Gene knockdown by EpCAM aptamer-siRNA chimeras suppresses epithelial breast cancers and their tumor-initiating cells

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Abbreviations: T-IC, tumor-initiating cell; AsiC, aptamer-siRNA chimera; TNBC, triple negative breast cancer; RNAi, RNA interference; siRNA, small interfering RNA; nt, nucleotide; SELEX, systematic evolution of ligands for exponential enrichment; RISC, RNA-induced silencing complex

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Supplementary Table
Abstract

Effective therapeutic strategies for in vivo siRNA delivery to knockdown genes in cells outside the liver are needed to harness RNA interference for treating cancer. EpCAM is a tumor-associated antigen highly expressed on common epithelial cancers and their tumor-initiating cells (T-IC, also known as cancer stem cells). Here we show that aptamer-siRNA chimeras (AsiC, an EpCAM aptamer linked to an siRNA sense strand and annealed to the siRNA antisense strand) are selectively taken up and knockdown gene expression in EpCAM+ cancer cells in vitro and in human cancer biopsy tissues. PLK1 EpCAM-AsiCs inhibit colony and mammosphere formation (in vitro T-IC assays) and tumor initiation by EpCAM+ luminal and basal-A triple negative breast cancer (TNBC) cell lines, but not EpCAM- mesenchymal basal-B TNBCs, in nude mice. Subcutaneously administered EpCAM-AsiCs concentrate in EpCAM+ Her2+ and TNBC tumors and suppress their growth. Thus EpCAM-AsiCs provide an attractive approach for treating epithelial cancer.
Introduction

RNA interference (RNAi) offers the opportunity to treat disease by knocking down disease-causing genes (1). Recent early phase clinical trials have shown vigorous (75-95%), sustained (lasting up to several months) and safe knockdown of a handful of gene targets in the liver using lipid nanoparticle-encapsulated or GalNAc-conjugated siRNAs (2-5). The liver, the body’s major filtering organ, traps particles and, hence, is relatively easy to transfect. The major obstacle to harnessing RNAi for treating most diseases however has yet to be solved – namely efficient delivery of small RNAs and gene knockdown in cells beyond the liver. In particular, the delivery roadblock is a major obstacle to harnessing RNAi to treat cancer (6).

Triple negative breast cancer (TNBC), a heterogeneous group of poorly differentiated cancers defined by the lack of estrogen, progesterone and Her2 receptor expression, has the worst prognosis of any breast cancer subtype (7-9). Most TNBCs have epithelial properties and are classified as basal-like, although a sizable minority are mesenchymal. TNBC afflicts younger women and is the subtype associated with BRCA1 genetic mutations. No targeted therapy is available. Although most TNBC patients respond to chemotherapy, within 3 years about a third develop metastases and eventually die. Thus new approaches are needed.

Here we develop a flexible, targeted platform for gene knockdown and treatment of basal-like TNBCs that might also be suitable for therapy against most of the common (epithelial) cancers. We deliver small interfering RNAs (siRNA) into epithelial cancer
cells by linking them to an RNA aptamer that binds to EpCAM, the first described tumor antigen, a cell surface receptor over-expressed on epithelial cancers, including basal-like TNBCs. Aptamer-linked siRNAs, known as aptamer-siRNA chimeras (AsiC), have been used in small animal models to treat prostate cancer and prevent HIV infection (10-18). We chose EpCAM for targeting basal-like TNBC because EpCAM is highly expressed on all epithelial cancers. A high affinity EpCAM aptamer was previously identified (19). EpCAM also marks tumor-initiating cells (T-ICs, also known as cancer stem cells) (20-27). Although the cancer stem cell hypothesis may not apply to all tumors, most solid cancers are heterogeneous and the less differentiated T-IC subpopulation is probably responsible for initiating tumors, resistant to conventional cytotoxic drugs and responsible for recurrence and metastases. Devising therapies to eliminate T-ICs is an important unmet goal of cancer research (28).

