Silencing β3 Integrin by Targeted ECO/siRNA Nanoparticles Inhibits EMT and Metastasis of Triple-Negative Breast Cancer

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Abstract

Metastatic breast cancer is the second leading cause of cancer-related deaths among women. Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer and currently lacks well-defined molecular targets for effective targeted therapies. Disease relapse, metastasis, and drug resistance render standard chemotherapy ineffective in the treatment of TNBC. Because previous studies coupled β3 integrin (ITGB3) to epithelial–mesenchymal transition (EMT) and metastasis, we exploited β3 integrin as a therapeutic target to treat TNBC by delivering β3 integrin siRNA via lipid ECO-based nanoparticles (ECO/siβ3). Treatment of TNBC cells with ECO/siβ3 was sufficient to effectively silence β3 integrin expression, attenuate TGFB-mediated EMT and invasion, restore TGFB-mediated cytostasis, and inhibit three-dimensional organoid growth.

Introduction

Breast cancer is the second leading cause of cancer-related deaths among women in the United States (1). The 5-year relative survival rate for women with localized disease is 98.6%; however, for those diagnosed with distant metastases, survival rates fall below 25% (2). Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer that lack the expression of estrogen receptor (ER) and progesterone receptor (PR), as well as HER2 amplification. Currently, there is a lack of targeted therapies for TNBCs, which renders chemotherapy as the standard of care (3–5). Although TNBC patients are initially responsive to chemotherapy, most patients relapse, and the recurrent tumor is usually highly metastatic and resistant to traditional chemotherapy, which leads to a disproportionately large number of deaths (3–6). Importantly, metastasis is associated with the aberrant activation of epithelial–mesenchymal transition (EMT; refs. 7–9), which endows cancer cells with elevated capabilities to invade and disseminate to distant sites (10). Various molecular and microenvironmental factors can induce EMT, including TGFB. TGFB is a pleiotropic cytokine that regulates virtually all aspects of mammary gland biology. TGFB induces the dramatic upregulation of β3 integrin, which is essential for EMT and breast cancer metastasis (11–13). Previous work has demonstrated that functional disruption of β3 integrin (ITGB3) inhibits TGFB-mediated cytostasis, EMT, and invasion in vitro (11) and reduces primary tumor burden in vivo (14). Given the essential roles that β3 integrin plays in mediating breast cancer tumor progression, we hypothesize that silencing β3 integrin expression has the potential to effectively treat TNBC.

RNAi is a natural biologic mechanism for modulating gene expression and can be exploited to effectively regulate the expression of β3 integrin in treating TNBC. Clinical application of RNAi requires efficient delivery of therapeutic siRNA into the cytoplasm of target cells (15, 16). We have recently developed a multifunctional cationic lipid-based carrier ([1-aminooethyl]iminobis[N-oleoylstearyl-1-aminooethyl]propionamide) (ECO), which can effectively mediate cytosolic siRNA delivery and facilitate efficient gene silencing in cancer cells (Fig. 1A; refs. 15, 17). ECO self-assembly with therapeutic siRNA forms stable nanoparticles that can be readily functionalized with targeting moieties to achieve targeted siRNA delivery to cancer cells. Considering the critical roles of β3 integrin in regulating EMT, proliferation and metastasis (11, 14, 18–20) and the unmet need for targeted therapies tailored to TNBC (3, 4, 21), we sought to evaluate silencing β3 integrin by targeted ECO–siRNA nanoparticles to treat metastatic breast cancer.

The present study demonstrates the efficacy of ECO/siβ3 nanoparticles in silencing β3 integrin expression and the subsequent...
inhibition of TGFβ-mediated EMT and invasion in breast cancer cells in vitro. The nanoparticles were modified with a cyclic RGD peptide via a PEG spacer to improve biocompatibility and systemic target-specific delivery of the therapeutic siB3 in vivo (11, 22). The efficacy of the targeted ECO/siB3 nanoparticles in alleviating primary tumor burden and inhibiting TNBC metastasis was determined in tumor-bearing mice following multiple i.v. injections.

