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Systemic Delivery of Tumor-Targeting siRNA Nanoparticles against an Oncogenic lncRNA Facilitates Effective Triple-Negative Breast Cancer Therapy

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Abstract

Long non-coding RNAs (lncRs), by virtue of their versatility and multilevel gene regulation, have emerged as attractive pharmacological targets for treating heterogeneous and complex malignancies like triple-negative breast cancer (TNBC). Despite multiple studies on lncRNA functions in tumor pathology, systemic targeting of these “undruggable” macromolecules with conventional approaches remains a challenge. Here, we demonstrate effective TNBC therapy by nanoparticle-mediated RNAi of the oncogenic lncRNA DANCR, which is significantly overexpressed in TNBC. Tumor-targeting RGD-PEG-ECO/siDANCR nanoparticles were formulated via self-assembly of multifunctional amino lipid ECO, cyclic RGD peptide-PEG, and siDANCR for systemic delivery. MDA-MB-231 and BT549 cells treated with the therapeutic RGD-PEG-ECO/siDANCR nanoparticles exhibited 80–90% knockdown in the expression of DANCR for up to 7 days, indicating efficient intracellular siRNA delivery and sustained target silencing. The RGD-PEG-ECO/siDANCR nanoparticles mediated excellent *in vitro* therapeutic efficacy, reflected by the significant reduction in the invasion, migration, survival, tumor spheroid formation, and proliferation of the TNBC cell lines. At the molecular level, functional ablation of DANCR

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Contributions

The conceptual design of the research was devised by AMV and ZRL. Experimental execution of all aspects of the research was done by AMV, ZS, NA, HJ, AS, XL, DS, and JS prepared the carrier and targeting peptide and formulated nanoparticles. NA, VQ, and, SH participated in cell culture, RT-PCR, western blot, and *in vitro* functional assays. HG reviewed the histology slides and WS assisted on TNBC tumor models. The manuscript was written and edited by AMV and ZRL and critically reviewed by WS.

Competing Interests

AMV and ZRL are co-inventors on a patent application. ZRL is a co-founder of Cleveland Theranostics, LLC, a startup company with a focus on developing nonviral gene therapies based on the multifunctional pH-sensitive amino lipids.

Supporting information

Raw data of colony assay, heat map of phospho-kinase profiles and combination plot depicting individual tumor volumes of mice treated with nanoparticle therapy

dynamically impacted the oncogenic nexus by downregulating PRC2-mediated H3K27-trimethylation and Wnt/EMT signaling, and altering the phosphorylation profiles of several kinases in the TNBC cells. Furthermore, systemic administration of the RGD-PEG-ECO/siDANCR nanoparticles at a dose of 1 mg/kg siRNA in nude mice bearing TNBC xenografts resulted in robust suppression of TNBC progression with no overt toxic side-effects, underscoring the efficacy and safety of the nanoparticle therapy. These results demonstrate that nanoparticle-mediated modulation of onco-lncRNAs and their molecular targets is a promising approach for developing curative therapies for TNBC and other cancers.

Graphical Abstract

Keywords

Targeted ECO/siRNA nanoparticles; systemic therapy; lncRNA; DANCR; TNBC

Introduction

Triple-negative breast cancer (TNBC) is highly heterogeneous with distinct molecular subtypes and refractory to endocrine and other targeted therapies.¹ Although chemotherapy forms the mainstay of clinical intervention for TNBC, it is known to further exacerbate tumor heterogeneity, resulting in disease relapse, metastases, and drug resistance in most patients.² In addition, conventional targeted therapies, including antibodies or small molecule inhibitors of oncoproteins, have met with limited success in achieving curative therapy for TNBC, due to the emergence of compensatory mitogenic pathways.^{3–4} The emerging consensus on the dynamic and evolving nature of triple-negative breast neoplasms has shifted the treatment paradigm to the development of new therapies against novel molecular targets that are associated with the complex biological characteristics of the disease.^{2, 5}

Long non-coding RNAs (lncRNAs) have recently emerged as critical players in the development of multiple oncogenic processes, including EMT, inflammation, cancer stemness, metastasis, and drug-resistance.^{6–8} Aberrant overexpression of oncogenic lncRNAs (onco-lncRNAs) is disease- and tissue-specific, and can influence global gene signatures and clinical outcomes by regulating multi-level gene expression.^{9–10} The onco-lncRNA DANCR (Differentiation Antagonizing Non-Coding RNA)¹¹ was recently implicated in the progression of multiple cancers, including gliomas,^{12–13} prostate,¹⁴ colorectal,^{15–16} gastric,¹⁷ liver,^{15, 18–19} breast,²⁰ and ovarian²¹ cancers. With a broad

spectrum of molecular targets and well-defined temporal and spatial expression,²² DANCER has the potential to overcome the limitations of single-target therapies and is a promising candidate for therapeutic targeting of multiple oncogenic TNBC networks with high efficacy and reduced risk of toxic side-effects in healthy tissues. Although DANCER was shown to be involved in TNBC,²⁰ its potential as a therapeutic target for systemic treatment and its dynamic functions in TNBC biology need to be explored.

