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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 EGFR-directed small inhibitory RNA (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 EGF-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 times, 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.

Keywords

Antisense control of gene expression; Growth factor receptors and other surface molecules as targets for therapy; Nonbiological delivery systems; Ultrasound contrast agents; Ultrasound imaging

INTRODUCTION

The inhibition of Epidermal Growth Factor Receptor (EGFR) signaling is an established strategy for treating a number of cancer types, including lung cancer, colorectal cancers, and head and neck squamous cell carcinoma. 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 regimes 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 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

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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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 utilization increases the possibility of off target effects and limits the option for multiple treatments (7). Delivery of genes that express RNA interference 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 non-viral vector systems capable of binding nucleic acids, and which 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 insonified 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 RNA interference as a strategy for tumor growth suppression. Specifically, we tested the hypothesis that UTMD-mediated delivery of anti-EGFR siRNA (EGFR-siRNA) will inhibit squamous cell carcinoma growth in mice. *In vitro* and *in vivo* studies were performed to confirm down regulation of EGFR by our delivery system. Thereafter, EGFR-siRNA loaded microbubbles were delivered to murine tumors via UTMD, and tumor growth was serially measured.

MATERIALS AND METHODS

Microbubble preparation and confirmation of RNA loading

Cholesterol solutions of 1,2-distearoyl-sn-glycero-3-phosphocholine, 1,2-distearoyl-sn-glycero-3-ethylphosphocholine, 1,2-distearoyl-sn-glycero-3-phosphoglycerol (Avanti Lipids), and polyethylene glycol-40 stearate (Sigma) at a mole ratio 100:43:1:4.5 were dried with argon gas. An aqueous micellar solution of lipid was then prepared by adding Phosphate Buffered Saline (PBS) containing 1 mM 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 head space 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 supernatant was clear (2-4 washes @53g), resulting in a microbubble concentration of 7×10^8 /ml and mean diameter of $2.1 \pm 1.1 \mu\text{m}$ (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#s65373 and ID#s65372) and Silencer Select negative control sequence #1 (control-siRNA). Silencer Select siRNAs are manufactured with LNA 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 by comparison to lanes loaded with known amounts of siRNA. To determine resistance to RNase, siRNA-loaded microbubbles were exposed to RNase A (0.04-4mg/ml in PBS, @37°C, 15 min), 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 squamous cell carcinoma cells (SCC-VII) cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% fetal bovine serum (FBS) (24). SCC-VII cells were directly prepared from SCC tumors harvested from freshly injected mice. Transfections of SCC-VII cells were performed in 60mm dishes using 100 pM siRNA (2 EGFR and one 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 assayed 3 days post by RT PCR. For UTMD, microbubbles ($1-2 \times 10^7$) bearing 100 pg siRNA (EGFR-siRNA ID#s65373 or control-siRNA) or no siRNA were added to a 60mm dish seeded with 1×10^5 SCC-VII cells. The dishes were inverted to permit MB/cell contact via MB buoyancy and continuously insonified at 1.3MHz 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 where 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 blots were performed 3 days post sonoporation of SCC-VII cells using anti-EGFR antibody (1:1500, BD Bioscience, cat#610017) and anti-mouse HRP conjugated antibody (1:8000, 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 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 5×10^5 cultured SCC-VII cells into C3H mice as previously described (10, 24).

Serial ultrasonic measurements of tumor volume

To quantify tumor volume, high resolution 3D ultrasound imaging of the tumor was performed 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 20mm^3 .

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 $20-40\text{mm}^3$. 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 ug per 7×10^8 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 1.3MHz and MI=1.6 (Sonos 7500, Philips) using an S3 probe mounted in a fixed position over the entire tumor (beam elevation ~15mm in the near field, 3cm focal distance) for 30 minutes. The ultrasound pulse sequence was adjusted to allow for complete tumor replenishment of MB between bursts (1.5-2.5 seconds) and for complete MB destruction (4-6 bursts), as guided by simultaneous Contrast Pulse Sequencing (Sequoia, Siemens Corp) at 7MHz and MI=0.2-0.4 (10). After each UTMD treatment, mice were recovered, and 3D ultrasound imaging of tumor volume was performed every 2-4 days thereafter.

Short term experiments were first performed to confirm UTMD-mediated knockdown of EGFR in tumors, followed by longer term experiments to determine treatment effects on tumor growth. One of the two EGFR siRNAs (ID#s65373) confirming EGFR knockdown in the lipofectamine transfection studies described above was chosen for the subsequent *in vivo* studies. Six mice were studied to detect UTMD-mediated knockdown of EGFR 3-4 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 3-4 days after UTMD-mediated delivery of either EGFR-siRNA (n=6) or control-siRNA (n=6).

