Ultrasound-Targeted Microbubble Destruction to Deliver siRNA Cancer Therapy

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Abstract

Microbubble contrast agents can specifically deliver nucleic acids to target tissues when exposed to ultrasound treatment parameters that mediate microbubble destruction. In this study, we evaluated whether microbubbles and ultrasound-targeted microbubble destruction (UTMD) could be used to enhance delivery of EGF receptor (EGFR)–directed siRNA to murine squamous cell carcinomas. Custom-designed microbubbles efficiently bound siRNA and mediated RNase protection. UTMD-mediated delivery of microbubbles loaded with EGFR-directed siRNA to murine squamous carcinoma cells in vitro reduced EGFR expression and EGFR-dependent growth, relative to delivery of control siRNA. Similarly, serial UTMD-mediated delivery of EGFR siRNA to squamous cell carcinoma in vivo decreased EGFR expression and increased tumor doubling time, relative to controls receiving EGFR siRNA-loaded microbubbles but not ultrasound or control siRNA-loaded microbubbles and UTMD. Taken together, our results offer a preclinical proof-of-concept for customized microbubbles and UTMD to deliver gene-targeted siRNA for cancer therapy. Cancer Res; 72(23); 6191–9. ©2012 AACR.

Introduction

The inhibition of EGF receptor (EGFR) signaling is an established strategy for treating a number of cancer types, including lung cancers, colorectal cancers, and head and neck squamous cell carcinoma (SCC). As such, several drugs have been developed to block growth factor binding, block receptor dimerization, decrease EGFR expression, or reduce downstream kinase activity (1). Pharmaceutical agents such as the monoclonal antibody cetuximab and the kinase inhibitor gefitinib inhibit EGFR signaling and have progressed through clinical trials (1). Despite their promise, these treatment regimens do not fully suppress EGFR signaling, and often must be combined with other treatment modalities to achieve clinical efficacy (2, 3). EGFR inhibition using RNA interference (RNAi) to treat solid tumors has also shown efficacy in some systems (4–6). These studies illustrate the potential potency of EGFR inhibition, but are disadvantaged by their reliance on direct intratumoral injection of therapeutic genes, which is not always technically feasible, and limits the possibility of repeated dosing. Viral vectors have been used to deliver therapeutic genes, but their use increases the possibility of off-target effects and limits the option for multiple treatments (7). Delivery of genes that express RNAi is limited by inefficient DNA transduction, particularly in nonviral systems that do not involve a direct injection into the target site (8–12). The direct injection of naked siRNA is attractive but can be limited by ubiquitous RNase activity (13). An ideal therapeutic approach would be capable of specifically delivering the therapeutic payload to tumors, protecting the payload from nuclease digestion, and amenable to multiple treatments.

Microbubble contrast agents are emerging nonviral vector systems capable of binding nucleic acids, and that may overcome some of the limitations of current siRNA delivery systems (10, 14, 15). The use of microbubbles as nucleic acid delivery vectors is based on the observation that the rupture of microbubbles via ultrasound-targeted microbubble destruction (UTMD) results in deposition of microbubble shell components and increased cell membrane permeability localized to the site of microbubble–ultrasound interaction. It is therefore hypothesized that if these microbubble shells carry therapeutic agents, the effects of the agents would be limited to the ionized area (16). This phenomenon has been exploited to deliver reporter genes both in vitro and in vivo. We and others have recently shown that UTMD can deliver therapeutic genes to tumors, pancreatic cells, and myocardium (10, 14, 17–20).

In the present study, we sought to use UTMD to achieve RNAi as a strategy for tumor growth suppression. Specifically, we tested the hypothesis that UTMD-mediated delivery of anti-EGFR siRNA (EGFR–siRNA) will inhibit SCC growth in mice. In vitro and in vivo studies were conducted to confirm downregulation of EGFR by our delivery system. Thereafter, EGFR–siRNA–loaded microbubbles were delivered to murine tumors via UTMD, and tumor growth was measured.
Materials and Methods