Materials and Methods

Cell lines and reagents

MDA-MB-231 cells were obtained from the ATCC and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco). NME cells are normal murine mammary gland (NuMuMG; obtained from the ATCC) cells that were engineered to overexpress EGFR by VSVG retroviral transduction of particles encoded from a pBabe plasmid (12). Both cell lines were engineered to stably express constitutively active NME and selection with Zeocin (500 μg/mL; Invitrogen). Cell lines were not independently authenticated. Early passage cells were used for all cell and tumor work. The following siRNAs were purchased from Integrated DNA Technologies (Coralville): Mouse integrin β3 sense: (GGCUAGAGAGAGUGUAUCAG-U), mouse integrin β3 antisense: (AGUAGAGAGAGGUCAUAGUC-U), human integrin β3 sense: (GGCUAGAGAGAGUGUCAGUC-U), and human integrin β3 antisense: (GGCUAGAGAGAGGUCAUAGUC-U).

Preparation of ECO–siRNA nanoparticles

The ECO lipid carrier was synthesized as described previously (15). ECO (MW = 1023) was dissolved in 100% ethanol at a stock concentration of 2.5 mmol/L for in vitro experiments and 50 mmol/L for in vivo experiments. Mouse and human siRNAs were reconstituted in RNase-free water to a concentration of 18.8 μmol/L for in vitro experiments and 25 μmol/L for in vivo experiments. For in vitro experiments, an siRNA transfection concentration of 100 nmol/L was used. ECO–siRNA nanoparticles were prepared at an N/P ratio of 7.7 by mixing predetermined volumes of ECO and siRNA for a period of 30 minutes in RNase-free water (pH 5.5) at room temperature under gentle agitation to enable complexation between ECO and siRNA. The total volume of water was determined such that the volume ratio of ethanol:water remained fixed at 1:20. For RGD- and RAD-modified ECO–siRNA nanoparticles, RGD–PEG3400-Mal or RGD–PEG3400–Mal (PEG, 3,400 Da, NANOCs) was mixed with ECO in RNase-free water at 2.5 mol% for 30 minutes under gentle agitation and subsequently mixed with siRNA in RNase-free water for an additional 30 minutes. RGD–PEG15400–Mal or RGD–PEG15400–Mal were prepared at a stock solution concentration of 0.32 mmol/L in RNase-free water. Again, the total volume of water was determined such that the volume ratio of ethanol:water remained fixed at 1:20. After the incubation, free peptide derivative was removed from RGD- and RAD-modified ECO–siRNA nanoparticles by ultrafiltration (Nanosep, MWCO = 100 K, 5,000 x g, 5 minutes; Sigma-Aldrich). Conjugation of RGD–PEG3400–Mal to ECO was determined and confirmed through matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a Bruker Autoflex III MALDI-TOF instrument. The size of RGD-modified ECO–siRNA nanoparticles in PBS was determined by dynamic light scattering (DLS) with a Brookhaven Zetasizer Particle Size Analyzer.

Western blot analyses

Immunoblotting analyses were performed as previously described (25). Briefly, NME and MDA-MB-231 cells were seeded into 6-well plates (1.5 × 10^5 cells/well) and allowed to adhere overnight. The cells were then incubated in the absence or presence of TGFβ1 (5 ng/mL) for 3 days and then treated with ECO–siRNA complexes for 4 hours in complete growth medium. At each indicated time point, detergent-solubilized whole-cell extracts (WCE) were prepared by lysing the cells in Buffer H (50 mmol/L β-glycerophosphate, 1.5 mmol/L EGTA, 1 mmol/L DTT, 0.2 mmol/L sodium orthovanadate, 1 mmol/L benzamidine, 10 mg/mL leupeptin, and 10 mg/mL aprotinin, pH 7.3). The clarified WCE (20 mg/ lane) were then treated with 0.25% trypsin containing 0.26 mmol/L EDTA (Invitrogen), collected by centrifugation at 1,000 rpm for 5 minutes, resuspended in 500 μL of PBS containing 5% parafomaldehyde, and finally passed through a 35-μm cell strainer (BD Biosciences). Cellular internalization of the nanoparticles was quantified by the fluorescence intensity measurement of Alexa Fluor 488 for a total of 1 × 10^6 cells per sample using a BD FACSAria flow cytometer. All the experiments were performed in triplicate and the data represent mean fluorescence intensity and standard deviation.