Therapeutic studies were performed on separate groups of mice that received ultrasound during two intravenous infusions of microbubbles loaded with EGFR-siRNA (n=7) or control-siRNA (n=6) on day 1 (when average tumor volume reached 20-40mm³) and on day 4-5. 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 2-4 day intervals for up to 3 weeks until tumor volume exceeded 1000mm³ or the tumor ulcerated.

Evaluation of inflammatory response to UTMD treatment

To determine if the treatment protocol causes an immune response, cytokine activation was assayed in a separate group of 6 non-tumor 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 post treatment using the RayBio Mouse Cytokine Antibody array (Cat#AAM-CYT-1, Raybiotech), which simultaneously assays for the expression of 21 cytokines, including TNF- α , IL-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 microns 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 FITC conjugate (1:5000, Cat#F0257, Sigma), and counterstained with DAPI (Sigma). All slides were visualized on an Olympus IX81 microscope interfaced with digital CCD camera (Olympus DP71). TUNEL analysis was performed using the ApopTag kit (Millipore) to identify apoptotic cells (26). At least 5 random 10 $\times$ fields were examined for each tissue sample, which represents at least 7500 total examined nuclei per tumor. Relative fluorescence was determined by comparison

of the number of pixels above threshold from 4-6 random 20× fields. Each analyzed field contained 3.5×10^5 total pixels and the number of pixels above threshold ranged from 8.9×10^1 to 3.0×10^4 .

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 Taqman reverse transcription kit (Applied Biosystems). EGFR expression was determined by using RT PCR primers 5'-CCCGGTCAGAGATGCGACC-3' and 5'-GGGTGAGCCTGTTACTTGTGCC-3'. The GAPDH gene (primers: 5'-GGCAAATTCAACGGCACAGT-3' and 5'-AGATGGTGATGGGCTTCCC-3') was used as a reference to normalize EGFR measurements. RT-PCR amplifications were performed with the Absolute blue Sybr Green/Rox kit (Thermo Scientific) and were performed on an Applied Biosystems 7900HT instrument. Analysis of EGFR expression was performed using the $2^{-\Delta\Delta C_t}$ method, and values were compared by Student's t-test (27).

Statistical methods

All data are expressed as mean ± standard deviation. Tumor volumes (y) were plotted as a function of time (t) and fit to the exponential function $y = X_0 \cdot e^{kt}$, where X_0 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's 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 ~7 µg siRNA per 1×10^9 microbubbles. Larger amounts of siRNA mixed into lipid microbubble components prior to 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 (Figure 1, lanes 5-8). Microbubble association also protected siRNA from RNase digestion (Figure 2, lanes 5-7) at RNase A concentrations sufficient for digestion of siRNA in the absence of microbubbles (Figure 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 to transfection of control-siRNA (ID#s65372: $59 \pm 20\%$ knockdown, $p=0.001$; ID#s65373: $67 \pm 19\%$ 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 to cells treated with control-siRNA loaded microbubbles +UTMD ($p=0.048$) or cells exposed to EGFR-siRNA loaded microbubbles without ultrasound ($p=0.027$) (Figure 3a). Additionally, UTMD-mediated delivery of EGFR-siRNA

reduced EGF-dependent growth compared to controls treated with control-siRNA or ultrasound only (Figure 3b) ($p < 0.002$).

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

UTMD-mediated delivery of EGFR-siRNA (ID#s65373) reduced EGFR expression *in vivo* as assayed by immunofluorescence (Figure 4 panels A, 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 to UTMD mediated delivery of control-siRNA (4700 ± 4300 vs. 22600 ± 5400 pixels, $p = 0.005$). EGFR knockdown was distributed widely throughout treated tumors. RT PCR analysis of tumor tissue revealed an EGFR knockdown of $35 \pm 21\%$ ($p = 0.035$) 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 (Figure 4, panels C and F) showed increased apoptosis in UTMD-treated tumors receiving EGFR-siRNA-loaded MBs vs. control-siRNA-loaded MBs ($12.2 \pm 1.7\%$ vs. $7.0 \pm 3.3\%$ TUNEL+ nuclei, respectively, $p = 0.016$).

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

Serial high resolution *in vivo* ultrasound 3-D reconstructions of representative SCC-VII tumors from 2 mice are illustrated in Figure 5 after UTMD-mediated delivery of microbubbles loaded with either control-siRNA (upper panels) or EGFR-siRNA (lower panels). Tumor volumes calculated from the scans (lower right corner of each panel) indicated rapid tumor growth in the control mouse, compared to 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 Figure 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 $25 \pm 18 \text{ mm}^3$, respectively. By day 9, control tumors had grown 19 ± 17 , 15 ± 6 , and 12 ± 7 fold ($p = \text{ns}$ vs each other). Tumors treated with EGFR-siRNA microbubbles+UTMD had initial average volumes $34 \pm 7 \text{ mm}^3$ and grew by only 4 ± 3 fold by day 9 ($p < 0.02$ EGFR-siRNA+UTMD vs. controls). Tumor doubling times (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 (Figure 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 ($p < 0.02$) (Figure 7).