Microbubble preparation and confirmation of RNA loading

Chloroform solutions of 1,2-distearyl-sn-glycero-3-phosphocholine, 1,2-distearyl-sn-glycero-3-ethylphosphocholine, 1,2-distearyl-sn-glycero-3-phosphoglycerol (Avanti Lipids), and polyethylene glycol-40 stearate (Sigma) at a molar ratio 100:43:1:4.5 were dried with argon gas. An aqueous micellar solution of lipid was then prepared by adding PBS containing 1 mmol/L EDTA to the dry lipid film to achieve a concentration of 1.7 mg total lipid/mL, and the mixture was sonicated using a Sonicator XL ultrasonic processor (Misonix) at power setting 5 to disperse lipids. The resulting lipid solution was diluted 1:4 in PBS/EDTA, placed in a vial with siRNA, the headspace was filled with perfluorobutane gas, and the solution was amalgamated using a dental amalgamator to form perfluorobutane gas-filled microbubbles (10). Microbubbles were washed until the subnatant was clear (2–4 washes at 53 relative centrifugal force), resulting in a microbubble concentration of 7 × 10⁶/mL and mean diameter of 2.1 ± 1.1 μm (range of 1.7–2.4 μm) as measured by Coulter counting (Beckman Coulter).

siRNA (Ambion) consisted of 2 distinct EGFR Silencer Select predesigned siRNAs (ID#65373 and ID#65372) and Silencer Select negative control sequence #1 (control siRNA). Silencer Select siRNAs are manufactured with locked nucleic acid modifications known to reduce or eliminate innate immune response to RNA (21, 22), and the sequence was selected to exclude immunostimulatory sequences (23). The manufacturer has reported a lack of off-target activity in control siRNA by microarray following transfection.

RNA binding to the microbubbles was confirmed by serial washing of microbubbles with PBS/EDTA, followed by extraction of RNA by phenol/chloroform, electrophoresis, and detection of siRNA by ethidium-bromide fluorescence. siRNA was quantified in comparison with lanes loaded with known amounts of siRNA. To determine resistance to RNAse, siRNA-loaded microbubbles were exposed to RNAse A (0.04–4 mg/mL in PBS, at 37° C, 15 minutes), in a separate set of experiments, followed by RNA extraction and electrophoresis.

In vitro studies of UTMD-mediated EGFR knockdown

EGFR knockdown by EGFR–siRNA following lipofectamine transfection or UTMD-mediated siRNA delivery was determined in vitro using mouse SCC-VII cells cultured in RPMI-1640 supplemented with 10% FBS (24). SCC-VII cells were directly prepared from SCC tumors harvested from freshly injected mice. Transfections of SCC-VII cells were conducted in 60-mm dishes using 100 pmol/L siRNA (2 EGFR and 1 control) and 5 μL lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions. Following transfection, cells were grown in RPMI with 1.5% FBS to limit growth, and EGFR knockdown was assessed 3 days after real-time polymerase chain reaction (RT-PCR). For UTMD, microbubbles (1–2 × 10⁷) bearing 100 pg siRNA (EGFR–siRNA ID#65373 or control siRNA) or no siRNA were added to a 60-mm dish seeded with 1 × 10⁶ SCC-VII cells. The dishes were inverted to permit microbubble (MB)/cell contact via MB buoyancy and continuously sonicated at 1.3 MHz and mechanical index (MI) of 1.6 (Sonos 7500, Philips) for 2 minutes with manual sweeping to ensure insonification of the entire sample. Following UTMD, cells were grown in RPMI with 1.5% FBS and 10 ng/mL EGF to create conditions in which growth was dependent upon supplemental EGF. EGFR knockdown at 2 days after UTMD was functionally assessed by quantifying the number of viable cells as defined by Trypan blue exclusion assay (25). Western blot analyses were conducted 3 days after sonoporation of SCC-VII cells using anti-EGFR antibody (1:1,500, BD Bioscience, cat#610017) and anti-mouse horseradish peroxidase–conjugated antibody (1:8,000, Bio-Rad, cat#170-6516). Protein bands were quantified from scanned autoradiography film and expression was quantified as fold over β-actin.

Murine squamous cell tumor model

The animal protocols were approved by the University of Pittsburgh (Pittsburgh, PA) Institutional Animal Care and Use Committee and conform to the Policy on Humane Care and Use of Laboratory Animals. Mouse squamous cell tumors were induced by subcutaneous injection of 5 × 10⁶ cultured SCC-VII cells into C3H mice as previously described (10, 24).