Semi-quantitative real-time PCR analyses

Real-time PCR studies were performed as described previously (18, 25). Briefly, NME or MDA-MB-231 cells (100,000 cells/well) were seeded overnight onto 6-well plates and treated with TGFβ1 (5 ng/mL) for 3 days upon delivery of ECO nanoparticles with a nonspecific siRNA or β3 integrin-specific siRNA. At each indicated time point, total RNA was isolated using the RNAeasy Plus Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad). Semi-quantitative real-time PCR was conducted using iQ SYBR Green (Bio-Rad) according to the manufacturer’s recommendations. In all cases, differences in RNA expression for each individual gene were normalized to their corresponding GAPDH RNA signals.

Invasion and proliferation assays

Invasion assays were conducted as described previously (14). Briefly, NME cells, unstimulated (pre-EMT) or stimulated with...
TGFβ for 3 days (post-EMT), were treated with the ECO–siRNA complexes for an additional 2 days. The cells were then trypsinized and their ability to invade reconstituted basement membranes (5 × 10^4 cells/well) was measured using modified Boyden chambers, as previously described (18). For the proliferation assay, the NME cells were cultured (1 × 10^4 cells/well) in the presence (post-EMT) or absence (pre-EMT) of TGFβ (5 ng/mL) for 3 days, and then treated with ECO–siRNA. Cell proliferation was determined by 

**Three-dimensional–organotypic cultures**

Three-dimensional (3D)–organotypic cultures using the “on-top” method were performed as described previously (25). NME or MDA-MB-231 cells, which were unstimulated (pre-EMT) or stimulated with TGFβ (5 ng/mL) for 3 days (post-EMT), were cultured in 96-well, white-walled, clear bottom tissue culture plates (2,000 cells/well) with 50 μL of Cultrex cushions (Trevigen) in media supplemented with 5% Cultiex. The cells were maintained in culture for 4 days with continuous ECO–siRNA treatment every 2 days. Growth was monitored by bright-field microscopy or bioluminescent growth assays (where indicated) using luciferin substrate (26, 27).

**Tumor growth and bioluminescent imaging**

All the animal studies were performed in accordance with the Institutional Animal Care and Use Committee for Case Western Reserve University. NME cells (1 × 10^6 cells/mouse) were engineered to stably express firefly luciferase, and were subsequently injected into the lateral tail vein of 4- to 6-week-old, female nude mice (nu/nu Balb/c background) after TGFβ stimulation (5 ng/mL) for 7 days. Pulmonary outgrowth was monitored and determined as described previously (28). MDA-MB-231 cells, also engineered to express firefly luciferase and stimulated with TGFβ for 7 days, were engrafted into the mammary fat pad of female nude mice. The primary tumor growth and metastatic burden were monitored and determined as described above.

**In vivo therapeutic treatment**

Tumor-bearing mice were i.v. injected with RGD- or RAD-modified ECO–siRNA nanoparticles with an siRNA dose of 1.5 mg/kg every 5 days starting at day 17 after tumor engraftment. Surface modified ECO–siRNA nanoparticles (N/P ratio of 7.7) were prepared directly before each treatment according to the two-step formulation process described above based on the siRNA dose (1.5 mg/kg siRNA, 18.6 mg/kg ECO, 2.5 mol% PEGylation of ECO with either RGD- or RAD-functionalized PEG1400-malendime). Each mouse (25 g body weight) received on average nanoparticles containing 37.5 μg siRNA, 464 μg ECO, and 47.7 μg of either RGD-PEG1400-mal or RAD-PEG1400-mal in 150 μL nuclease-free water at each injection.

**Immunofluorescence and immunohistochemical staining**

For visualization of the actin cytoskeleton, immunofluorescence analysis was performed as previously described (14). NME cells (5 × 10^4 cells/well) were plated onto glass-bottom confocal dishes and allowed to adhere overnight, after which they were simultaneously stimulated with TGFβ (5 ng/mL) and treated with ECO–siRNA nanoparticles, either siβ3 or siNS at 100 nmol/L siRNA concentration. After 48 and 72 hours of simultaneous TGFβ stimulation and nanoparticle treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabili-

lized in 0.1% Triton-X 100, stained with Alexa Fluor 488 phalloidin (25 μmol/L, Invitrogen), and visualized under a fluorescent confocal microscope.