In normal epithelia, EpCAM is only weakly expressed on basolateral gap junctions, where it may not be accessible to drugs (29). In epithelial cancers it is not only more abundant (by several orders of magnitude), but is also distributed along the cell membrane. Ligation of EpCAM promotes adhesion and enhances cell proliferation and invasivity. Proteolytic cleavage of EpCAM releases an intracellular fragment that increases stem cell factor transcription (30, 31). EpCAM’s oncogenic properties may make it difficult for tumor cells to develop resistance by down-modulating EpCAM. In one study about 2/3 of TNBCs, presumably the basal-like subtype, stained strongly for EpCAM(25). The number of EpCAM+ circulating cells is linked to poor prognosis in breast cancer (32-36). An EpCAM antibody has been evaluated clinically for epithelial
cancers, but had limited effectiveness on its own (37-39). EpCAM expression identifies circulating tumor cells in an FDA-approved method for monitoring metastatic breast, colon and prostate cancer treatment (32-36). Moreover, about 97% of human breast cancers and virtually 100% of other common epithelial cancers, including lung, colon, pancreas and prostate, stain brightly for EpCAM (23), suggesting that the platform developed here could be adapted for RNAi-based therapy of common solid tumors.

Here we show that epithelial breast cancer cell lines uniformly stain brightly for EpCAM, while immortalized normal breast epithelial cells, fibroblasts and mesenchymal tumor cell lines do not. EpCAM-AsiCs cause targeted gene knockdown in luminal and basal-A TNBC cancer cell lines (which resemble basal-like TNBC primary tumors), and human breast cancer tissues in vitro, but not in normal epithelial cells, basal-B TNBC cell lines (which resemble mesenchymal TNBC primary tumors) or normal human breast tissues. Knockdown is proportional to EpCAM expression. Moreover EpCAM-AsiC-mediated knockdown of PLK1, a gene required for mitosis, suppresses in vitro T-IC functional assays (colony and mammosphere formation) of epithelial breast cancer lines. Ex vivo treatment specifically abrogates tumor initiation. Subcutaneously injected PLK1 EpCAM-AsiCs are taken up specifically by EpCAM+ xenografts of poor prognosis basal-A and Her2 breast cancers and cause rapid tumor regression.

**Material and Methods**

**Cells**
MDA-MB-468 (MB468) cells transduced with a Firefly luciferase reporter (MB468-luc) were kindly provided by Andrew Kung (Columbia University, New York NY, USA). BPE and BPLER cells, provided by Tan Ince (University of Miami, Miami, FL, USA), were maintained in supplemented WIT-T medium (Stemgent). Other human cell lines were obtained from ATCC and grown in MEM (MCF7, BT474), McCoy’s 5A (SKBR3), RPMI1640 (HCC1806, HCC1143, HCC1937, HCC1954, HCC1187, MB468, T47D) or DMEM (MB231, BT549, MB436) media supplemented with 10% fetal bovine serum (Gemini Bioproducts), 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate, 6 mM HEPES, 1.6 mM L-glutamine, 50 μM β-mercaptoethanol (Sigma-Aldrich). MCF10CA1a cells, provided by Karmanos Cancer Institute (Detroit, Michigan, USA), were grown in supplemented DMEM. MB231 cells stably expressing Firefly luciferase and mCherry (MB231-luc-mCherry) were selected after infection with pLV-Fluc-mCherry-Puro lentivirus (provided by Andrew Kung) using puromycin. The authors have not authenticated the cell lines used. Cell lines were obtained in 2012 and were expanded briefly before aliquoting and freezing. Cells used for experiments were thawed from low passage aliquots. All cell lines were verified to be free of mycoplasma.

**RNAs**

The long strand of the AsiC synthesized with 2′-fluoropyrimidines (TriLink Biotechnologies San Diego CA) was annealed to the short antisense strand (Integrated DNA Technologies (IDT) Coralville, Iowa) using a 2 fold molar excess of the long strand. The long strand was heated to 95°C for 10 min before adding the short strand which was annealed at 65°C for 7 min. The mixture was allowed to cool at room
temperature for 20 min. The annealed AsiC duplexes were purified further using Illustra MicroSpin G-25 columns (GE Healthcare Life Sciences). siRNAs, conjugated or not with cholesterol on the 3’ end as described (15) were from IDT. RNA sequences are provided in Supplementary Table 1. For some experiments the 3’-end of the antisense strand was conjugated to Cy3, Alexa Fluor 647 or 750. The stability of RNAs incubated in human or mouse serum was assessed with aliquots removed during a 36 h incubation at 37°C in 50% serum. Samples were analyzed by densitometry after polyacrylamide gel electrophoresis and ethidium bromide staining.