Serial ultrasonic measurements of tumor volume

To quantify tumor volume, high-resolution 3-dimensional (3D) ultrasound imaging of the tumor was conducted using automated scanning (Vevo 2100, VisualSonics) at 21 MHz, with a 0.197 step size between cross-sections. Tumor cross-sections were manually outlined in representative image frames and volumes were calculated from 3D reconstructions of the outlined areas. This method of volume estimation is similar to our previously described approach (10), with improved resolution and volume calculation. This technique was chosen as it does not assume an elliptical tumor shape, detects the depth of the tumor, and can measure tumors as small as 20 mm³.

UTMD-mediated siRNA delivery to tumors

Tumor volumes were measured daily beginning 7 days after SCC-VII cell injection. Microbubble treatments commenced when tumor volumes reached 20 to 40 mm³. On the first day of treatment, an indwelling catheter made of PE-10 tubing was placed in the internal jugular vein to facilitate multiple injections. siRNA-loaded microbubbles (5 μg per 7 × 10⁶ MB) were suspended in 0.4 mL PBS and infused through the internal jugular via the indwelling catheter for 20 minutes during concurrent pulsed UTMD at 1.3 MHz and MI = 1.6 (Sonos 7500, Philips) using an S3 probe mounted in a fixed position over the entire tumor (beam elevation ~15 mm in the near field, 3 cm focal distance) for 30 minutes. The ultrasound pulse sequence was adjusted to allow for complete tumor replenishment of microbubbles between bursts (1.5–2.5 seconds) and for complete microbubble destruction (4–6 bursts), as guided by simultaneous Contrast Pulse Sequencing (Sequoia, Siemens Corp.) at 7 MHz and MI = 0.2–0.4 (10). After each UTMD treatment, mice were recovered, and 3D ultrasound imaging of tumor volume was conducted every 2 to 4 days thereafter.

Short-term experiments were first carried out to confirm UTMD-mediated knockdown of EGFR in tumors, followed by...
longer term experiments to determine treatment effects on tumor growth. One of the 2 EGFR siRNAs (ID#65373) confirming EGFR knockdown in the lipofectamine transfection studies described earlier was chosen for the subsequent in vivo studies. Six mice were studied to detect UTMD-mediated knockdown of EGFR 3 to 4 days after delivery of microbubbles loaded with EGFR–siRNA (n = 3) or control siRNA (n = 3) by immunofluorescence. A separate group of 12 mice was studied to detect EGFR knockdown using RT-PCR 3 to 4 days after UTMD-mediated delivery of either EGFR–siRNA (n = 6) or control siRNA (n = 6).

Therapeutic studies were conducted on separate groups of mice that received ultrasound during 2 intravenous infusions of microbubbles loaded with EGFR–siRNA (n = 7) or control siRNA (n = 6) on day 1 (when average tumor volume reached 20–40 mm³) and on days 4 to 5. Addition control groups consisted of mice injected with empty microbubbles + UTMD (n = 5) and EGFR–siRNA-loaded microbubbles without UTMD (n = 5). Tumor volumes from these 4 groups were measured at 2 to 4 day intervals for up to 3 weeks until tumor volume exceeded 1,000 mm³ or the tumor ulcerated.

Evaluation of inflammatory response to UTMD treatment

To determine whether the treatment protocol causes an immune response, cytokine activation was assayed in a separate group of 6 nontumor-bearing mice. Three were intravenously injected with EGFR–siRNA–loaded MBs and received UTMD over the dorsal surface identical to the treatment protocol given to the mice bearing tumors. Another 3 mice received sham surgery alone (catheter insertion, no siRNA/MB injection, no UTMD). Cytokine expression was assayed in diluted (1:5) serum harvested from mice 24 hours posttreatment using the RayBio Mouse Cytokine Antibody array (Cat# AAM-CYT-1, Raybiotech), which simultaneously assays for the expression of 21 cytokines, including TNF-α, interleukin-12, IFN-γ, with sensitivities at pg/mL levels. Arrays were then processed as per manufacturer’s instructions and exposed to autoradiography film and digitized images quantitated (pixels) to assess cytokine levels in the serum.