For immunohistochemistry, primary tumor samples were embedded in optimum cutting temperature compound (Tissue-Tek) in preparation for cryostat sectioning and immediately frozen. The samples were then sectioned, fixed in paraformin, and maintained at −80°C. The samples were stained with hematoxylin and eosin (H&E) to evaluate the presence of tumor tissue. Briefly, the samples were fixed in 10% formalin, rehydrated in 70% ethanol, and rinsed in deionized water before hematoxylin staining. Samples were then rinsed in tap water, decolorized in acid alcohol, immersed in lithium carbonate and rinsed again in tap water. Next, the eosin counterstain was applied and slides were dehydrated in 100% ethanol, rinsed in Xylene and finally mounted on a coverslip with Biomount. For immunofluorescence detection of fibronectin, the paraffin-embedded slides were first deparaffinized using a series of washes in xylene and decreasing concentrations of ethanol. Heat-induced antigen retrieval was performed using a pressure cooker in sodium citrate buffer (10 mMol/L sodium citrate, 0.05% Tween 20, pH 6.0) for 20 minutes. Following heat-induced antigen retrieval, the samples were blocked in TBST solution containing donkey serum and rinsed three times with TBST. The primary antibody (Abcam) was applied at dilution of 1:100 in blocking solution for 1 hour
followed by three rinses with TBST. The Alexa Fluor 488 secondary antibody (The Jackson Laboratory) was applied at a dilution of 1:5,000 in blocking solution for 1 hour followed by three washes with TBST and counterstained with DAPI at a dilution of 1:2,500 in blocking solution. After washing with TBST and mounting in an anti-fade mounting solution (Molecular Probes), the samples were imaged using a confocal microscope.

**Statistical analyses**

Statistical values were defined using the unpaired Student t test, with a P value of <0.05 considered to be statistically significant.

**Results**

**ECO/siβ3 nanoparticles induce sustained silencing of β3 integrin**

We examined the ability of ECO/β3 integrin-specific siRNA nanoparticles (ECO/siβ3) to silence β3 integrin expression in mouse NME breast cancer cells (23, 24), which are reminiscent of a basal-like breast cancer cell line (24), and human MDA-MB-231 breast cancer cells, a TNBC cell line (29). The expression of β3 integrin was elevated in both cell lines after stimulation with TGFβ for 72 hours (14). Subsequent treatment of the stimulated cells with ECO/siβ3 nanoparticles resulted in the rapid loss of β3 integrin mRNA within the first 16 hours following treatment (Fig. 1B). β3 integrin expression was reduced by approximately 75% and this downregulation was sustained for up to 7 days in NME cells treated with TGFβ (Fig. 1B and C). ECO/siβ3 treatment of MDA-MB-231 cells reduced β3 integrin expression level to that of the unstimulated cells (Fig. 1B and D). Importantly, treatment with ECO/nonspecific siRNA nanoparticles (ECO/siNS) failed to alter β3 integrin expression in both cell lines (Fig. 1B–D). Collectively, these results demonstrate the ability of ECO/siβ3 nanoparticles to induce efficient and prolonged silencing of β3 integrin expression in breast cancer cells.

**ECO/siβ3 nanoparticles attenuate TGFβ-mediated EMT, invasion, and proliferation**

Next, we investigated the effects of ECO/siβ3 nanoparticles on EMT, invasion, and proliferation of breast cancer cells. Phalloidin