**Fluorescence microscopy**

Cells were incubated with 1 µM Cy3-labeled EpCAM aptamer in microscopy chamber wells for indicated times and then counter stained with CellMask Deep Red Plasma Membrane Stain (Life Technologies) and imaged live. Images were acquired with a spinning disk confocal head (Yokogawa) coupled to a fully motorized epifluorescence microscope (Axio Observer) equipped with a 63x lens (Plan Apochromat, 1.4 NA, Carl Zeiss). Three 50 mW solid-state lasers (491, 561 and 660 nm; Cobalt Laser) were used as light sources. The imaging system operates under control of SlideBook 5.0 and an EM-CCD camera (Quant-EM, Hamamatsu) was used to acquire images.

**Gene knockdown**

For in vitro gene silencing experiments, cells were used immediately after plating at 10,000 cells/well in 96-well plates. For transfections, cells were incubated with lipoplexed siRNAs or AsiCs without lipid at 100 nM or 4 µM concentrations, respectively,
unless otherwise indicated, in WIT-T or OptiMEM medium, respectively. For lipid transfections, cells were transfected with Dharmafect I according to the manufacturer’s protocol. Gene knockdown was assessed by measuring protein levels using flow cytometry 72-96 h after treatment and by measuring mRNA by qRT-PCR 24-48 h after treatment. Cell viability was assessed by CellTiter-Glo (Promega) or by Trypan-Blue staining 24-48 h after treatment.

**Ago immunoprecipitation**

MB468 cells (10⁶ cells/well of a 6-well plate) were treated with 4 μM PLK1 AsiCs or unconjugated PLK1 siRNA for 48 h. The cytoplasmic lysates of AsiC/siRNA treated cells were incubated with protein G Dynabeads (Life Technologies) that were coated with 2 μg anti-pan-Ago antibody (clone 2A8, Millipore) or mouse IgG overnight at 4°C in the presence of RNase inhibitors (Life Technologies) and protease inhibitors (Roche). The beads were washed 5 times with NT2 buffer (50 mM Tris pH 7, 150 mM NaCl, 0.05% NP-40) and eluted by incubation for 30 min at 55°C with 200 μl SDS-TE buffer (5 mM Tris pH 7.5, 0.5 mM EDTA, 0.5% SDS). RNA was extracted from the unbound supernatants and bead-bound protein-RNA complexes eluted from the beads using Trizol LS (Life Technologies). Taqman small RNA assay kits that included pre-designed primers (Life Technologies) were used to quantify PLK1 siRNA and miR-16 by qRT-PCR according to the manufacturer’s manual. The amount of PLK1 siRNA in the supernatant and immunoprecipitates was normalized to miR-16.

**qRT-PCR**
Total RNA was extracted with Trizol (Invitrogen) and cDNA prepared from 1000 ng total RNA using Thermoscript RT kit (Invitrogen) as per the manufacturer’s directions. qPCR of cDNAs was performed using SYBR Green Master Mix (Applied Biosystems) and a BioRad C1000 Thermal Cycler (Biorad). Relative CT values were normalized to GAPDH. Primers are provided in Supplemental Table 1.

**Flow cytometry**

Cells were stained in PBS containing 0.5% FCS, 1 mM EDTA, and 25 mM HEPES with the following antibodies: EpCAM (Clone EBA-1 from BD Biosciences or clone 9C4 from BioLegend), AKT1 (clone 55/PKBa/Akt from BD Biosciences). Flow cytometry analysis was performed on a FACS-Canto II (BD Biosciences) using FlowJo (Treestar Inc.) software.

