT was attached to this linker via the sulfhydryl reactive pyridyl disulfide residue in a 100 mmol/L sodium phosphate solution (pH 8.1).

The siRNA oligos were then conjugated to MN-EPPT using a protocol modified from previous reports (20, 27). 5′-Sense thiol-modified birc5-targeting and scrambled siRNA duplexes were designed and synthesized by Dharmacon. For conjugation, the siRNA was deprotected before coincubation with the MNs, as recommended by the supplier (Dharmacon). Briefly, the duplex was deprotected with a solution of 3% Tris (2-carboxy-ethyl)-phosphin-HCl followed by a 9.5 mol/L ammonium acetate/ethyl alcohol precipitation. Following deprotection and precipitation, the siRNA was dissolved in 50 mmol/L NaCl and 10 mmol/L EDTA (pH 8) and incubated with the MNs overnight at 4°C. The final nanodrug was purified using a G-50 Sephadex Quick Spin column (Roche Applied Science).

**Nanodrug characterization**

The nanoparticle concentration was determined based on iron concentration and measured spectrophotometrically, as described (28). The conjugation ratio of Cy5.5 and EPPT-FITC to MN was quantified spectrophotometrically, as described (16). The number of EPPT-FITC peptides and Cy5.5 molecules per nanoparticle was obtained from absorbance at 494 nm [extinction coefficient, 68,000 (mol/L) cm⁻¹] and 678 nm [extinction coefficient, 250,000 (mol/L) cm⁻¹], respectively. To characterize the conjugation efficiency and dissociation stoichiometry of siRNA from MN, we performed gel electrophoresis of MN-EPPT-siRNA, following incubation in the reducing buffer DTT (100 mmol/L DTT for 1 hour at 37°C). The siRNA standard and the nanodrug treated with the reducing agent were electrophoresed on a 15% Tris-borate 0.1 mol/L glycerol gel (Invergent) at 200 V for 45 minutes. After electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide for 30 minutes and visualized using a Molecular Imager FX scanner (Bio-Rad). The images were analyzed using the software Quantity One, version 4.4.0 (Bio-Rad).

**Cell line**

All of the studies were performed using the BT-20 and/or CAPAN-2 and LS-174T cell lines [human breast, pancreatic, and colorectal adenocarcinoma; American Type Culture Collection (ATCC) HTB-19, HTB-80, and CL-188, respectively]. The cell lines were authenticated based on viability, recovery, growth, morphology, and isoenzymology by the supplier (ATCC).

**Flow cytometry**

Nanodrug uptake by BT-20, CAPAN-2, and LS-174T cells was analyzed using flow cytometry. Cells were incubated with MN-EPPT-siBIRC5 for 48 hours at 37°C, washed, and analyzed in the FL4 channel (Cy5.5, MN) and the FL2 channel (Dy547, siRNA). To correlate nanodrug uptake to uMUC-1 expression, after incubation with the nanodrug, the cells were fixed in 2% paraformaldehyde, incubated for 1 hour at 4°C with a uMUC-1–specific monoclonal antibody (FITC-labeled mouse anti-human CD227; BD Biosciences), and analyzed in the FL4 channel (Cy5.5, MN) and the FL1 channel (FITC, uMUC-1–specific antibody). Flow cytometry was performed using a FACSCalibur (Becton Dickinson) equipped with the CellQuest software package (Becton Dickinson).

**MRI of cell phantoms**

BT-20 cells were treated as described for flow cytometry. The cells were then pelleted in 0.2 mL PCR tubes and imaged. Imaging was performed using a 9.4-T Bruker horizontal bore scanner equipped with ParaVision 3.0 software. The imaging protocol consisted of coronal T2-weighted spin echo (SE) pulse sequences with the following parameters: SE repetition time/echo time (TE) = 3,000/[8, 16, 24, 32, 40, 48, 56, 64]; field of view = 32 × 32 mm; matrix size = 128 × 128 pixels; slice thickness = 0.5 mm; in-plane resolution = 250 × 250 μm². Image reconstruction and analysis were performed using the Marevisi 3.5 software (Institute for Biodiagnostics, National Research Council, Canada). T2 maps were constructed according to established protocol by fitting the T2 values for each of the eight TEIs to a standard exponential decay curve.

T2 relaxation times were calculated by manually segmenting out the cell pellet on MR images.