In addition, DANCER is an “undruggable” macromolecule that cannot be targeted with conventional pharmacological approaches, necessitating the use of RNA interference (RNAi). However, the primary challenge for RNAi therapy is specific delivery of therapeutic siRNA into the cytosol of target cells via systemic administration. The clinical translation of most non-viral siRNA delivery systems is impeded, particularly by their limited cellular uptake, low transfection efficiency, toxicity, and transient gene expression,²³ underscoring the urgent need for a safe and efficient gene delivery platform for cancer therapy. To address these concerns, we implemented the multifunctional amino lipid carrier ECO, (1-aminoethyl)iminobis[N-oleicylcysteinyl-1-aminoethyl]propionamide], which can form stable self-assembly nanoparticles with therapeutic nucleic acids, including siRNA, DNA, and CRISPR/Cas.^{24–27} Fig. 1 illustrates the schematic for the formation and working of the nanoparticle system. ECO undergoes electrostatic complexation with siRNA to form ECO/siRNA nanoparticles that are further stabilized by the hydrophobic condensation of the oleic acid tails and auto-oxidation of cysteine residues to form disulfide bonds. The ECO/siRNA nanoparticles can also be functionalized by conjugation with polyethylene glycol (PEG) for improved biocompatibility and with cyclic RGD peptide for tumor targeting for *in vivo* gene delivery (Fig. 1a).^{25–26} The nanoparticles are known to mediate efficient cytosolic siRNA delivery and robust knockdown of target mRNAs through the PERC mechanism: pH-sensitive amphiphilic endosomal escape, followed by reductive dissociation and cytosolic release of the siRNA cargo, to enable effective RNAi (Fig. 1b).^{23, 28}

Here, we demonstrate efficient and effective nanoparticle-mediated targeting of the onco-lncRNA DANCER for TNBC therapy. We established the significant overexpression of DANCER in human breast cancer (BCa) cells, tumors, and The Cancer Genome Atlas (TCGA) database. We then investigated DANCER as a therapeutic target for effective TNBC therapy in two cell lines and mouse models by silencing its expression using tumor-targeting RGD-PEG-ECO/siDANCER nanoparticles to facilitate efficient cytosolic delivery of siDANCER. Transfections of RGD-PEG-ECO/siDANCER nanoparticles showed significant and prolonged DANCER silencing and inhibited invasion and proliferation of TNBC cells. Systemic injections of the RGD-PEG-ECO/siDANCER nanoparticles led to suppression of TNBC proliferation in mice with high efficacy and no overt side-effects. We also investigated the mechanism of action of DANCER and observed its pleiotropic roles in regulating multiple cancer-associated signaling pathways in TNBC.

Results

DANCER is overexpressed in triple-negative breast cancer (TNBC)

We examined the endogenous levels of DANCER in a wide range of human BCa cells, tumors, and healthy tissues. Using qRT-PCR, we compared the expression of DANCER in

BCa cell lines with distinct molecular profiles: hormone receptor (HR)-positive MCF7 and ZR-75, and TNBC Hs578T, BT549, and MDA-MB-231 cells.²⁹ Compared to normal human mammary epithelial cells (HMECs), the BCa cells showed significantly elevated (4–9 fold) levels of DANCER (Fig. 2a). Among the cancer lines, the TNBC cells (particularly, BT549 and MDA-MB-231) overexpressed DANCER more (2-fold) than the HR-positive cells. Next, we quantified DANCER levels using a cDNA array and found that DANCER is significantly upregulated (40-fold) in TNBC tumors than in normal breast tissues (Fig. 2b). Analysis of the RNA-Seq data from TCGA database for DANCER expression in 104 normal and 790 BCa samples showed consistent results, where DANCER was significantly overexpressed in BCa patients (Fig. 2c), and particularly in TNBC patients (Fig. 2d), compared to healthy controls. The heat map in Fig. 2e shows a diverse range of DANCER expression in the individual normal (N) and TNBC (T) patients, indicating the heterogeneity of the lncRNA. An additional comparison revealed higher DANCER expression in TNBC samples than in the ER⁺, PR⁺, and Her2⁺ tumors (1.34-fold, 1.33-fold, and 1.27-fold, respectively) (Fig. 2f–h). These results demonstrate that DANCER is substantially upregulated in BCa, and especially more so in TNBC.

Targeted RGD-PEG-ECO/siDANCER nanoparticles facilitate robust and prolonged DANCER silencing in TNBC cells

To effectively downregulate DANCER expression in TNBC, we formulated stable targeted nanoparticles using the lipid ECO and unmodified siDANCER via self-assembly. The nanoparticles were functionalized with cyclic RGD peptide via a PEG spacer (3,400 Da) for tumor targeting and improved biocompatibility during systemic delivery. These RGD-PEG-ECO/siDANCER nanoparticles mediate efficient cytosolic siRNA delivery and robust knockdown of target mRNAs through the PERC mechanism (Fig. 1).^{23, 28} RGD-PEG-ECO/siDANCER nanoparticles were formulated ([siRNA]=100 nM, N/P=10, RGD-PEG/ECO=2.5 mol%,) as previously described.²⁵ Control RGD-PEG-ECO/NC (NC) nanoparticles were similarly prepared using siLuciferase as the non-specific siRNA (NC). The targeted nanoparticles possessed a narrow size and charge distribution, as characterized by dynamic light scattering (Fig. 3a,b). The RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCER nanoparticles had a zeta potential of 20 ± 4.6 mV and 24.67 ± 3.6 mV and hydrodynamic diameter of 107.83 ± 19.28 nm and 109.12 ± 6.34 nm, respectively, Fig. 3c. The siRNA loading efficiency and encapsulation in the nanoparticles were evaluated by gel retardation assay, Fig. 3d. Both the nanoparticles demonstrated efficient siRNA encapsulation, with negligible free siRNA bands. The structure and morphology of the nanoparticles were visualized by transmission electron microscopy (Fig. 3e) and consistent sizes (ca. 100 nm) were observed.

The efficiency and kinetics of DANCER silencing by RGD-PEG-ECO/siDANCER nanoparticles were tested in MDA-MB-231 and BT549 cells with high DANCER expression on days 1, 4, and 7 post-treatment. Compared to the control, RGD-PEG-ECO/siDANCER nanoparticles induced 80–90% silencing of DANCER in both the cell lines for at least 7 days after a single transfection (Fig. 3f). These results demonstrate that RGD-PEG-ECO/siDANCER nanoparticles can facilitate efficient, robust, and prolonged silencing of DANCER in TNBC cells.