![Figure 2. ECO/siβ3 nanoparticles attenuated TGFβ-mediated EMT, invasion, and proliferation. A, immunofluorescence images of actin cytoskeleton visualized with rhodamine-conjugated phalloidin in mouse NME cells with different treatments (scale bar, 100 μm; inset scale bar, 50 μm). B, semiquantitative real-time PCR analysis (n = 3) of EMT markers in NME cells (***P < 0.001). C, Western blot analysis of E-cadherin and N-cadherin in NME cells. D, invasion assay of quiescent (white bars) or TGFβ-stimulated (gray bars) NME cells (n = 3; **P < 0.005; ***P < 0.001). E, proliferation as measured by [3H]thymidine incorporation of either quiescent (white bars) or TGFβ-stimulated (gray bars) NME cells (n = 3; *, P < 0.05; ***, P < 0.001). For all experimental groups, NME cells were pretreated with TGFβ (5 ng/mL; 72 hours) followed by ECO–siRNA nanoparticle treatment using 100 nmol/L siRNA. For B, D, and E, data, mean ± SE. Results for D and E are representative of three independent experiments.**
staining of the actin cytoskeletal architecture (18) revealed that quiescent NME cells displayed the epithelial hallmark of densely packed and well-organized cortical actin network (18), whereas those stimulated with TGFβ exhibited dissolved junctional complexes and acquired an elongated morphology consistent with stress fiber formation that are characteristic of mesenchymal cells (Fig. 2A). Treatment of NME cells with ECO/siβ3 nanoparticles at the time of TGFβ stimulation inhibited dissolution of the junctional complexes and stress fiber formation, whereas treatment with ECO/siNS nanoparticles failed to affect TGFβ-induced morphologic changes (Fig. 2A). Moreover, the phenotypic changes in post-EMT cells were accompanied by alterations in the expression of EMT-related genes (30). Silencing of β3 integrin inhibited TGFβ-mediated upregulation of the mesenchymal markers, N-cad and PAI-1, and inhibited TGFβ-mediated downregulation of the epithelial markers, E-cad and CK-19 (Fig. 2B and C). ECO/siNS nanoparticles did not alter the effect of TGFβ on the aforementioned EMT markers.

TGFβ-mediated EMT is also associated with increased invasiveness (10, 30) and cell-cycle arrest (31). TGFβ-stimulated NME cells treated with ECO/siNS readily reconstituted basement membrane, whereas ECO/siβ3 nanoparticles significantly inhibited invasion (Fig. 2D). Conversely, treatment of quiescent NME cells with ECO/siβ3 nanoparticles had no effect on basal invasiveness, an event that is uncoupled from β3 integrin expression. Previous studies demonstrate that parental NMuMG cells readily undergo proliferative arrest when stimulated with TGFβ (31). We found that the NME cells override these cytostatic effects of TGFβ (Fig. 2E), whereas treatment with ECO/siβ3 partially restores TGFβ-mediated cytosis (Fig. 2E). Collectively, these findings indicate that ECO/siβ3 nanoparticles-mediated silencing of β3 integrin attenuates TGFβ-induced EMT and invasion, and partially restores TGFβ-mediated cytosis.

**ECO/siβ3 nanoparticles attenuate outgrowth of murine and human MECs in 3D-organotypic culture**

To study the effects of ECO/siβ3 nanoparticles in a physiologically relevant system, we cultured NME and MDA-MB-231 cells in 3D-organotypic cultures to recapitulate the elastic modulus of a distant metastatic site such as the pulmonary microenvironment (32). This culture method presented additional obstacles in the delivery and uptake of nanoparticles, because these organoids were compact and surrounded by a dense matrix. Using confocal microscopy, we confirmed that ECO–siRNA nanoparticles formulated with fluorescently labeled siRNA (AF488) readily gained access to NME organoids by first penetrating into the periphery within 30 minutes after treatment, and further dispersing throughout the entirety of the organoid to reach a near-uniform distribution within 24 hours (Fig. 3A). The dispersion of ECO–siRNA nanoparticles into the inner cell layers of the organoids suggests that ECO–siRNA uptake by these cells may result from diffusion through intercellular spaces or through transcytosis (33). Fig. 3B and C show that NME and MDA-MB-231 organoids stimulated with TGFβ exhibited increased growth as compared with their quiescent counterparts. Treatment with ECO–siβ3 nanoparticles inhibited the growth of both quiescent and TGFβ-stimulated NME and MDA-MB-231 organoids (Fig. 3B and C) in comparison with treatment with ECO/siNS nanoparticles. These results demonstrate the effectiveness of ECO/siβ3 nanoparticles in attenuating the 3D outgrowth of post-EMT breast cancer cells.