**RNA uptake by human breast tissues**

Fresh breast cancer and normal breast tissue biopsies, obtained from the UMASS Tissue Bank, were cut into 3x3x3 mm cubes and placed in 96 well plates containing 100 μl RPMI. Samples were incubated for 24 h with 4 μM Alexa647-labeled siRNA, 100 nM Alexa647-labeled, chol-siRNA or 4 μM Cy3-labeled EpCAM-AsiC, all targeting eGFP, and photographed. To make single cell suspensions, triplicate samples were pooled, sonicated using a gentleMACS dissociator (Miltenyi, spleen program for 30 min at 37°C), incubated at 37°C for 30 min with shaking in 10 ml RPMI containing 1 mg/ml collagenase II (Sigma-Aldrich), and then sonicated again. The resulting cell suspension was passed through a 70-μm cell strainer (BD Falcon), washed with 30 ml RPMI, and stained for flow cytometry.
Tumor-initiating cell in vitro assays

For colony forming assay, 1,000 cells were treated for 6 h with medium, 4 μM AsiC or 100 nM paclitaxel (Sigma) in round bottom 96-well plates and transferred to 10-cm plates in serum-containing medium. Medium was replaced every 3 d. After 8-14 d, cells were fixed in methanol (-20°C) and stained with crystal violet. For sphere formation assay, cells (1,000/ml), treated as above for 6 h in round bottom 96-well plates, were cultured in suspension in serum-free DMEM/F12 1:1 (Invitrogen), supplemented with EGF (20 ng/ml, BD Biosciences), B27 (1:50, Invitrogen), 0.4% bovine serum albumin (Sigma) and 4 μg/ml insulin (Sigma). Spheres were counted after 1-2 weeks.

Mouse experiments

All animal experiments were performed with the approval of the Harvard Medical School and Boston Children’s Hospital Animal Care and Use Committees. 8-week old female Nu/J mice (Stock # 002019, Jackson Laboratories) were used for all experiments.

To assess tumor initiation, mice were injected subcutaneously with MB468-luc (5x10⁶ viable cells) that had been pretreated for 24 h with medium or 4 μM EpCAM-AsiCs targeting eGFP or PLK1. Cells were trypsinized with TrypLE Express (Invitrogen), resuspended in WIT media and injected subcutaneously in the flank. After intraperitoneal injection of 150 mg/kg D-luciferin (Caliper Life Sciences), luminescent images of the whole body were taken every 5 d for 15 d using an IVIS Spectrum using Living Image® software (Caliper Life Sciences). The mice were sacrificed on day 15.
To assess AsiC uptake, MB468-luc (5x10^6) and MB231-luc-mCherry (5x10^5) cells trypsinized with TrypLE Express (Invitrogen), were resuspended in 1:1 WIT-Matrigel and injected subcutaneously in opposing flanks. Five days later, when tumors were easily palpated, mice were injected subcutaneously in the neck with Alexa750-EpCAM-AsiC-GFP (0.5 mg/kg in PBS). Animals were maintained on an alfalfa-free diet (Research Diets, Inc) to reduce autofluorescence and imaged using the IVIS Spectrum 15 min after the injection and twice thereafter at 2 d intervals. Animals were sacrificed 4 d after treatment and tumors were excised and imaged.

To assess tumor inhibition, MB468-luc (5x10^6) and MB231-luc-mCherry (5x10^5) cells trypsinized with Tryple Express (Invitrogen), resuspended in 1:1 WIT-Matrigel, were injected subcutaneously in opposite flanks. Five days later when tumors were palpable, mice bearing tumors of comparable size were randomized into groups and treated subcutaneously every 3 d in the scruff of the neck with PBS containing nothing or 5 mg/kg EpCAM-AsiCs directed against eGFP or PLK1, EpCAM aptamer, or PLK1 siRNA. Mice were sacrificed on day 13. A similar protocol was followed in mice injected with 4x10^4 MCF10-CA1a-Luc cells, except that treatment was initiated 1 d after tumors were implanted, the injections were performed in the flank near the tumor, but not intratumorally, and the mice were sacrificed on day 15.

**Statistical analysis**
Student’s t-tests were used to analyze the significance between the treated samples and the controls where the test type was set to 2-tailed distribution and two-sample equal variance. To assess innate immune stimulation, one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test was performed using GraphPad Prizm 4 software (GraphPad Software, San Diego, CA). p<0.05 was considered significant. Correlations were analyzed by Pearson coefficient.