RGD-PEG-ECO/siDANCR nanoparticles suppress the invasion and proliferation of TNBC cells

To determine if DANCR depletion plays a role in TNBC suppression, we performed loss-of-function studies in MDA-MB-231 and BT549 cells treated with RGD-PEG-ECO/siDANCR and RGD-PEG-ECO/NC to evaluate functional changes. After confirming ~80% knockdown of DANCR expression after 24 h of siDANCR-nanoparticle treatment (Fig. 4a), the transfected cells were tested for their migratory and invasive capacity. The RGD-PEG-ECO/siDANCR-treated cells showed a significant reduction in their ability to invade Matrigel™-coated membranes in standard transwell assays compared to the control-treated cells, evidenced by the reduced number of migrated cells stained using crystal violet (Fig. 4b) and counted using ImageJ (Fig. 4c). The siDANCR-treated cells also showed a significant decrease in their ability to close the wounds scratched into a confluent monolayer, as compared to those treated with the control (Fig. 4d). These results demonstrate that DANCR downregulation inhibits the invasion and migration of TNBC cells.

Next, we determined the effect of RGD-PEG-ECO/siDANCR-mediated DANCR silencing on the survival, growth, and proliferative abilities of MDA-MB-231 and BT549 cells. The cells were treated with the nanoparticles for 48 h, and their viability was measured using the MTT assay. Compared to control, DANCR knockdown caused a significant decrease (30–70%) in the viability of both the cell lines (Fig. 4e), which was further validated by BrdU incorporation assay. RGD-PEG-ECO/siDANCR-treated cells showed significant reduction in BrdU incorporation (Fig. 4f), indicating that DANCR knockdown suppresses the proliferation of TNBC cells.

The effect of RGD-PEG-ECO/siDANCR treatment in MDA-MB-231 and BT549 cells was also assessed in 3D Matrigel culture. Silencing of DANCR significantly reduced the ability of the cells to form 3D tumor spheroids, as compared to control-treated cells (Fig. 4g), at 5 days post-treatment. Finally, the ability of single TNBC cells to grow and proliferate into colonies following DANCR silencing was evaluated by standard clonogenic assay. Control and siDANCR-treated cells were serially-diluted and allowed to grow for 10–15 days. Individual colonies formed were stained with 0.01% crystal violet and counted. As shown in Fig. 4h and Fig. S1, RGD-PEG-ECO/siDANCR-treated cells showed significant reduction of colonies than the NC-treated cells, indicating that loss of DANCR can inhibit the ability of TNBC cells to establish new colonies.

RGD-PEG-ECO/siDANCR nanoparticles suppress TNBC invasion and proliferation by influencing multiple oncogenic targets

The mechanism of biological functions of DANCR was investigated by evaluating the expression of multiple oncoproteins in MDA-MB-231 and BT549 cells treated with RGD-PEG-ECO/siDANCR or ECO-PEG-RGD/NC. These cell lines represent the claudin-low mesenchymal TNBC subtype, which is particularly aggressive, and is characterized by highly active EMT and Wnt signaling.^{3, 29} As shown in Fig. 5a, DANCR silencing downregulated the expression of the Wnt signaling protein β -catenin, the EMT markers ZEB1 and N-cadherin, as well as the anti-apoptotic marker survivin. DANCR is known to mediate its effects through EZH2-mediated epigenetic regulation.^{14, 20, 30} Since EZH2 is a

part of Polycomb repressive complex 2 (PRC2) that facilitates target gene silencing through trimethylation of H3K27 residues,³¹ we tested the expression of EZH2 and SUZ12 in siDANCR-treated cells and found a significant decrease in the expression of the two proteins. This was also accompanied by a reduction in the total levels of H3K27-trimethylation (Fig. 5b), suggesting that DANCR may play a role in PRC2-mediated trimethylation and repression of target genes.

Since siDANCR strongly inhibits PRC2 expression, we performed RNA immunoprecipitation (RIP) for EZH2. As shown in Fig. 5c, DANCR directly binds to EZH2 in both MDA-MB-231 and BT549 cells, showing a 12-fold and 8-fold enrichment with anti-EZH2 antibody pull-down, respectively. This enrichment was lost when the cells were treated with an EZH2-inhibitor, DZNep. This direct binding coupled with the methylation changes suggests that DANCR influences the expression of its target genes by epigenetic control.

Because lncRNAs are known to play dynamic roles in multiple oncogenic signaling pathways,³² we evaluated the effect of DANCR silencing on molecular alterations in the TNBC phosphorylation network using a Phospho-kinase array. The functional status of most oncogenes and tumor suppressors is governed by post-translational modifications like phosphorylation, which are widely dysregulated in TNBC.^{33–34} Among these, the phosphorylation status of over 40 kinases expressed in MDA-MB-231 and BT549 cells were analyzed after treatment with RGD-PEG-ECO/siDANCR and RGD-PEG-ECO/NC, Fig. 5d and Fig. S2. The kinases that showed a significant change in phosphorylation with siDANCR treatment are highlighted by numbered boxes and depicted in Fig. 5e. A common subset of tumor-promoting kinases, including p38 α , ERK1/2, AMPK α 1, Src, RSK, FAK, and PRAS40 (#1–7), was significantly less phosphorylated with DANCR silencing in both cell lines.^{35–36}

Interestingly, DANCR knockdown showed a differential phosphorylation pattern between the 2 TNBC cell lines, with reduced phosphorylation of 2 distinct residues of AKT1/2/3 (T308 in MDA-MB-231 vs S473 in BT549 cells, #11), contrasting S133 phosphorylation levels of CREB (#9), phosphorylation of JNK1/2/3 and PDGF-R β in MDA-MB-231 (#12,13) only, and phosphorylation of Fyn, Yes, WNK1, and PYK in BT549 (#12–15) only. DANCR silencing reduced the total level of β -catenin (*) in both cell lines, independently validating the results in Fig. 5a, and also altered the phosphorylation patterns of tumor suppressors like p53, p27 and transcription factors (TFs) like Stat proteins. The endogenous kinase profiles and the siDANCR-mediated changes in these profiles are distinct between the two cell lines (Fig. S2), highlighting the heterogeneity of the disease and broad functions of the lncRNA. Our results underscore the dynamic and complex role of DANCR in impacting multiple oncogenic and tumor suppressor proteins in different TNBC models.

