Multifunctional ECO nanoparticles delivering β3 integrin siRNA for the treatment of metastatic breast cancer

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ABSTRACT SUMMARY
Successful siRNA-mediated cancer therapy requires the use of multifunctional nanoparticle systems to overcome the barriers associated with delivery. We have recently developed a cationic lipid-based carrier, ECO, which efficiently delivers siRNA intracellularly to induce potent gene silencing. In breast cancer, β3 integrin is linked to metastasis via TGF-β-induced epithelial-to-mesenchymal transition (EMT). As such, this integrin serves as an attractive and novel target for nanoparticle-mediated gene therapy against metastatic breast cancer.

INTRODUCTION
While RNA interference holds promise as a novel therapeutic modality for cancer, a number of obstacles have revealed the need for safe and effective siRNA delivery systems in order to translate these technologies into the clinic. Recently, we developed a multifunctional cationic lipid-based carrier, ECO, which can induce siRNA-mediated RNAi in various cancer cell lines.1,2 ECO ionically condenses siRNA forming stable nanoparticles to protect the siRNA against degradation and facilitate cellular uptake. Once internalized, ECO nanoparticles are trafficked to late-endosomes whereupon ECO’s pH-sensitive amphiphilicity promotes endosomal escape. Cytosolic glutathione reduces disulfide linkages created during nanoparticle formation to release the siRNA and achieve potent gene silencing.2 As nanoparticles can be formed with any siRNA, ECO serves as a promising siRNA delivery platform for the treatment of a wide range of human diseases. In breast cancer, β3 integrin has been identified as a key mediator in the acquisition of a metastatic phenotype through TGF-β-induced epithelial-mesenchymal transition (EMT).3 Inactivation of β3 integrin has been shown to disrupt the oncogenic properties of TGF-β and as such, ECO nanoparticles delivering β3 integrin siRNA (ECO/siβ3) may prove effective against metastatic breast cancer.

EXPERIMENTAL METHODS
Preparation of Nanoparticles: ECO nanoparticles complexed with siRNA were prepared at an N/P ratio of 10, where N/P ratio is defined as the ratio of protonable amines on ECO to the phosphate groups of the siRNA. ECO and siRNA were diluted in equal volumes of RNase-free water and were mixed and incubated at room temperature for 30 minutes.

In vitro β3 Integrin Silencing: Normal murine epithelial (NME) cells were stimulated with TGF-β (5ng/mL) for 72 hours to induce EMT. ECO/siβ3 nanoparticles or ECO nanoparticles carrying a non-specific siRNA (ECO/siNS) were delivered at a final siRNA concentration of 100 nM in complete growth media with TGF-β. The expression of β3 integrin, along with E-cadherin and N-cadherin, was evaluated with quantitative real-time PCR for up to 7 days post-treatment. Gene expression levels were normalized to GAPDH and compared to unstimulated and untreated NME cells.

Actin Phalloidin Immunofluorescence: NME cells were grown to partial confluency on glass-bottom dishes. At the time of treatment with either ECO/siβ3 or ECO/siNS nanoparticles, TGF-β (5ng/mL) was added concurrently to induce EMT. At 48 and 72 hours following treatment, the rearrangement of the actin cytoskeleton was directly visualized with rhodamine-conjugated Phalloidin, a peptide that binds selectively to F-actin. At each time point, cells were washed with PBS, fixed and permeabilized with 3.7% paraformaldehyde and 0.1% Triton X100. The cells were then blocked in 1% BSA in PBS for 1 hour then incubated with Phalloidin in 1% BSA in PBS for an additional hour. Control cells did not receive TGF-β or nanoparticles.

3D Organotypic Growth: To setup a 3D organotypic culture, 50μL of Cultrex basement membrane extract was added to wells of a 96-well plate. NME cells were stimulated with TGF-β (5ng/mL) for 72 hours and then plated (2000 cells/well) within the 96-well plate in 150 μL of media to bring the total well volume to 200 μL (Day 0). On day 4, 6, and 8, cells received treatment with either ECO/siβ3, ECO/siNS nanoparticles or no treatment at all. Growth of the cells was monitored every 2 days by the addition of 2 μL of luciferin to each well and the luminescence was quantified on a luminometer. Unstimulated NME cells served as an additional control.

RESULTS AND DISCUSSION
In NME cells stimulated with TGF-β to induce EMT, ECO/siβ3 nanoparticles were able to induce potent silencing of β3 integrin for up to 7 days post-transfection following a single treatment (Figure 1, top). A rapid decline of β3 integrin mRNA levels was observed within the first 24 hours reaching a maximum silencing of 84% compared to non-treated TGF-β-stimulated cells. A steady silencing of ~80% was then maintained for the remainder of the 7 day period.

During EMT, E-cadherin expression is diminished while N-cadherin becomes overexpressed, allowing for an increase in cell invasiveness. Depletion of β3 integrin by the ECO nanoparticles consequently reduced the acquired...
mesenchymal marker N-cadherin while restoring the epithelial marker E-cadherin (Figure 1, bottom). The phenotypic changes that arise during EMT drive a morphological change in which the actin cytoskeleton rearranges into stress fibers to facilitate cell motility. Delivery of siβ3 with the ECO nanoparticles suppressed TGF-β-mediated stress fiber formation to closely resemble the epithelial-like control cells which were not treated with TGF-β, i.e. well-organized and densely packed (Figure 2). In contrast, the group treated with ECO/siNS nanoparticles underwent EMT as is evident by the disorganized and elongated morphologies and stress fibers at both the 48 and 72 hour time point.

A 3D organotypic culture rich in collagen can better mimic the growth of cells within the primary tumor and pulmonary microenvironment. As such, the response of TGF-β-stimulated NME cells grown as mammospheres in 3D culture to treatment with ECO/siβ3 nanoparticles provides an indication as to how this therapy will translate in vivo. Treatment with the ECO/siβ3 nanoparticles was found to inhibit growth of stimulated NME cells compared to the non-specific and TGF-β-stimulated control groups (Figure 3, left). Treatments were administered on day 4, 6 and 8 and a significant decrease in growth was observed on day 6, 8 and 10. Of importance, by day 8, treatment with ECO/siβ3 nanoparticles inhibited growth to levels of non-stimulated, or non-metastatic, NME cells.

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CONCLUSION

Collectively, the in vitro results suggest that ECO/siβ3 nanoparticles are a promising therapeutic strategy against metastatic breast cancer. ECO nanoparticles were able to induce potent and sustained gene silencing of β3 integrin to combat the acquisition of metastasis by restoring basal levels of EMT markers and inhibiting actin cytoskeletal rearrangement and growth.

REFERENCES


ACKNOWLEDGMENTS

This material is based upon work partially supported by the National Science Foundation Graduate Research Fellowship under Grant No. DGE-0951783 and Grant Number R000489 from the National Institute of Health.