**Surface modification of ECO–siRNA nanoparticles with RGD peptide promotes cellular uptake and sustains gene silencing**

An essential goal of in vivo siRNA delivery is to increase siRNA localization at the disease site while minimizing its accumulation in nontarget tissues (34). We modified ECO–siRNA nanoparticles with a cyclic RGD peptide, which binds to αvβ3 integrin, or a nontargeting control cyclic RAD peptide via PEG spacers (3,400 Da; Fig. 4A and B; refs. 22, 31). The size of RGD-modified ECO–siRNA nanoparticles (RGD–ECO–siRNA) was 88.3 ± 5.2 nm, as determined by DLS (Fig. 4C). Their cellular uptake was then examined in both unstimulated and TGFβ-stimulated NME cells. TGFβ stimulation had no effect on the cellular uptake of unmodified ECO–siRNA nanoparticles, whereas cellular uptake of RGD–ECO–siRNA nanoparticles was robustly enhanced (Fig. 4D and E), leading to effective silencing of β3 integrin in TGFβ-treated cells (Fig. 4F and G). Because αvβ3 is a major receptor that recognizes the RGD-targeting peptide, we sought to determine whether β3 integrin silencing affects cellular uptake of RGD–ECO–siRNA nanoparticles. Although cellular uptake of RGD-targeted nanoparticles was diminished upon β3 integrin silencing, uptake was nonetheless elevated consistently, because of the presence
of other receptors for the peptide (Fig. 4H; ref 35). Taken together, these results show that RGD-targeted ECO–siRNA nanoparticles efficiently promote cellular uptake and robust gene silencing, particularly in post-EMT and metastatic breast cancer cells.

RGD–ECO/siβ3 nanoparticles inhibit pulmonary outgrowth of mouse MECs in vivo

To evaluate the effect of β3 integrin silencing on pulmonary outgrowth, we inoculated TGFβ-treated NME cells into the lateral tail vein of nude mice and subsequently monitored pulmonary
outgrowth. Systemic injections of RGD-targeted ECO/siβ3 nanoparticles dramatically inhibited pulmonary outgrowth of post-EMT NME cells (Fig. 5), as compared with nonspecific RAD–ECO/siβ3 and RGD–ECO/siNS treatment groups. These results demonstrate that RGD-targeted ECO/siβ3 nanoparticles with PEG spacers can effectively inhibit pulmonary outgrowth of TGFβ-stimulated NME cells, when targeted for in vivo delivery applications.

**RGD–ECO/siβ3 nanoparticles effectively inhibit primary tumor growth and metastasis of malignant human MECs**

To further evaluate the in vivo effect of our targeted ECO/siβ3 nanoparticles, MDA-MB-231 cells pretreated with TGFβ were engrafted into the mammary fat pad of nude mice. Mice were treated with RGD–ECO/siβ3 (1.5 mg/kg siRNA, 18.6 mg/kg ECO) every 5 days, starting at day 17 (Fig. 6A). Primary tumor burden was monitored by bioluminescence imaging (BLI) and caliper measurements. Compared with the untreated control, RGD–ECO/siNS or RAD–ECO/siβ3 treatment groups, RGD–ECO/siβ3–treated mice exhibited significantly reduced primary tumor burden (Fig. 6B–D). The primary tumors were resected at week 9 (Fig. 6A) and weighed. Figure 6E shows that RGD–ECO/siβ3 treatment resulted in significantly reduced tumor weights as compared with the control groups. Importantly, the therapeutic efficacy of RGD–ECO/siβ3 was reflected by decreased mRNA expression of β3 integrin in the primary tumors, relative to that in the control groups (Fig. 6F). RAD–ECO/siβ3 treatment resulted in marginally reduced β3 integrin expression (Fig. 6F), which was consistent with the marginally reduced primary tumor burden, which were not statistically significant (Fig 6D and E). These data reflect partial uptake of the RAD–ECO/siβ3 nanoparticles by primary tumors as a result of passive tumor accumulation attributed to tumor vascular hyperpermeability. H&E staining of tissue sections demonstrated similar histopathologic patterns in RGD–ECO/siβ3-treated and control groups, whereas untreated mice developed tumors that were more vascularized than RGD–ECO/siβ3–treated tumors (Fig. 6G). Further immunostaining of tissue sections indicated that RGD–ECO/siβ3–treated primary tumors exhibited decreased expression of a mesenchymal marker, fibronectin (Fig. 6H), which is associated with poor overall survival (36).