Systemic administration of RGD-PEG-ECO/siDANCR nanoparticles mediates effective therapy of TNBC in vivo

The efficacy of RGD-PEG-ECO/siDANCR nanoparticles in treating TNBC was investigated in mice bearing MDA-MB-231 and BT549 xenografts. For the *in vivo* experiments, RGD-PEG-ECO/siDANCR and RGD-PEG-ECO/NC nanoparticles were formulated at N/P=8,

RGD-PEG/ECO = 2.5 mol%, and siRNA dose=1.0 mg/kg. Given the higher siRNA concentration, the nanoparticles were larger than those formed for *in vitro* transfections (Fig. 3), (average diameter 189.46 ± 14.3 nm and 188.11 ± 6.82 nm for RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles, respectively) (Fig. 6a). Compared to unmodified ECO/NC and ECO/siDANCR nanoparticles (51.15 ± 2.15 mV and 53.1 ± 1.7 mV), the RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles showed a reduction in the zeta potential (29.7 ± 0.6 mV and 40.35 ± 1.65 mV, respectively), indicating proper RGD-PEG conjugation (Fig. 6b). Mice were intravenously injected with RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles weekly for 6 weeks. The treatment with RGD-PEG-ECO/siDANCR resulted in a complete suppression of tumor proliferation and reduced tumor volumes (90.3 ± 18.77 to 59.79 ± 18.73 mm³ for MDA-MB-231 and 72.9 ± 14.75 to 58.79 ± 18 mm³ for BT549), while the RGD-PEG-ECO/NC-treated mice showed a rapid tumor growth (91.5 ± 32 to 718 ± 237 mm³ for MDA-MB-231 and 77.77 ± 17.4 to 868.1 ± 238.3 mm³ for BT549), Fig. 6c–e and Fig. S3.

H&E staining of the tumors showed poorly differentiated high-grade malignancy (Fig. 6f). While the NC-treated tumors showed large areas of zonal necrosis, the siDANCR-treated tumor sections showed only focal areas of necrosis. The efficacy of RGD-PEG-ECO/siDANCR was also validated by measuring DANCR expression in total RNA extracted from the primary tumors. Significant downregulation of DANCR was observed in the siDANCR-treated tumors, compared to the NC-treated tumors (Fig. 6g), confirming that RGD-PEG-ECO/siDANCR effectively delivered siDANCR into the cancer cells to mediate efficient DANCR silencing and anti-tumor activity.

As quantified by qRT-PCR, significant reduction of EZH2 (Fig. 6h) and survivin (Fig. 6i) was observed in the siDANCR-treated tumors, suggesting that the tumor suppression and regression are likely due to epigenetic changes and apoptotic/cytostatic effects of systemic siDANCR therapy.

No changes in the body weight were observed in the siDANCR- and NC-treated mice during the entire treatment period (Fig. 7a). The vital organs of the mice were assessed by H&E-staining. As shown in Fig. 7b–d, the liver, kidneys, and lungs showed normal hepatocyte, renal, and alveolar histology, with no evidence of inflammation and fibrosis/interstitial disease, respectively. No significant difference in morphology was observed between the siDANCR- and NC-treated mice, indicating that repeated injections of the nanoparticles did not adversely affect the vital organs and health of the mice.

Discussion

Formerly considered fragments of “junk DNA”, lncRNAs are contributors of pervasive transcription that simultaneously influence the transcriptome, proteome, and epigenome.^{10, 37–39} Onco-lncRNAs can function as decoys, scaffolds, sponges, and tethers in *cis* or *trans* through dynamic binding to their DNA, RNA, and protein partners,^{10, 37, 40} making them attractive candidates for highly efficient cancer therapy with minimal risk of toxic side-effects in healthy tissues. However, systemically targeting these “undruggable” macromolecules with conventional approaches remains a challenge, which can be

circumvented through RNAi, a promising strategy for specifically downregulating aberrantly overexpressed onco-lncRNAs for systemic therapy. This work demonstrates that DANCER is highly overexpressed and acts as an oncogenic lncRNA in TNBC, and treatment with targeted RGD-PEG-ECO/siDANCER nanoparticles mediates robust DANCER silencing and therapeutic effects in TNBC, with concomitant gene expression changes in numerous cancer-driving pathways.

RGD-PEG-ECO/siDANCER nanoparticles were developed using multifunctional pH-sensitive amino lipid, ECO, for systemic targeted siRNA delivery.^{23, 41–42} ECO self-assembles with siRNA to form stable ECO/siRNA nanoparticles, which possess unique features that protect the siRNA cargo during systemic delivery and facilitate pH-sensitive endosomal escape and reductive cytosolic siRNA release (PERC) in target cells.^{24, 43} RGD-PEG-ECO/siDANCER nanoparticles mediate robust silencing of DANCER in TNBC cells for at least a week. DANCER knockdown induces apoptotic/cytostatic effects and inhibits invasion, migration, and colony formation, which are important characteristics of tumorigenesis, proliferation, and aggression in TNBC.² Consequently, weekly systemic injections of RGD-PEG-ECO/siDANCER nanoparticles at a low dose result in tumor suppression and regression in TNBC models. It can be speculated that the tumor growth arrest or cytostasis may, in part, be due to increased apoptosis, suppression of tumor-promoting proteins, and as yet unidentified epigenetic changes. The therapeutic efficacy of DANCER silencing can be attributed to the diverse gene expression changes we observed in the treated TNBC cells.