**Real-time quantitative reverse transcription-PCR**

To assess birc5 knockdown by the nanodrug, BT-20, CAPAN-2, and LS-174T cells were incubated with MN-EPPT-siBIRC5 or the control MN-EPPT-siSCR (100 μg/mL iron) for 48 hours at 37°C. Total RNA was extracted using the RNeasy Mini kit, according to the manufacturer’s protocol (Qiagen, Inc.). Relative levels of uMUC-1 mRNA were determined by real-time quantitative reverse transcription-PCR (qRT-PCR; Taqman protocol). Taqman analysis was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems). The PCR primers and Taqman probe specific for MUC-1 mRNA were designed using Primer Express software 1.5. Primer and probe sequences were as follows:

- forward primer, 5′-ACAGGTTCTGTGATGCAAGC-3′ (nucleotides 64–84 in the 5′ nonrepetitive region);
- reverse primer, 5′-CTCACAGGTCTCTCAGTGGAC-3′ (nucleotides 139–164 in the 5′ nonrepetitive region); and
- Taqman probe, 5′-FAM-TGGAGAAAAGGACTTCGGC-TACCAGA-TAMRA-3′ (nucleotides 96–124 in the 5′ nonrepetitive region).

Eukaryotic 18S rRNA Taqman PDAR Endogenous Control reagent mix (PE Applied Biosystems) was used to amplify 18S rRNA as an internal control, according to the manufacturer’s protocol.

**Tumor model**

Five- to 6-week-old female nu/nu mice (n = 8; Massachusetts General Hospital Radiation Oncology breeding facilities) were injected s.c. with 3 × 10⁸ BT-20 tumor cells (ATCC). Animals were used in experiments on days 10 to 14 after the inoculation, when tumors were ∼0.5 cm in diameter.
All animal experiments were performed in compliance with institutional guidelines and according to the animal protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

**Animal treatment**

Treatment with MN-EPPT-siBIRC5 or the control MN-EPPT-siSCR probe involved systemic administration through the tail vein at a dose of 10 mg/kg Fe and 400 nmol/kg siRNA once a week over the course of 2 weeks. Based on our previous biodistribution data, the amount of probe/siRNA delivered to the tumor with each injection is ∼2 mg/kg Fe, which is equivalent to ∼8 nmol/kg siRNA.

**In vivo MRI**

MRI was performed before and 24 hours after each nanodrug injection using a 9.4-T Bruker horizontal bore scanner equipped with ParaVision 3.0 software. The imaging sequences were the same as for in vitro MRI. Image reconstruction and analysis were performed as described for in vitro MRI.

Tumor volumes and T2 relaxation times were calculated by manually segmenting out the tumor on MR images. Quantitative evaluation of differential tumor growth by MRI was based on multislice T2-weighted images. The volume was estimated by adding up the number of voxels occupied by tumor and multiplying by voxel volume. For T2 map analysis of relaxation times, the terminal slices were not included to avoid interference from partial volume effects.

**In vivo and ex vivo optical imaging**

In vivo optical imaging was performed immediately after each MRI session.

Animals were placed prone into a whole-body animal imaging system (IVIS Spectrum, Caliper Life Sciences), equipped with 10 narrow band excitation filters (30-nm bandwidth) and 18 narrow band emission filters (20-nm bandwidth) that assist in significantly reducing autofluorescence by the spectral scanning of filters and the use of spectral unmixing algorithms (Caliper Life Sciences). Imaging was performed using a 675-nm excitation and a 720-nm emission filter. The fluorescence imaging settings (exposure time, 0.5 seconds; F-stop, 2; binning, medium) were kept constant for comparative analysis. Grayscale white-light photographs and epifluorescent images were acquired and superimposed. For ex vivo imaging, excised tumors and adjacent muscle tissue were placed in the optical imaging system and imaged as above. The images were reconstructed using the Living Image software version 3.1 (Caliper Life Sciences).

![Figure 2.](image-url)
**Immunohistochemistry and *in situ* apoptosis detection**

To detect the nanodrug in tumor tissues, tumors were embedded in Tissue-Tek OCT compound (Sakura Finetek) and snap frozen in liquid nitrogen. Tumors were then cut into 7-μm frozen sections, fixed in 2% paraformaldehyde, washed, counterstained with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and analyzed by fluorescence microscopy. Microscopy was performed using a Nikon Eclipse 50i fluorescence microscope equipped with an appropriate filter set (Chroma Technology Corp.). Images were acquired using a charge-coupled device camera with near-IR sensitivity (SPOT 7.4 Slider RTKE; Diagnostic Instruments) and analyzed using SPOT 4.0 advanced version software (Diagnostic Instruments). Fluorescence was collected in the green channel for detection of the FITC label on EPPT peptides, the blue channel for DAPI, and the NIR channel for detection of the Cy5.5 label on MN nanoparticles.