Post mortem histology

Tumor tissue was frozen in optimal cutting temperature media and sectioned at 5 μm for immunofluorescent staining to detect EGFR. Slides were blocked, washed, and incubated with primary antibody for murine EGFR (1:200, Cat#610017, BD Biosystems). After washing, slides were incubated with secondary anti-mouse fluorescein isothiocyanate conjugate (1:5,000, Cat# F0257, Sigma), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). All slides were visualized on an Olympus BX51 microscope interfaced with digital charge-coupled device camera (Olympus DP71). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis was conducted using the ApopTag kit (Millipore) to identify apoptotic cells (26). At least 5 random ×10 fields were examined for each tissue sample, which represents at least 7,500 total examined nuclei per tumor. Relative fluorescence was determined in comparison of the number of pixels above threshold from 4 to 6 random ×20 fields. Each analyzed field contained 3.5 × 10⁴ total pixels and the number of pixels above threshold ranged from 8.9 × 10⁴ to 3.0 × 10⁵.

Real-time quantitative PCR

RNA was extracted from tissue samples using Trizol reagent (Invitrogen), and complementary DNA was prepared from 2 μg of total RNA (n = 6 per group) using TagMan Reverse Transcription Kit (Applied Biosystems). EGFR expression was determined by using RT-PCR primers 5′-CCCGGTCAAGATGGCACC-3′ and 5′-GGGTTAGCCCTTGTTACTTG-3′. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (primers: 5′-GGCAATTCAGGGACAGT-3′ and 5′-AGATGGTGATGGGCTTCCC-3′) was used as a reference to normalize EGFR measurements. RT-PCR amplifications were conducted with the Absolute blue SYBR Green/Rox Kit (Thermo Scientific) and were conducted on an Applied Biosystems 7900HT instrument. Analysis of EGFR expression was conducted using the 2⁻ΔΔCt method, and values were compared by Student t test (27).

Statistical methods

All data are expressed as mean ± SD. Tumor volumes (y) were plotted as a function of time (t) and fit to the exponential function y = x₀eᵏᵗ, in which x₀ represents initial tumor volume. Doubling times (DT) were calculated as DT = ln(2)/k (28), and compared using ANOVA on ranks (growth rate of treated tumors was not normally distributed), with post hoc testing using Dunn method (Sigma-Stat, Aspire Software). Tumor volume measurements were also used to create a Kaplan–Meier curve that reports days to “critical volume” for each individual mouse tumor and compared using the log-rank stat, followed by Bonferroni-corrected pairwise analysis. A critical volume of 8-fold growth was chosen as this represents the earliest point in which an ulceration appears, which necessitates euthanization.

Results

siRNA binding by microbubbles

The microbubble formulation bound approximately 7 μg siRNA per 1 × 10⁹ microbubbles. Larger amounts of siRNA mixed into lipid microbubble components before amalgamation resulted in similar levels of siRNA binding, indicating saturation of this microbubble formulation with RNA. When stored at 4°C in PBS, little siRNA dissociation was seen up to 8 hours after siRNA attachment (Fig. 1, lanes 5–7) at RNAse A concentrations sufficient for digestion of siRNA in the absence of microbubbles (Fig. 2, lanes 2–4).

EGFR knockdown in SCC VII cells in vitro

Transfection of the 2 different EGFR–siRNA molecules significantly reduced the expression of EGFR in SCC-VII cells by RT-PCR compared with transfection of control siRNA (ID#65372: 59% ± 20% knockdown, P = 0.001; ID#65373: 67% ± 19% knockdown, P = 0.001). Although the magnitude of
EGFR knockdown was statistically not different between the 2 EGFR–siRNA formulations, EGFR–siRNA ID#s65373 was chosen for the subsequent in vitro and in vivo studies due to its numerically higher degree of EGFR silencing. UTMD-mediated delivery of EGFR–siRNA (ID#s65373) to SCC-VII cells resulted in reduced EGFR protein expression compared with cells treated with control siRNA–loaded microbubbles + UTMD (P = 0.048) or cells exposed to EGFR–siRNA–loaded microbubbles without ultrasound (P = 0.027; Fig. 3A). In addition, UTMD-mediated delivery of EGFR–siRNA reduced EGFR-dependent growth compared with controls treated with control siRNA or ultrasound only (Fig. 3B; P < 0.002).