Although the overexpression of DANCER has been shown to be associated with poor prognosis and tumor progression in multiple cancers,^{14–15, 17, 19, 44} little is known about the pathways that upregulate DANCER in TNBC. During epidermal and osteoblast development, DANCER is required to maintain the progenitor cells in the undifferentiated state.^{11, 30} Since TNBC consists of many poorly differentiated cell types, and a high content of cancer stem cells, it is possible that the maintenance of self-renewal and cancer stemness or the transformation of well-differentiated mammary epithelial cells into the highly aggressive, mesenchymal and poorly differentiated phenotype requires or triggers the overexpression of DANCER.⁴⁵ Irrespective of the underlying causes, we demonstrate that DANCER functions pleiotropically at epigenetic, transcriptional, translational, and post-translational levels of gene expression.^{9–10, 37} DANCER silencing results in inhibition of EZH2 and PRC2-mediated H3K27-trimethylation at the epigenetic level; downregulation of TFs β -catenin, ZEB1, and Stat proteins, and proteins N-cad and survivin; and decreased phosphorylation of Src, FAK, and other oncoproteins. These proteins are upregulated in TNBC; they play critical roles in disease progression, metastasis and drug resistance,^{2–3, 29, 46–47} and some of them are common targets for developing targeted therapies.^{3, 35, 48} Unfortunately, limited curative outcomes have been achieved with these targeted therapies individually. The pleiotropic effects of DANCER silencing with RGD-PEG-ECO/siDANCER may overcome the limitations of conventional approaches of targeting an individual pathway or protein to achieve better therapeutic outcomes. DANCER silencing and the consequent downregulation of the EZH2-SUZ12 axis of the PRC2 complex likely alters the recruitment or binding of EZH2 to its target genes or proteins, in turn impacting the transcriptional silencing of tumor promoters or activation of tumor suppressors.^{14, 20, 30, 46} Interestingly, although both cell lines show

reduced expression of phospho-AKT, the phosphorylated residues were different, e.g., S473 in BT549 vs T308 in MDA-MB-231, indicating that DANCER silencing impacts the cell lines differently, underscoring the broad therapeutic functions of the lncRNA. It can be hypothesized that DANCER downregulation enables collective repression of multiple TNBC-promoting pathways, including Wnt and EMT signaling, anti-apoptosis, phosphorylated FAK, Src, RSK1/2/3, AKT1/2/3, and other oncoproteins, resulting in efficacious therapeutic outcome. Thus, it can be speculated that the functional downregulation of a single onco-lncRNA can simultaneously impair several different tumor-promoting pathways, potentially circumventing the problem of compensatory mitogenic pathways arising from drug resistance.^{5, 48}

This study demonstrates the promise of systemically silencing the oncogenic lncRNA DANCER with targeted RGD-PEG-ECO/siRNA nanoparticles for treating TNBC. Further studies are needed to evaluate the precise role of DANCER in the expression and modifications of multiple proteins at the same time, and to explore the role of DANCER in tumorigenesis, metastasis, and drug resistance. Because DANCER also promotes other types of cancers,²² this nanoparticle platform can be potentially expanded as a promising therapy for broader clinical applications. Although no overt toxicity was observed with systemic treatments of RGD-PEG-ECO/siDANCER, comprehensive studies on pharmacokinetics and toxicity of the nanoparticles are needed to assess potential off-target effects, activation of immune responses, and other shortcomings of lncRNA-targeted therapy⁴⁹ for clinical translation.

In summary, we demonstrate that DANCER is overexpressed in TNBC and plays pleiotropic roles by regulating multiple molecular pathways. DANCER silencing mediates effective regulation of an array of oncogenic activities and inhibits invasion and proliferation of TNBC cells. Additionally, systemic treatment with targeted nanoparticles results in tumor suppression and regression in two independent TNBC models. This research opens new avenues for developing novel therapeutics by systemically regulating onco-lncRNAs to effectively treat TNBC and other aggressive cancers.

Materials and Methods

Cell lines and culture

Normal human mammary epithelial cells (HMECs), hormone receptor positive breast cancer cell lines MCF7 (ER⁺, PR^{+/-}, Her2⁻) and ZR-75-1 (ER⁺, PR^{+/-}, Her2⁺), and triple-negative breast cancer cell lines, namely, MDA-MB-231, Hs578T, and BT549 (claudin-low ER⁻, PR⁻, Her2⁻), were purchased from ATCC (Manassas, VA). HMECs were passaged in Mammary Epithelial Cell Basal Medium supplemented with components of the Mammary Epithelial Cell Growth Kit (Lonza, Allendale, NJ). MCF7 and ZR-75-1 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin and 0.01 mg/mL recombinant human insulin (Sigma, St. Louis, MO). MDA-MB-231, Hs578T, and BT549 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. All the cells were grown at 37°C and 5% CO₂. The cell lines were

tested for the absence of mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza).

TCGA analysis

RNA-Seq data from TCGA database for DANCER transcript (ENST00000411630) was mined from the lncRNAtor website.⁵⁰ Graphpad Prism was used to perform differential gene expression analysis between 104 normal and 790 breast cancer samples (comprising 80 TNBC, 584 ER⁺, 508 PR⁺, 128 Her2⁺ samples). Graphpad Prism was also used to construct dot plots and heat maps, and perform statistical analyses for the expression data.

cDNA array

Breast cancer cDNA Array IV (BCRT104) was purchased from Origene Technologies (Rockville, MD). DANCER expression was quantified by qRT-PCR using SyBr Green PCR Master Mix (Applied Biosystems, CA) and DANCER-specific primers, with 18S primers as controls. Gene expression was analyzed by the 2^{-C_t} method with 18S expression as the control.