**Measurement of in vivo innate immune stimulation**

Mice were injected sc with eGFP EpCAM-AsiCs (5 mg/kg) or ip with Poly(I:C) (5 or 50 mg/kg). Serum samples, collected at baseline and 6 and 16 hr after treatment, were stored at -80°C before measuring IFNβ, IL-6 and IP-10 using the ProcartaPlex Multiplex Immunoassay (Affymetrix/eBioscience, San Diego, CA). Spleens, harvested at sacrifice 16 hr post treatment, were stored in RNAlater (Qiagen) before extracting RNA by adding TRIZOL (Invitrogen) to single cell suspensions generated using the gentleMACS Dissociator (MACS Miltenyi Biotec, San Diego, CA). cDNA was synthesized using Superscript III and random hexamers (Invitrogen) and PCR was performed using SsoFast EvaGreen Supermix and a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA) using the following primers:

- *Gapdh* forward: 5’- TTCACCACCATGGAGAAGGC-3’,
- *Gapdh* reverse: 5’- GGCATGGACTGTGGTCATGA-3’,
- *ifnb* forward: 5’-CTGGAGCAGCTGAATGGAAAG-3’,
- *ifnb* reverse: 5’- CTTGAAGTCCGCCCTGTAGGT-3’,
- *il-6* forward: 5’-TGCCCTTCATTTATCCCTTGAA-3’,
Results

EpCAM is highly expressed on epithelial breast cancer cell lines

We first examined EpCAM expression in breast cancer cell lines. Based on gene expression data in the Cancer Cell Line Encyclopedia (40), EpCAM mRNA is highly expressed in basal-A TNBC and luminal breast cancer cell lines, but poorly in basal-B (mesenchymal) TNBCs (Figure 1a). (In all figure labels in this paper, basal-A TNBC cell lines are labeled in red, basal-B TNBC in green and luminal cell lines in blue.) Surface EpCAM staining, assessed by flow cytometry, was 2-3 logs brighter in all luminal and basal-like cell lines tested, than in normal epithelia immortalized with hTERT (BPE) (41), fibroblasts or mesenchymal TNBCs (Table 1). Thus EpCAM is highly expressed in epithelial breast cancer cell lines compared to normal cells or mesenchymal tumors.

EpCAM-AsiCs selectively knock down gene expression in EpCAM+ breast cancer cells

A 19 nucleotide (nt) aptamer that binds to human EpCAM with 12 nM affinity (19) was identified by SELEX (42, 43). A handful of EpCAM-AsiCs that linked either the sense or
antisense strand of the siRNA to the 3'-end of the aptamer by several linkers were designed and synthesized with 2’-fluoropyrimidine substitutions and 3’-dTdT overhangs to enhance in vivo stability, avoid off-target knockdown of partially complementary genes, and limit innate immune receptor stimulation. To test RNA delivery, gene knockdown and anti-tumor effects, siRNAs were incorporated to knockdown a marker gene (eGFP), a ubiquitous, endogenous nonessential gene (AKT1), and PLK1, a kinase required for mitosis, whose knockdown is lethal to dividing cells (Supplemental Table 1). The AsiC that performed best in dose response studies of gene knockdown joined the 19 nt EpCAM aptamer to the sense (inactive) strand of the siRNA via a U-U-U linker (Figure 1b). The EpCAM-AsiC was produced by annealing the chemically synthesized ~42-44 nt long strand (19 nt aptamer + linker + 20-22 nt siRNA sense strand) to a 20-22 nt antisense siRNA strand. The 2’-fluoropyrimidine modification led to RNase resistance and stability in 50% human serum (T₁/₂ >>36 hr, Supplemental Figure 1). EpCAM-AsiCs did not trigger innate immunity when injected sc into tumor-bearing mice (Supplemental Figure 2).