RGD–ECO/siβ3 treatment resulted in robust inhibition of tumor metastases (Fig. 7A and C) and primary tumor recurrence (Fig. 7B), as compared with control groups at week 12 after engraftment. Primary tumor recurrence was evaluated by restricting the region of interest of the bioluminescent image to that of the area originally occupied by the resected primary tumor. Interestingly, RAD–ECO/siβ3 treatment also mediated significant inhibition of tumor metastases and primary tumor recurrence as compared with RGD–ECO/siNS treatment, but to a lesser extent than RGD–ECO/siβ3. This decrease in the efficacy of RAD–ECO/siβ3 could be attributed to the lack of specific targeting and binding of the nanoparticles to the cancer cells. At 12 weeks after engraftment, the RGD–ECO/siβ3 group was released from nanoparticle treatment to evaluate the lasting effects of therapeutic β3 integrin silencing on tumor recurrence and metastases in comparison with the untreated control group. At 4 weeks after treatment release (16 weeks after engraftment), the RGD–ECO/siβ3–treated mice remained tumor-free, whereas the tumor burden of untreated mice continued to increase (Fig. 7D and E). Finally, throughout the entire course of treatment, no significant difference was observed in the body weights across the different treatment groups, demonstrating the low toxicity of the i.v. administered, targeted, and PEGylated ECO–siRNA nanoparticles (Fig. 7F). Collectively, these data highlight the effectiveness and safety of the systemic administration of RGD–ECO/siβ3 nanoparticles for the inhibition of TNBC tumor progression and metastases.

**Discussion**

Cancer metastasis involves a cascade of events, including EMT and local invasion, intravasation, survival in circulation, extravasation, and outgrowth of disseminated cells at the secondary site. Cancer cell EMT is considered to be a critical step for the initiation of cancer metastasis. To alleviate metastasis, it is essential to prevent EMT and to eliminate the dissemination and outgrowth of cells that have already undergone EMT. Silencing EMT-related genes by RNAi has the potential to revolutionize current treatment standards. β3 integrin has been implicated as a powerful inducer of EMT (11, 14), potentiating the oncogenic effects of TGFβ by inducing invasion and metastases of MECs. Here, we demonstrated that silencing the expression of β3 integrin with RGD–ECO/siβ3 nanoparticles prevented TGFβ-mediated EMT and inhibited TNBC metastasis.

Although functional disruption of β3 integrin has been shown to attenuate TGFβ-mediated EMT and tumor progression (11, 14), the utilization of β3 integrin siRNA as a therapeutic regimen has been limited because of the lack of a clinically feasible approach. In the present study, we demonstrated ECO to be a versatile, safe, and effective siRNA delivery vehicle that forms stable RGD-targeted siRNA nanoparticles for systemic siRNA delivery, which silenced β3 integrin expression, and subsequently eliminated
post-EMT cells responsible for metastases. The inhibition of TGFβ-mediated EMT with ECO/siβ3 nanoparticles was evident by the obstruction of TGFβ-mediated morphologic changes, downregulation of epithelial markers, and upregulation of mesenchymal markers in vitro. Moreover, silencing β3 integrin also decreased invasiveness and reduced outgrowth of breast cancer cells in 3D culture and in vivo. The effectiveness of RGD-targeted ECO/siβ3 nanoparticles for treating metastatic TNBC was evident by reduced primary tumor burden in tumors with diminished expression of β3 integrin. More importantly, ECO/siβ3 treatment abrogated metastases and primary tumor recurrence after treatment release.