Nanoparticle formulation and transfections

ECO-PEG-RGD/siRNA nanoparticles were formulated as previously described.²⁸ Briefly, the amino lipid ECO (5 mM stock in ethanol) was gently agitated with RGD-PEG-Mal (RGD-PEG/ECO=2.5 mol%) in nuclease-free ultra-pure water for 30 min at RT. This was followed by complexation with siDANCER or siLuc (as negative control NC) at a final siRNA concentration of 100 nM and N/P = 10 for an additional 30 min to enable self-assembly formation of ECO-PEG-RGD/siDANCER or ECO-PEG-RGD/NC nanoparticles, respectively. For transfections, the nanoparticle formulation was mixed with culture media and added on to plated cells for 24–48 h, depending on the relevant experiments. The siDANCER duplex⁵¹ [sense 5'-GGU CAU GAG AAA CGU GGA UUA CAdCdC-3' and antisense 5'-GGU GUA AUC CAC GUU UCU CAU GAC CUC-3'] was purchased from IDT (Coralville, IA). The siLuc duplex [sense 5'-CCU ACG CCG AGU ACU UCG AdTdT-3' and antisense 5'-dTdT GGA UGC GGC UCA UGA AGC U-3'] was purchased from Dharmacon (Lafayette, CO).

Nanoparticle characterization

The nanoparticles were diluted in nuclease-free water and characterized for particle size and zeta potential on the LiteSizer™ 500 (Anton Paar), according to manufacturer's instructions. The morphology of the nanoparticles was determined using Transmission electron microscopy (TEM), as described previously.²⁶ In short, the nanoparticle suspension (20 µL) was pipetted onto a 300-mesh copper grid coated with a thin amorphous carbon film (20 nm). After blotting away excess sample with a filter paper, 3 µL of 2% uranyl acetate solution was added to stain the samples. After two rounds of staining, the samples were dried and imaged via TEM.

RNA extraction and analysis

Total RNA was extracted from cells and tissues using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD), according to manufacturer's instructions. Reverse transcription was performed using the miScript II RT Kit (Qiagen) and qPCR was performed using the SyBr Green PCR Master Mix. Gene expression was analyzed by the 2^{-C_t} method with 18S expression as the control. The following primer sequences were used- DANCER: Fwd 5'-GCGCCACTATG TAGCGGGTT-3' and Rev 5'-TCAATGGCTTGTGCCTGTAGTT-3'; 18S: Fwd 5'-TCAAGAAC GAAAGTCGGAGG-3' and Rev 5'-GGACATCTAAGGGCATCACA-3'; EZH2: Fwd 5'-AGGA CGGCTCCTCTAACCAT-3' and Rev 5'-CTTGGTGTGCACTGTGCTT-3'; Survivin: Fwd 5'-ATGGCCGAGGCTGGCTTCATC-3' and Rev 5'-ACGGCGCACTTTCTTCGCAGTT-3'

Viability assay

MTT assay was performed according to manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). Briefly, 5000 cells were plated on 96-well plates. After 24 h, the cells were transfected with therapeutic and negative control nanoparticles. After 48 h, MTT agent (5 mg/mL) was added into the wells and incubated for 4 h. The purple crystals were dissolved in SDS-HCl by incubation for 4 h. The absorbance was measured on SpectraMax microplate reader at 570 nm. Viability of control cells was normalized to 100%, for comparison to treated cells.

BrdU incorporation assay

BrdU incorporation was measured using the BrdU Cell Proliferation ELISA Kit from Abcam (Cambridge, MA), according to manufacturer's instructions. Briefly, 5000 cells were plated on a 96-well plate for 24 h and then transfected with the nanoparticles. After 40 h, diluted BrdU reagent was added to the cells. After 8 h, the cells were fixed for 30 min and incubated with anti-BrdU antibody for 1 h. After 3 washes, the cells were then incubated with Peroxidase Goat Anti-mouse IgG conjugate for 30 min. Finally, TMB Peroxidase Substrate was added for color development (30 min), followed by the Stop Solution. Absorbance was measured at 450 nm using SpectraMax Microplate reader. Viability of control cells was normalized to 100%, for comparison to treated cells.

Scratch wound and Transwell migration assays

For the scratch wound assay, approximately 5×10^5 TNBC cells were plated on 6-well plates for 24 h. A single straight scratch was made in the monolayer with a small pipet tip. After washing the detached cells with PBS, the cells were transfected with therapeutic and control nanoparticles and monitored for up to 48 h, or until the scratch wounds were closed. Images were taken using the Moticam T2 camera.

For Transwell migration assays, $1-2 \times 10^5$ cells transfected with nanoparticles were plated on Transwell inserts (VWR, Radnor, PA) coated with 0.28 mg/mL Corning™ Matrigel™ Membrane Matrix (Corning, NY). The next day, the un-migrated cells in the inserts were removed by swabbing with Q-tips. The migrated cells were fixed by treating the inserts with 10% formalin for 10 min. After washing with PBS three times, the inserts were placed in 0.05% crystal violet stain for 20 min to stain the migrated cells. The inserts were washed

under running water to remove excess stain and set to dry overnight. Images of the purple migrated cells were taken using the Moticam T2 camera. The cells in the images were counted using the ImageJ software.

3D tumor spheroid growth

For the Matrigel growth assay, $3\text{--}4 \times 10^5$ TNBC cells were transfected with the nanoparticles, suspended in 5% Matrigel™-containing media and plated on to 24-well plates coated with a thick layer of Corning™ Matrigel™ Membrane Matrix. The ability of the cells to form tumor spheroids in the 3D Matrix was monitored and photographed for up to 7 days using the Moticam T2 camera.

Western blot

Total cellular protein was extracted as previously described.^{52–53} Protein extracts (40 µg) were separated by SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted with primary antibodies overnight. The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA): anti-β-catenin, anti-ZEB1, anti-N-cadherin, anti-EZH2, anti-SUZ12, anti-me³-H3K7, and anti-survivin. After secondary antibody incubation, the membranes were developed using ChemiDoc™ XRS+ Imager (Biorad, Hercules, CA). β-Actin was used as the loading control.

RNA Immunoprecipitation (RIP)

Magna RIP kit and DZNep were purchased from Millipore Sigma (Burlington, MA). RIP was performed according to manufacturer's instructions. Briefly, cell lysates were incubated with antibody-bead mix overnight at 4°C. Following this, proteins were degraded and the RNA was isolated by the phenol-chloroform extraction method. The RNA was precipitated using Kit components and ethanol at –80°C overnight. For EZH2 inhibition, cells were treated with 500 µM DZNep for 72 h before harvesting for RIP assay.