To verify selective uptake by EpCAM+ tumor cells, we first used confocal fluorescence microscopy to compare internalization of the EpCAM aptamer, fluorescently labeled at the 5’-end with Cy3, in EpCAM+ MDA-MB-468 TNBC cells and BPE, EpCAMdim immortalized breast epithelial cells (Figure 1c). Because AsiCs contain only one aptamer, they do not crosslink the receptor they recognize. As a consequence, cellular internalization is slow since it likely occurs via receptor recycling, rather than the more rapid process of activation-induced endocytosis. Only MDA-MB-468 cells took up the
aptamer. Uptake was clearly detected at 22 hr, but increased greatly after 43 hr. To test whether EpCAM-AsiCs are specifically taken up by EpCAM bright cell lines, the 3’ end of the antisense strand of the AsiC was fluorescently labeled. EpCAM+ BPLER, a basal-A TNBC cell line transformed from BPE by transfection with human TERT, SV40 early region and H-RASV12, took up Alexa-647 EpCAM-AsiCs when analyzed after a 24 hr incubation, but BPE cells did not (Figure 1d). Previous studies have shown that AsiCs are processed within cells by Dicer to release the siRNA from the aptamer (10, 12, 15). To verify that the released siRNA was taken up by the RNA induced silencing complex (RISC), we used qRT-PCR to amplify that PLK1 siRNA immunoprecipitated with Ago when MDA-MB-468 cells were incubated with PLK1 EpCAM-AsiCs (Supplemental Figure 3). No PLK1 siRNA bound to Ago when the same cells were incubated with PLK1 siRNAs.

Next to assess whether gene knockdown was specific to EpCAM+ tumors, we compared eGFP knockdown by eGFP EpCAM-AsiCs and lipid transfection of eGFP siRNAs in these same cell lines, which stably express eGFP (Figure 1e). Although transfection of eGFP siRNAs knocked down gene expression equivalently in BPE and BPLER, incubation with EpCAM-AsiCs in the absence of any transfection lipid selectively knocked down expression only in BPLER. AsiC knockdown was uniform and comparable to that achieved with lipid transfection. Next we compared the knockdown of AKT1 by AKT1 AsiCs and transfected AKT1 siRNAs in 6 breast cancer cell lines and normal human fibroblasts (Figure 1e). AKT1 was selectively knocked down by EpCAM-AsiCs targeting AKT1 only in EpCAMbright luminal and basal-A TNBCs, but not in
mesenchymal basal-B TNBCs or fibroblasts. As expected, AsiCs targeting eGFP had no effect on AKT1 levels and transfection of AKT1 siRNAs comparably knocked down expression in all the cell lines studied. Moreover, EpCAM-AsiC knockdown of AKT1 correlated with EpCAM expression (Figure 1f). Similar results were obtained when AKT1 protein was analyzed by flow cytometry in stained transfected cells (Figure 1g,h). Thus in vitro knockdown by EpCAM-AsiCs is effective and specific for EpCAM bright tumor cells.

**PLK1 EpCAM-AsiCs selectively kill EpCAM bright tumor cells in vitro**

To explore whether EpCAM-AsiCs could be used as anti-tumor agents in breast cancer, we examined by CellTiterGlo assay the effect of AsiCs directed against PLK1, a kinase required for mitosis, on survival of 10 breast cancer cell lines that included 5 basal-A TNBCs, 2 luminal cell lines, and 3 basal-B TNBCs. EpCAM-AsiCs targeting PLK1, but not control AsiCs directed against eGFP, decreased cell proliferation in the basal-A and luminal cell lines, but did not inhibit basal-B cells (Figure 2a). Lipid transfection of PLK1 siRNAs suppressed the growth of all the cell lines. The anti-proliferative effect strongly correlated with EpCAM expression (Figure 2b). To determine whether ligation of the EpCAM aptamer contributed to the anti-proliferative effect of the EpCAM-AsiC, we compared survival of cells treated with PLK1 EpCAM-AsiCs with cells treated with the aptamer on its own (Figure 2c). The aptamer by itself did not reproducibly affect survival of any breast cancer cell lines, possibly because as a monomeric agent it does not cross-link the EpCAM receptor to alter EpCAM signaling. Thus the PLK1 EpCAM-AsiC
asserts its specific anti-tumor effect on EpCAM+ breast cancer cells by gene knockdown.

To determine whether EpCAM-AsiCs specifically target EpCAM+ cells when mixed with EpCAM\textsuperscript{dim} non-transformed epithelial cells, we incubated co-cultures of GFP- TNBC cells and GFP+ BPE cells with \textit{PLK1} EpCAM-AsiCs or medium and used GFP fluorescence to measure their relative survival by flow cytometry 3 days later (Figure 2d, e). EpCAM-AsiCs targeting \textit{PLK1} greatly reduced the proportion of surviving EpCAM+ basal-A tumor cells, but had no effect on survival of an EpCAM- basal-B cell line. Thus \textit{PLK1} EpCAM-AsiCs are selectively cytotoxic for EpCAM+ tumor cells when mixed with normal cells.