Several unique features of ECO and ECO–siRNA nanoparticles render the delivery system effective for safe and systemic delivery of a therapeutic siRNA in treating metastatic TNBC. ECO possesses pH-sensitive amphiphilicity, which is essential to promote endo-lysosomal escape and avoid lysosomal siRNA degradation. The amino groups within ECO become protonated when exposed to the increasingly acidic environment of the endo-lysosomes, thereby increasing the cationic charge of the nanoparticles to promote endo-lysosomal membrane fusion for escape. Thiol groups of the cysteine residues are autoxidized into disulfide bridges during nanoparticle formulation, which stabilize the nanoparticles during circulation and become reduced by cytosolic glutathione, resulting in siRNA release from ECO to initiate RNAi in cytoplasm (17). The thiol groups also facilitate surface modification of ECO–siRNA nanoparticles with PEG or targeting peptides. This modification enables targeted in vivo siRNA delivery into tumor tissues and minimizes potential toxic side effects. This multifunctionality uniquely resides within a simple small molecular lipid, making ECO a versatile carrier for highly efficient cytosolic siRNA delivery. The formation of targeted ECO–siRNA nanoparticles is straightforward and reproducible, and can be readily scaled up for clinical development.

We demonstrated that β3 integrin is a powerful therapeutic target for treating metastatic TNBC, and that RGD–ECO/siβ3 nanoparticles are an effective vehicle to systemically silence β3 integrin in post-EMT cancer cells. Specifically, RGD–ECO/siβ3 may be beneficial for TNBC patients who currently lack targeted...
treatments. Administering RGD–ECO/siβ3 nanoparticles in combination with chemotherapy also has the potential to resensitize drug-resistant TNBC cells to chemotherapy. Moreover, αβ3 integrin is highly expressed in the angiogenic vasculature of many cancer types, and the RGD peptide is a well-established strategy for honing the delivery of therapeutics to the tumor (37–39). These studies highlight the potential of our RGD–ECO/siβ3 regimen in targeting not only the primary tumor, but also endothelial cells in angiogenic tumor vasculature for treating breast cancer. However, β3 integrin silencing may cause potential side effects in wound healing (10), which remain to be addressed. The effectiveness of ECO/siβ3 for treating established metastatic lesions and some types of TNBC without elevated expression of β3 integrin is not clear and needs further investigation. Previous studies suggest that established metastatic breast lesions use β1 integrin to sustain outgrowth (40, 41), which we currently hypothesize to limit the effectiveness of our RGD–ECO/siβ3 regimen in the treatment of late stage metastatic breast cancer. Because of the versatility of our multifunctional ECO–siRNA nanoparticles, targeted nanoparticles with different targeting ligands and siRNAs, including β1 integrin siRNA, can be readily formulated to address this issue. We are currently exploring the effectiveness of dual β1 and β3 integrin targeting using our ECO–siRNA nanoparticles (14). In summary, silencing of β3 integrin expression with our targeted multifunctional ECO/siβ3 nanoparticles is a promising therapeutic strategy for the effective treatment of metastatic TNBC associated with elevated β3 integrin.

Disclosure of Potential Conflicts of Interest
J.G. Parvani, M.D. Gujrati, and W.P. Schiemann have ownership interest (including patents) in Case Western Reserve University. Z.-R. Lu has ownership interest (including patents) in Cleveland Theranostics LLC. No potential conflicts of interest were disclosed by the other authors.

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Figure 7. RGD–ECO/siβ3 nanoparticles inhibited breast cancer metastasis and primary tumor recurrence. A, BLI images of mice at week 12 revealed differences in metastasis and primary tumor recurrence for the different treatment groups after primary tumor resection on week 9. B, quantification of primary tumor recurrence. (data represents mean ± SE, n = 5; *P < 0.05). C, quantification of thoracic metastasis by BLI (data represents mean ± SE, n = 5; *P < 0.05; †P < 0.01). Mice were released from ECO–siRNA therapeutic regimen at week 12. D, representative BLI of mice on week 16. E, quantification of whole body tumors from D; data represents mean ± SE, n = 5; *P < 0.05. F, change in the body weight of mice bearing MDA-MB-231 primary tumors across various treatment groups over the course of 16 weeks. The body weight was measured weekly and reported as mean ± SE (n = 5) for each group. No significant difference was observed between the various treatment groups at any time point.
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