Phosphokinase array

The differential phosphorylation status of 43 different kinases and 2 whole proteins with and without DANCER silencing was detected using the Proteome Profiler Human Phospho-Kinase Array Kit (RnD Systems, Minneapolis, MN). MDA-MB-231 and BT549 cells were transfected with RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCER nanoparticles. After 48 h, the cells were lysed and the lysates were analyzed for the relative phosphorylation profiles, according to the manufacturer's instructions. The expression was quantified by subtracting the background signal from the mean pixel intensity of the duplicate spots and plotted as bar graphs.

Animal models

Nude athymic mice (6-week-old nu/nu females) were purchased from the Athymic Animal and Preclinical Therapeutic Facility of the Case Comprehensive Cancer Center and housed in the Case Center for Imaging Research at CWRU. All animal experiments were performed according to the protocol and guidelines laid down by the IACUC and ARC of CWRU. For tumor xenografts, 2×10^6 MDA-MB-231 cells and 4×10^6 BT549 cells suspended in

Matrigel-PBS (6 mg/mL) were injected into the mammary fat pads of each nude mouse. Tumor volumes were monitored and measured once a week using a Vernier caliper. When the average tumor volumes reached 70–90 mm³, mice were randomized into control and treatment groups (n = 6 for MDA-MB-231 and n = 4 for BT549).

Tumor xenograft treatments

For *in vivo* therapy, nanoparticles were formulated as follows: ECO (50 mM stock) was conjugated to RGD-PEG-Mal (RGD-PEG/ECO=2.5 mol%) for 30 min, followed by complexation with siDANCR or siLuc (siRNA dose=1 mg/kg, [siRNA]=0.3 mg/mL) at N/P=8 for 30 more min, to obtain ECO-PEG-RGD/siDANCR or ECO-PEG-RGD/NC nanoparticles, respectively. The formulations were made in nuclease-free ultra-pure water and injected into the tail vein at siRNA dose of 1.0 mg/kg (100 µL per mouse) once a week for 6 weeks. Tumor volumes and body weights of mice were monitored once a week for 6 weeks. At the end of the experiment, the animals were euthanized, and the tumors and vital organs were harvested for analysis of morphology, histology, and metastasis. Portions of the tissues were fixed in 10% neutral buffered formalin, followed by paraffin embedding, sectioning, and H&E staining. Staining and IHC services were provided by the Tissue Resources Core Facility of CWRU. All the slides were reviewed by a certified pathologist.

Statistical analyses

All the experiments were independently replicated at least 3 times (n = 3), unless otherwise stated. Data are represented as mean ± s.e.m. Statistical analysis was performed using Graphpad Prism. Data between two groups was compared using unpaired Student's *t*-test. Data between three groups was compared using One-way ANOVA. *p* < 0.05 was considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of formation and working of RGD-PEG-ECO/siRNA nanoparticles. (a) Amino lipid carrier ECO (E) is mixed with the targeting moiety RGD-PEG-Mal followed by electrostatic condensation with siRNA molecules. Oxidation of cysteine residues (C) to form disulfide bonds and hydrophobic condensation of the oleic acid tails (O) further stabilize the formation of RGD-PEG-ECO/siRNA nanoparticles. (b) RGD-PEG-ECO/siRNA nanoparticles mediate efficient siRNA delivery by the PERC mechanism. Nanoparticles are internalized into cells by receptor-mediated endocytosis and travel through

the intracellular trafficking pathway. As the pH in the endosomes decreases, the pH-sensitive amphiphilicity of the nanoparticles enables them to induce endosomal membrane destabilization and endosomal escape into the cytosol. The nanoparticles then undergo reductive dissociation, releasing the siRNA cargo into the cytoplasm where it encounters the RNAi machinery to silence the target lncRNA.

Figure 2.

DANCER is significantly overexpressed in TNBC. (a) Endogenous expression of DANCER is significantly elevated in all breast cancer cell lines: MCF7 (luminal A; ER⁺, PR^{+/-}, Her2⁻), ZR-75 (luminal B, ER⁺, PR^{+/-}, Her2⁺), and Hs578T, BT549, and MDA-MB-231 (claudin-low ER⁻, PR⁻, Her2⁻), compared to HMECs, and even more so in the TNBC cell lines. (b) Quantification of DANCER expression from a breast cancer array containing cDNA from normal subjects (n=4) and breast cancer patients shows that DANCER is highly upregulated in tumor samples from TNBC patients (n=12), in comparison with breast tissues from

normal subjects. **(c)** Differential gene expression analysis for DANCER performed for 104 normal and 790 breast cancer samples from TCGA database shows a significant upregulation of DANCER in breast cancer samples, particularly in 80 TNBC samples **(d)**, compared to normal levels. **(e)** Heat map comparing and demonstrating the heterogeneity of DANCER expression in the individual normal (N) and TNBC (T) samples from TCGA database. DANCER expression is significantly higher in TNBC samples than breast cancer samples expressing **(f)** ER (n=584), **(g)** PR (n=508), and **(h)** HER2 (n=128) receptors (error bars denote s.e.m., *p<0.05, ***p<0.0005).

Figure 3.

RGD-PEG-ECO/siDANCR nanoparticles mediate robust and sustained silencing of DANCR expression in MDA-MB-231 and BT549 cells. RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles were formulated at N/P = 10, RGD-PEG/ECO = 2.5 mol%, and [siRNA] = 100 nM and characterized on LiteSizer. The particles show (a) and (b) single intensity peaks indicating uniform distribution, with (c) predicted size of about 108 nm and zeta potential of 22 mV. (d) Gel retardation assay of RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles shows high encapsulation efficiency, with free siRNA as control. (e) Morphology of RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles is visualized by transmission electron microscopy. (f) MDA-MB-231 and BT549 cells transfected with RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles were harvested on days 1, 4, and 7, for measurement of DANCR knockdown by qRT-PCR. DANCR expression by siDANCR treatment is normalized to NC treatment for both the cell lines (scale bar = 200 nm, error bars denote s.e.m., ** $p < 0.00005$, * $p < 0.005$).