**EpCAM-AsiCs concentrate in EpCAM+ breast tumor biopsy specimens**

We next examined whether EpCAM-AsiCs concentrate in human breast tumors relative to normal breast samples within intact tissues. Paired normal tissue and breast tumor biopsies from 3 breast cancer patients (2 ER\textsuperscript{+}PR\textsuperscript{+}HER2\textsuperscript{-} luminal breast cancers, 1 TNBC) were cut into \~3 mm sided cubes and placed in 96 well plates. The tumor sample cells were all EpCAM\textsuperscript{bright} and the normal tissue cells were EpCAM\textsuperscript{dim} (Figure 3a). Fluorescently labeled Alexa647-siRNAs (not expected to be taken up by either normal tissue or tumor), Alexa647-cholesterol-conjugated siRNAs (chol-siRNAs, expected to be taken up by both), or Cy3-EpCAM-AsiCs were added to the culture medium and the tissues were incubated for 24 h before harvest. The Cy3 signal of the AsiC, visible to the naked eye, concentrated only in the tumor specimens and was not
detected in normal tissue (Figure 3b). To quantify RNA uptake, flow cytometry analysis was performed on washed single cell suspensions of the tissue specimens (representative tumor-normal tissue pair (Figure 3c), mean ± SEM of triplicate biopsies from 3 EpCAM\textsuperscript{bright} paired breast tumor-normal tissue samples (Figure 3d)). The EpCAM-AsiC was significantly taken up by the tumors, but not normal tissue, while neither took up the unconjugated siRNA and both weakly took up the chol-siRNA. Thus, within intact tissue, EpCAM-AsiCs are selectively delivered to EpCAM\textsuperscript{bright} tumors relative to normal tissue.

**PLK1 EpCAM-AsiCs inhibit T-ICs of EpCAM+ tumors**

EpCAM was chosen for targeting in part because EpCAM marks T-ICs and metastasis-initiating cells (20, 22, 26, 27, 31). To investigate whether EpCAM-AsiCs inhibit T-ICs, we compared colony and mammosphere formation (T-IC functional in vitro assays) after mock treatment, treatment with paclitaxel or with EpCAM-AsiCs against eGFP or PLK1. PLK1 EpCAM-AsiCs more strongly inhibited colony and mammosphere formation of EpCAM+ basal-A TNBCs and luminal cell lines than paclitaxel, but were inactive against EpCAM- basal-B TNBCs (Figures 4a-c). T-IC inhibition was specific, since eGFP AsiCs had no effect. To evaluate the effect of EpCAM-AsiCs on tumor initiation, EpCAM+ MB468 cells stably expressing luciferase were treated overnight with medium or PLK1 or eGFP EpCAM-AsiCs and equal numbers of viable cells were then implanted sc in nude mice (Figure 4d). PLK1 EpCAM-AsiCs completely blocked tumor formation assessed by in vivo luminescence (Figure 4e,f). Thus PLK1 EpCAM-AsiCs inhibit in vitro T-IC assays and tumor initiation selectively for EpCAM+ breast cancers.
Subcutaneously administered EpCAM-AsiCs are selectively taken up by distant EpCAM+ TNBCs

To be clinically useful, EpCAM-AsiCs need to be taken up by disseminated tumor cells. Intravenously injected AsiCs do not accumulate efficiently within subcutaneous tumors implanted in the flanks of nude mice, probably because their size (~25 kDa) is below the threshold for kidney filtration and they are rapidly excreted. Linkage to polyethylene glycol greatly enhanced the circulating half-life, tumor accumulation and antitumor therapeutic effect of PSMA-AsiCs in a mouse xenograft model of prostate cancer (11). However, to see if this modification could be bypassed, we examined by live animal epifluorescence imaging whether sc injection of Alexa750-labeled eGFP EpCAM-AsiCs in the scruff of the neck of 7 mice led to accumulation in distant EpCAM+ MB468 and EpCAM- MB231 TNBCs implanted sc in each flank (Figure 5). Within a day of injection, EpCAM-AsiCs concentrated in the EpCAM+ tumor and persisted there for at least 4 days. The EpCAM-AsiCs were detected around the injection site on day 2, but were only found within the EpCAM+ tumor on day 4.