Effect of UTMD-mediated delivery of siRNA on serum cytokine levels

The cytokine microarray demonstrated 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 to 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 effect a therapeutic outcome. Our study demonstrates a novel application of microbubbles as non-viral carriers of siRNA with the ability to suppress gene expression in a specific tissue and the unique capacity for multiple non-invasive 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, ~400 pMoles siRNA are infused per treatment compared to 13-25 pMoles plasmid DNA at this same MB 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 appear 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 ~7 μ g RNA per 1×10^9 microbubbles, which is over 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 compared to 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 demonstrated in Figure 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 two separate EGFR-siRNA molecules was first performed to confirm effective, specific EGFR knockdown, and one 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 EGFR-siRNA to SCC-VII cells. UTMD-mediated delivery of EGFR-siRNA (but not control-siRNA) strongly inhibited growth under conditions where 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 two days after UTMD-mediated

delivery of EGFR-siRNA, tumor growth was slowed, arrested, or even reversed in EGFR-siRNA- treated mice, while control-siRNA treated tumors grew at an exponential rate (Figure 6). There was a 3-5 fold increase in the doubling time of treated tumors compared to controls, which was maintained in all treated tumors throughout the treatment period. Some (3/7) treated tumors returning to exponential growth after 7-9 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 (Figure 7) compared to controls.

It should be noted there was no detectable increase in cytokine expression 24 hours after injection of EGFR-siRNA-loaded microbubble+UTMD, compared to 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 demonstrated 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 Figure 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 non-tumor tissue (heart, lung, liver) 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 utilize 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 to that demonstrated 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 intra-tumor 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 to 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 anti-cancer 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 demonstrated 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 demonstrated no tumor growth inhibition,

and no cytokine expression was induced 24 hour post treatment. 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 US-mediated nucleic acid delivery. RNA interference 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 utilizing 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 it is 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 RNA interference strategies in other disease systems amenable to specific gene silencing.

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Figure 1.

Acrylamide-Urea gel electrophoresis and ethidium-bromide staining demonstrate siRNA binding to microbubbles (MB) and lack of siRNA dissociation from the MB over time. MB loaded with siRNA were washed immediately to remove unbound siRNA (0 hours, lane 4) and washed again at 1, 2, 4, and 8 hrs post attachment (lanes 5-8). Washed MB were destroyed and bound siRNA was recovered after washes were complete ("Bound", lane 9). Lanes 1-3 contain siRNA as loading controls.

Figure 2. Acrylamide-Urea gel electrophoresis demonstrates the resistance of siRNA bound to microbubbles against RNase digestion. Free siRNA (lanes 2-4) or microbubbles loaded with siRNA (lanes 5-7) were subjected to a range of RNase A concentrations from 0.04 to 4 mg/ml. Lane 1 contains siRNA as a loading control.

A. **B.** **Figure 3.**

A) EGFR Western blot in SCC-VII cells 3 days post UTMD-mediated delivery of control siRNA (lanes 1-3), EGFR siRNA (lanes 4-6), or EGFR siRNA without UTMD (lanes 7-9). **B)** Viable SCC-VII cell counts 2 days post UTMD of microbubbles loaded with EGFR-siRNA, control-siRNA, or no siRNA grown in low serum with 10 mg/ml epidermal growth factor. Data represent 5 independent experiments. * $p < 0.002$ vs. control-siRNA or no siRNA.

Figure 4. Immunofluorescent staining of EGFR in murine tumors 3 days after intravenous delivery of control-siRNA (A,B) or EGFR-siRNA (D,E) bound to microbubbles and treatment with ultrasound. Panels C and F are representative TUNEL stains demonstrating apoptosis after delivery of either control-siRNA (C) or EGFR-siRNA (F). Panels A,B,D,E; blue, DAPI(nuclei); green, anti-EGFR. Panels C, F; blue, hematoxylin (nuclei), brown, TUNEL+.

Figure 5. Representative 3D reconstruction and calculated tumors volumes using high frequency ultrasound scanning in mice receiving UTMD-mediated delivery of control-siRNA (upper panels) or UTMD-mediated delivery of EGFR-siRNA (lower panels) on days 1 and 5.

Figure 6.

Individual tumor volumes, average growth plots, and doubling times (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 day 4-5. Lower panel 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 MBs, control-siRNA loaded microbubbles, or EGFR-siRNA loaded microbubbles on day 1 and day 4-5.