**Effect of UTMD-mediated delivery of EGFR–siRNA on tumor EGFR expression**

UTMD-mediated delivery of EGFR–siRNA reduced EGFR expression in vivo as assayed by immunofluorescence (Fig. 4A, B, D, and E). Quantification of EGFR immunofluorescent images revealed a knockdown of 80% in EGFR-positive area after UTMD-mediated delivery of EGFR–siRNA compared with UTMD-mediated delivery of control siRNA (4,700 ± 4,300 vs. 22,600 ± 5,400 pixels, P = 0.005). EGFR knockdown was distributed widely throughout treated tumors. RT-PCR analysis of tumor tissue revealed an EGFR knockdown of 35% ± 21% (P = 0.035) after UTMD-mediated delivery of EGFR–siRNA relative to delivery of control siRNA to tumors. No significant EGFR knockdown was seen in heart, lung, or liver tissues after UTMD delivery of EGFR–siRNA (n = 3; P > 0.45 vs. control siRNA delivery). TUNEL assay (Fig. 4C and F) showed increased apoptosis in UTMD-treated tumors receiving EGFR–siRNA–loaded MBs versus control siRNA–loaded MBs (12.2% ± 1.7% vs. 7.0% ± 3.3% TUNEL+ nuclei, respectively; P = 0.016).

**Effect of UTMD-mediated delivery of EGFR–siRNA on tumor growth**

Serial high-resolution in vivo ultrasound 3D reconstructions of representative SCC-VII tumors from 2 mice are illustrated in Fig. 5 after UTMD-mediated delivery of microbubbles loaded with either control siRNA (top) or EGFR–siRNA (bottom). Tumor volumes calculated from the scans (bottom right corner of each panel) indicated rapid tumor growth in the control mouse, compared with slower growth, and even initial regression, in the mouse treated with EGFR–siRNA–loaded microbubbles + UTMD.

Individual tumor volumes over time were plotted for all mice and averaged to generate the exponential growth curves...
illustrated in Fig. 6. Control tumors treated with empty microbubbles + UTMD, control siRNA microbubbles + UTMD, or EGFR–siRNA microbubbles without UTMD had initial mean volumes of 37 ± 18, 35 ± 23, and 25 ± 18 mm³, respectively. By day 9, control tumors had grown 19-±17-, 15-±6-, and 12-±7-fold (P = ns vs. each other). Tumors treated with EGFR–siRNA microbubbles + UTMD had initial average volumes 34 ± 7 mm³ and grew by only 4-±3-fold by day 9 (P<0.02 EGFR–siRNA + UTMD vs. controls). Tumor DTs (calculated from exponential best-fit lines generated from individual tumor growth curves) were significantly longer in mice treated with EGFR–siRNA microbubbles + UTMD (8.5 ± 6.1 days) than mice receiving empty microbubbles + UTMD (1.9 ± 0.3 days; P = 0.003), control siRNA microbubbles + UTMD (2.1 ± 0.2 days; P = 0.001), or EGFR–siRNA microbubbles without UTMD (2.7 ± 0.6 days; P = 0.005).

All tumors from control groups achieved critical volume (>8-fold growth) by 13 days, with most (14/16) achieving critical volume by day 10 (Fig. 7). In contrast, no EGFR–siRNA microbubbles + UTMD-treated tumors achieved critical volume by day 10, and only 2 of 7 achieved critical volume by day 13. Tumors treated with EGFR–siRNA microbubbles + UTMD took significantly longer to reach critical volume than any control group (P<0.02; Fig. 7).

Effect of UTMD-mediated delivery of siRNA on serum cytokine levels
The cytokine microarray showed no significant increase (P>0.4 for each cytokine) in the serum levels of the 21 assayed cytokines in siRNA-loaded microbubbles + UTMD-treated mice compared with sham surgery-treated mice at 24 hours after treatment.

Discussion
The main finding of this study is that custom-designed microbubbles can load therapeutic siRNA and that ultrasound can be used to burst these microbubbles in murine tumors,
resulting in a significant reduction of tumor growth. These data establish proof-of-concept that custom-designed microbubbles and UTMD can deliver siRNA in sufficient quantities to affect a therapeutic outcome. Our study shows a novel application of microbubbles as nonviral carriers of siRNA with the ability to suppress gene expression in a specific tissue and the unique capacity for multiple noninvasive treatments.