Figure 4.

RGD-PEG-ECO/siDANCR nanoparticles inhibit TNBC invasion, migration, and proliferation. (a) siDANCR-treated MDA-MB-231 and BT549 cells show 80–90% knockdown in DANCR expression after 24 h, compared to NC-treated cells (**p<0.0005). (b) DANCR silencing leads to substantial reduction in the invasive potential of MDA-MB-231 and BT549 cells plated in Matrigel-coated Transwell inserts in serum-free media for 24 h. The migrated cells were fixed with 10% formalin, stained with 0.05% crystal violet, and counted using ImageJ software (**p<0.0005). (c & d) Standard scratch-wound assays demonstrate that siDANCR-treated MDA-MB-231 and BT549 cells have reduced ability to close the scratched wounds in 24 h, while the NC-treated cells readily migrate and close the wounds. (e) MTT assay demonstrates significant reduction in cell viability of MDA-MB-231 and BT549 cells treated with siDANCR nanoparticles, compared to NC (*p<0.01). (f) DANCR silencing in MDA-MB-231 and BT549 cells results in significant

decrease in cell proliferation, as indicated by the decreased BrdU incorporation, in comparison to NC (** $p < 0.0001$). (g) MDA-MB-231 and BT549 cells treated with RGD-PEG-ECO/siDANCR nanoparticles show considerable reduction in their ability to form 3D tumor spheroids, compared to NC-treated cells. (h) Clonogenic assay shows significant suppression of proliferative potential of MDA-MB-231 and BT549 cells treated with RGD-PEG-ECO/siDANCR nanoparticles, compared to NC nanoparticles (* $p < 0.01$). Colonies formed after plating 2000 siDANCR- and NC-treated cells were stained with 0.01% crystal violet after 10 days, counted, and represented as % of survived colonies. Error bars denote s.e.m., scale bar = 100 μ m.

Figure 5.

RGD-PEG-ECO/siDANCR nanoparticles suppress the expression of proteins involved in multiple oncogenic pathways. (a) DANCR silencing in MDA-MB-231 and BT549 cells induces significant decrease in the expression of β-catenin (Wnt signaling); ZEB1 and N-cadherin (EMT markers); and the anti-apoptotic protein, survivin. β-Actin is used as the loading control. (b) RGD-PEG-ECO/siDANCR nanoparticle-mediated silencing of DANCR in MDA-MB-231 and BT549 cells downregulates the expression of the PRC2 complex proteins EZH2 and SUZ12, along with decreased H3K27 tri-methylation marks. (c) DANCR

regulates PRC2 complex by directly binding to EZH2 in MDA-MD-231 and BT549 cells (* $p < 0.005$). (d) Phospho-kinase arrays demonstrate the differential phosphorylation patterns of 43 kinases with treatment of RGD-PEG-ECO/siDANCR nanoparticles in MDA-MB-231 and BT549 cells. Proteins highlighted in the boxes show significant differences in phosphorylation status between NC and DANCR knockdown. (e) The bar graphs represent the corresponding kinases/proteins that show significant changes ($p < 0.05$) in expression between control and DANCR silencing, calculated by comparing the mean pixel intensities of the duplicate spots.

Figure 6.

Systemic therapy of RGD-PEG-ECO/siDANCR nanoparticles significantly reduces primary tumor burden in TNBC xenografts. RGD-PEG-ECO/NC and RGD-PEG-ECO/siDANCR nanoparticles were formulated at N/P = 8, PEG-RGD/ECO = 2.5 mol% and [siRNA] = 0.3 mg/mL, and characterized for (a) particle size and (b) zeta potential (*p<0.05). (c) Weekly intravenous injections of RGD-PEG-ECO/siDANCR nanoparticles at a siRNA dose of 1.0 mg/kg for 6 weeks result in significant reduction in tumor volumes (*p<0.05). MDA-MB-231 and BT549 xenografts (2×10^6 and 4×10^6 cells, respectively) were implanted into the

mammary fat pads of athymic mice and treatment was started at average tumor volume of 70–90 mm³. **(d)** Compared to RGD-PEG-ECO/NC, RGD-PEG-ECO/siDANCR nanoparticles mediate significant inhibition of tumor progression. **(e)** Tumor status at the end of 6 weeks. **(f)** H&E staining of paraffin-fixed tumor samples from mice treated with RGD-PEG-ECO/siDANCR and NC (scale bar = 100 µm) shows large zonal necrosis (arrows) in NC but only focal necrosis with siDANCR treatment. Compared to RGD-PEG-ECO/NC-treated tumors, RGD-PEG-ECO/siDANCR-treated tumors demonstrate **(g)** significantly reduced DANCR levels (**p<0.005), and significantly lower mRNA expression of **(h)** EZH2 and **(i)** survivin, indicating that the targeted RGD-PEG-ECO-siDANCR therapy likely causes epigenetic alterations and apoptosis in the TNBC tumors (*p<0.05).

Figure 7.

Systemic administrations of RGD-PEG-ECO/siDANCR nanoparticles do not cause evident side-effects in the treated mice. **(a)** Both siDANCR- and NC-treated mice show no changes in their general health and no significant differences in the body weights during the 6 weeks of nanoparticle injections. H&E staining of the tumor sections demonstrates no significant changes in the morphology of the vital organs in NC- and siDANCR-treated mice, with the **(b)** liver, **(c)** kidney, and **(d)** lungs showing normal hepatocyte, renal, and alveolar histology, respectively. (error bars denote s.e.m., scale bar = 100 μ m).