To evaluate levels of apoptosis in tumor cells, we performed a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (ApopTag Fluorescein In situ Apoptosis Detection kit, Chemicon International) according to the manufacturer’s protocol. The nuclei were counterstained with DAPI and examined in the DAPI (nuclei) and FITC (apoptotic nuclei) channels. For quantitative analysis of apoptotic levels, a total of 100 nuclei over two separate slides were taken into account. To evaluate levels of necrosis, we stained sections with H&E and analyzed them by light microscopy.

**Statistical analysis**

All data were represented as means ± SD. Statistical analysis was performed using two-tailed Student’s *t* test and linear regression where indicated. A *P* value of ≤0.05 was considered statistically significant.

**Results**

**MN-EPPT-siRNA synthesis**

The MN-EPPT-siRNA (BIRC5/SCR) probes consisted of dextran-coated MNs, triple conjugated to Cy5.5, EPPT, and siRNA (Fig. 1B). A ratio of four Cy5.5 molecules and eight EPPT peptides per nanoparticle was obtained. To characterize the conjugation efficiency and dissociation stoichiometry of siRNA from MN, we performed gel electrophoresis of MN-EPPT-siRNA, following incubation in the reducing buffer DTT. The resultant siRNA/MN molar ratios reached 7 for MN-EPPT-siBIRC5 and 7.15 for MN-EPPT-siSCR (Fig. 1C).

**In vitro uptake and silencing efficacy of MN-EPPT-siBIRC5**

To assess the cellular uptake of MN-EPPT-siBIRC5 by human BT-20 breast adenocarcinoma cells, we performed flow cytometry following incubation with the nanodrug. A representative dot plot is shown in Fig. 2A. A considerable 97.9 ± 0.8% of the cells were labeled with MN-EPPT-siBIRC5. The good cellular colocalization between fluorescence in the FL2 channel (Dy547, siRNA) and the FL4 channel (Cy5.5, MN) indicated stability of the nanodrug on cellular uptake. In addition, to show that the uptake is governed by the expression of MUC-1, we costained the cells with a MUC-1–specific monoclonal antibody. The cellular colocalization between fluorescence in the FL1 channel (MUC-1, FITC) and the FL4 channel (Cy5.5, MN) suggested MUC-1–specific uptake (Fig. 2B).

Similar results were obtained with human pancreatic and colorectal adenocarcinoma cell lines (Supplementary Fig. S1A and B), illustrating the broad applicability of our approach. Because one of our primary goals is to detect nanodrug bioavailability by MRI, we performed *in vitro* T2-weighted MRI on breast cancer cells, following incubation with MN-EPPT-siBIRC5. Quantitative analysis revealed a significant (*P* = 0.001, *n* = 4) shortening of the T2 relaxation times of the cells following incubation with the nanodrug, indicating that the uptake can be visualized and quantified by MRI.
Finally, to see if MN-EPPT-siBIRC5 can mediate effective silencing of the target gene, we isolated total RNA from the breast adenocarcinoma cell lines 48 hours after incubation with the nanodrug and performed quantitative gene expression analysis (qRT-PCR). As seen in Fig. 2D, treatment with MN-EPPT-siBIRC5 led to a significantly lower expression of \textit{birc5} (survivin) relative to the siRNA scrambled, MN-siSCR, probe \((P = 0.004, n = 4)\). Analogous levels of silencing were also seen in human pancreatic and colorectal adenocarcinoma cells (Supplementary Fig. S1C). These findings imply that the MUC-1 tumor antigen–targeted approach to siRNA delivery mediates a robust gene silencing effect.

\textbf{In vivo imaging of MN-EPPT-siBIRC5 delivery to tumors}

To monitor the delivery of the nanodrug to tumors, we performed \textit{in vivo} MRI of mice, implanted s.c. with human breast adenocarcinoma tumors. Superparamagnetic iron oxide nanoparticles are characterized by their strong T2 magnetic susceptibility effects. Their presence in tissue is reflected by marked shortening of T2 relaxation times, resulting in a loss of signal (darkening) on MR images. In this experiment, tumors appeared characteristically bright on precontrast T2 images. Following injection of the nanodrug, there was a notable decrease in T2 relaxation time (signal) associated with the tumors (Fig. 3A), as a result of MN-EPPT-siBIRC5 accumulation. We also monitored tumor T2 relaxation times as a quantitative measure of nanodrug abundance in tumor tissue during the entire course of therapy. Treatment with the nanodrug involved weekly i.v. injections over the course of two weeks. Imaging was performed before and after each injection for a total of four data points. As shown in Fig. 3B, injection of the nanodrug resulted in a significant decrease of tumor T2 relaxation times \((P = 0.02, n = 4)\). Furthermore, after injection, tumor T2 relaxation times remained significantly lower than the preinjection values at all time points (preinjection, \(44.2 \pm 0.8\); postinjection range, \(37.5 \pm 0.6\) to \(28.1 \pm 4.2\); \(P < 0.02; \ n = 4\)). This indicated that any phenotypic effects associated with MN-EPPT-siBIRC5 administration are a function of siRNA activity and are not influenced by a potential differential probe bioavailability.