The use of microbubbles combined with ultrasound to deliver nucleic acids was first investigated by delivering genes at the DNA level (11, 12, 29, 30). The delivery of therapeutic siRNA has several potential advantages over delivery of plasmid DNA. First, siRNA is a much smaller molecule than plasmid DNA, and can more easily diffuse throughout the cell after it crosses the plasma membrane (31). Second, because of the small-molecular weight of siRNA, approximately 400 pmol/L siRNA are infused per treatment compared with 13 to 25 pmol/L plasmid DNA at this same microbubble concentration (10). Third, siRNA can mediate a knockdown effect both in the cytoplasm and in the nucleus (32) and does not have to traffic to the nucleus to be biologically active, unlike plasmid DNA.

One potential disadvantage of RNA as a therapeutic is that RNA is routinely degraded by ubiquitous RNAses, resulting in shorter-lived gene knockdowns on the order of days (32). We have addressed this concern by using a commercially prepared siRNA, which is modified by the manufacturer using a proprietary process to increase the stability of the siRNA. Furthermore, the microbubbles themselves seem to confer protection against RNase digestion, as exposure of microbubble–siRNA complexes to doses of RNase that degrade free siRNA produced minimal siRNA breakdown.

We sought to custom design microbubbles capable of delivering large amounts of RNA by incorporating positively charged lipids into the microbubble shell. The resulting microbubble formulation bound approximately 7 mg RNA per 1 × 10^9 microbubbles, which is more than 10-fold lower than the binding capacity of related microbubbles designed for plasmid DNA on a weight basis, albeit about 20-fold higher on a molar basis (10). This is likely due, at least in part, to the stoichiometry of the microbubble-nucleic acid–binding event. Plasmid DNA has at least 100 times more phosphate residues per molecule than siRNA and there is a higher probability that stable charge–charge interactions will form between a molecule of plasmid DNA and cationic microbubbles as compared with a molecule of siRNA and those same microbubbles. We expect the negatively charged siRNA to associate tightly with cationic lipids present in the microbubble shell, and we expect this association results in the RNAse protection shown in Fig. 2. A deep or complete burial of siRNA within the lipid shell would also provide RNAse protection and is possible with the binding of free lipid and siRNA to microbubbles via charge–charge interactions or hydrophobic interactions.

Lipofectamine transfection of SCC-VII cells with 2 separate EGFR–siRNA molecules was first conducted to confirm effective, specific EGFR knockdown, and 1 formulation (ID#s65373) was selected for the subsequent in vitro and in vivo studies. To confirm the functional activity of EGFR–siRNA in tissue culture, microbubbles and ultrasound were used to deliver the

Figure 6. Individual tumor volumes, average growth plots, and DT, of tumors after intravenous injection of empty microbubbles + UTMD (green), control siRNA–loaded microbubbles + UTMD (purple), EGFR–siRNA–loaded microbubbles without ultrasound (blue), or EGFR–siRNA–loaded microbubbles + UTMD (red) on day 1 and days 4 to 5. Bottom compares the growth curves for the 4 groups, color-coded as above.

Figure 7. Time to critical volume (>8-fold growth) of individual murine SCC tumors after UTMD during intravenous injection of empty microbubbles, control siRNA–loaded microbubbles, or EGFR–siRNA–loaded microbubbles on day 1 and days 4 to 5.
EGFR–siRNA to SCC-VII cells. UTMD-mediated delivery of EGFR–siRNA (but not control siRNA) strongly inhibited growth under conditions in which growth is dependent upon supplemental EGF. These data confirm the strong growth suppression effects of EGFR inhibition, which is well documented (1, 3–5, 33).

We also determined the effect of UTMD-mediated siRNA delivery to murine tumors in vivo using a mouse model of SCC. Beginning approximately 2 days after UTMD-mediated delivery of EGFR–siRNA, tumor growth was slowed, arrested, or even reversed in EGFR–siRNA–treated mice, whereas control siRNA–treated tumors grew at an exponential rate (Fig. 6). There was a 3- to 5-fold increase in the DT of treated tumors compared with controls, which was maintained in all treated tumors throughout the treatment period. Some (3/7) treated tumors returning to exponential growth after 7 to 9 days and others never returning to a pattern of exponential growth. The treatment effect delayed the time required to achieve an 8-fold increase in volume by several days (Fig. 7) as compared with controls.

It should be noted there was no detectable increase in cytokine expression 24 hours after injection of EGFR–siRNA–loaded microbubble + UTMD, compared with sham surgery controls. Although it is possible that there may be cytokine activation at early time points, there is no evidence that the long-term growth inhibition shown here is associated with immune activation. Despite this finding, it is possible that there are additional mechanisms of tumor growth inhibition resulting from UTMD-mediated delivery of EGFR–siRNA, beyond the increased apoptosis noted in Fig. 4; further studies of other mechanisms of tumor growth inhibition would be an important next step.

It is also important to note that EGFR knockdown was not seen in nontumor tissue (heart, lung, liver, etc.), which was nonetheless exposed to circulating EGFR–siRNA–loaded microbubbles. This finding underscores the critical role of ultrasound in this delivery system, in that the ultrasound beam not only acts to release the siRNA via destruction of microbubbles, but also functionally targets the therapeutic effect solely to the intended site of treatment. This site-specific delivery capability of UTMD provides a distinct advantage over systemic cancer treatments for tumors that are accessible to ultrasound.

To our knowledge, this is the first report describing the delivery of siRNA via custom-designed intravascular microbubbles and UTMD to treat tumors in vivo, and the first therapeutic microbubble study to use serial UTMD treatments. Other studies, including a previous report by our group, have used UTMD-mediated gene therapy to treat solid tumors with plasmid DNA (10, 19, 34, 35). These studies do not achieve levels of tumor growth inhibition comparable with that shown in the current study, likely due to low levels of transduction using plasmid DNA (4, 10). The magnitude of tumor growth inhibition in the current study is also greater than that reported following direct intratumor injections of microbubbles loaded with DNA (36–38). The increased efficacy seen here might be due to higher molar dose of siRNA, increased bioeffects, or the use of serial treatments. Indeed, the ability to administer multiple treatment doses in our study is an inherent advantage of this minimally invasive, nonviral system.

UTMD-mediated delivery of EGFR–siRNA also compares favorably with the clinically available anti-EGFR drug cetuximab. Cetuximab, when used singly, does not significantly affect the growth of SCC-VII tumors and only begins to inhibit growth when used together with bevacizumab and/or cisplatin (39). It should be noted that the efficacy of UTMD-mediated EGFR–siRNA treatment will likely vary by tumor type. Although we have not tested the ability of our EGFR–siRNA to act synergistically with established anticancer agents, such combined approaches could further increase the efficacy of standard clinical regimens.

Several limitations to this study should be mentioned. Although the level of tumor growth inhibition when EGFR–siRNA is delivered via UTMD is significant, the underlying mechanism is not entirely understood. While we have shown that EGFR expression is inhibited by this commercially available siRNA, it is possible that some tumor growth inhibition is mediated by direct or indirect immune responses to siRNA (21–23), although we found that mice treated with control siRNA showed no tumor growth inhibition, and no cytokine expression was induced 24 hours posttreatment. Also, the lack of increased cytokine expression after UTMD of siRNA-loaded microbubbles suggests that the cationic lipids, which comprised the microbubble shell are not immunogenic. Although the degree of tumor growth inhibition is indeed quite large in this study, there is potential for even greater degrees of tumor growth inhibition with optimization of microbubble parameters, such as inclusion of targeting ligands (40–42), enhancement of ultrasound conditions, further modification of the treatment schedule, or the use of alternate siRNA molecules. Additional modifications may also become clear with increased understanding of the underlying mechanism of ultrasound-mediated nucleic acid delivery. RNAi strategies using UTMD require accessibility of the target to ultrasound, which can be scattered or blocked by bowel gas, lungs, ribs, etc., so UTMD may not be applicable to all solid tumors. Finally, the absolute tumor sizes in our study were well within the size of the beam elevation of our transducer, allowing the entire tumor to be insonified from a static position. Tumors larger than the beam elevation would require sweeping the ultrasound beam over the entire tumor, an adaptation that we envision to be relatively straightforward.

In conclusion, microbubble and ultrasound mediated delivery of siRNA is a promising strategy for treatment of tumors that could prove more effective than other siRNA delivery strategies. By using a systemic intravenous injection and externally applied ultrasound, the technique is minimally invasive and amenable to serial treatment. The ability to focus treatment by navigation of the ultrasound beam is a unique advantage of this system. As is it possible to insonify almost any location in the body, specific delivery of siRNA is possible regardless of tumor location. Our data establish that UTMD-directed siRNA therapeutics is effective at suppressing tumor growth, and these data might provide insights into further optimization and development of RNAi
strategies in other disease systems amenable to specific gene silencing.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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