Finally, to analyze the uptake of the nanodrug by tumor cells following \textit{in vivo} delivery, we performed fluorescence microscopy of frozen tumor sections. The good colocalization between signal originating from the Cy5.5 label on MN and the FITC label on the EPPT peptide indicated that the nanodrug remained intact after persistence in the circulation (Fig. 4C). Taken together, these findings attested to the favorable...
bioavailability of MUC-1–targeted MN-siRNAs delivered as part of the MN complex. They showed that the MN-EPPT-siRNA nanodrug successfully targeted tumors and that this process could be detected by in vivo MRI and optical imaging.

**Therapeutic effects of MN-EPPT-siBIRC5 delivery to tumors**

Because birc5 encodes the antiapoptotic survivin proto-oncogene, silencing of birc5 can mediate a therapeutic effect by inducing necrotic/apoptotic tumor cell death. We tested the therapeutic potential of MN-EPPT-siBIRC5 by H&E staining (necrosis) and TUNEL assay (apoptosis) of tumor sections derived from breast tumor–bearing animals treated with the nanodrug. We observed the induction of considerable levels of both necrosis (Fig. 5A) and apoptosis (Fig. 5A and B). There was a 5-fold increase in the fraction of apoptotic nuclei in experimental tumors versus controls injected with MN-EPPT-siSCR (P = 0.003; Fig. 5B). This translated into a ∼2-fold decrease in tumor growth rate (Fig. 5C). Because there were no differences in the uptake of MN-EPPT-siBIRC5 and MN-EPPT-siSCR (Supplementary Fig. S2), we concluded that the therapeutic effect observed here was due to siBIRC5 action. These data indicated that the siRNA oligo remained functional in vivo as part of the MN-EPPT-siRNA complex, and illustrated the potential of this technology for tumor therapy.

**Discussion**

The discovery of the RNAi mechanism of posttranscriptional gene regulation (29) and its relevance to mammalian gene silencing (30) opened up a new possibility for intervention against disease. Therapeutic strategies that rely on RNAi are unique because they would involve the modular design of RNA duplexes capable of mediating powerful phenotypic reprogramming over a broad range of disease applications and with specificity sufficient to correct single-nucleotide mutations. The interest in developing RNAi into a therapeutic modality has engendered a variety of ideas and approaches that have as their final goal the transformation of the siRNA duplex into a molecular drug. Several of these approaches are undergoing clinical trials (31).

Our review of the literature, however, reveals the apparent lack of clinically relevant image-guided strategies for siRNA-based therapy. Although versatile, most nanoparticle delivery systems do not possess imaging capability. We feel that the “image-guided” property is important because imaging could...
provide key information about the persistence of the agent in the circulation, its relative accumulation in the target tissue, and the associated phenotypic or therapeutic effect.

Here, we illustrate the potential of this approach by designing a tumor-targeted, imaging-capable nanodrug and applying it for MRI-guided breast tumor treatment in a murine xenograft model of the disease. MRI serves the dual purpose of reporting on the relative accumulation of the nanodrug in tumor tissue, as a guide to selecting an optimal treatment time course, and quantifying the change in tumor volume over the course of treatment, as an indicator of therapeutic response.

In addition to being detectable by MRI, our nanodrug is tumor targeted. Tumor targeting is deemed necessary to achieve optimized tumor bioavailability and a minimized nonspecific uptake by vital organs. The tumor selectivity of our nanodrug derives from the tumor-associated uMUC-1 antigen, which is underglycosylated and overexpressed in >90% of breast cancers and in >50% of all cancers in humans (32), attesting to the broad applicability of this delivery strategy.

As a therapeutic moiety, we have used siRNA to the human BIRC5 gene. BIRC5 encodes survivin, a member of the inhibitor of apoptosis protein family, which is highly expressed by most cancers and associated with chemotherapy resistance, increased tumor recurrence, and shorter patient survival, making anti-survivin therapy an attractive cancer treatment strategy (33).

Because of the modular nature of our agent, the described method is not only novel but also highly relevant to a variety of neoplastic and other diseases that can be manipulated at the level of gene expression. Ultimately, we believe that the core elements of the developed technology would be applicable in a clinical setting because related iron oxides are already in clinical use (34).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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