*PLK1* EpCAM AsiCs cause regression of basal-A TNBC and Her2 breast cancer xenografts

Because sc injected EpCAM-AsiCs concentrated in distant EpCAM+ tumors, we next looked at whether sc injection of *PLK1* EpCAM-AsiCs could selectively inhibit the growth of an EpCAM+ TNBC xenografted tumor. EpCAM+ MB468-luc cells were implanted in Matrigel in one flank of a nude mouse and EpCAM- MB231-luc-mCherry
cells were implanted on the opposite flank. Once the luciferase signal of both tumors was clearly detected above background, groups of 5-6 mice were mock treated or injected sc with 5 mg/kg of EpCAM-AsiCs targeting \( PLK1 \) or \( eGFP \) every 3 d for 2 wks. (We chose biweekly dosing because gene knockdown in siRNA-transfected rapidly dividing cancer cells usually only persists for about 5 d.) Tumor growth was followed by luminescence. All the EpCAM+ tumors rapidly completely regressed only in mice that received the \( PLK1 \)-targeting AsiCs (Fig. 6). The EpCAM+ tumors in mice treated with eGFP-targeting AsiCs and all the EpCAM- tumors continued to grow. This experiment was repeated with similar results after injection of \( PLK1 \) AsiCs into mice bearing Her2+ MCF10A-CA1a (Supplemental Figure 4). Thus sc injected \( PLK1 \) EpCAM-AsiCs show specific antitumor activity against EpCAM+ human xenografts.

**Discussion**

Here we show that EpCAM-AsiCs can be used to knockdown genes selectively in epithelial breast cancer cells and their stem cells, sparing normal epithelial cells and stroma, to cause tumor regression and suppress tumor initiation. In one very aggressive TNBC xenograft model, the EpCAM-AsiCs caused complete tumor regression after only 3 injections. However, we did not keep the mice alive off therapy to see if the tumors recurred after treatment was stopped. This is a flexible platform for targeted therapy, potentially for all the common epithelial cancers, which uniformly express high levels of EpCAM. Although we used EpCAM-AsiCs targeting \( PLK1 \), in principle the siRNA could be varied to knockdown any tumor dependency gene that would be customized to the tumor subtype or the molecular characteristics of an individual patient’s tumor. AsiC
cocktails targeting more than one gene would be ideal for cancer therapeutics to lessen
the chances of developing drug resistance. Targeted cancer therapy so far has relied on
using tumor-specific antibodies or small molecule inhibitors to oncogenic kinases.
Although the AsiC platform is not new, using EpCAM as an AsiC ligand and developing
RNAi therapy to target cancer stem cells is novel. No one before has shown that an
unconjugated AsiC can have potent antitumor effects or that AsiCs could be
administered sc. (Moreover, preliminary studies of sc administered CD4-AsiCs in
humanized mice (L.A.W. and J.L.) showed strong knockdown in CD4 cells in the spleen
and distant lymph nodes, suggesting that AsiCs targeting receptors on cells located
elsewhere in the body could also be administered sc.) There is currently no targeted
therapy for TNBC or for T-ICs. Targeted delivery has the advantage of reduced dosing
and reduced toxicity to bystander cells. Developing targeted therapy for TNBC and
developing ways of eliminating T-ICs are important unmet goals of cancer research.

Multiple groups have already used AsiCs to demonstrate impressive therapeutic effects
(so far only in mice) to knockdown gene expression in challenging types of cells,
including cancer cells and lymphocytes. AsiCs are cleaved within cells by Dicer to
liberate the siRNA from the aptamer (10, 12, 15). The U-U-U linker used here may be a
particularly good Dicer substrate. AsiCs are an attractive method for gene knockdown
outside the liver. It is a flexible platform that by modifying the aptamer can be used to
target any cell type and by changing the siRNA can be used to knockdown any gene or
combinations of genes. Over a thousand aptamers to human proteins have already
been identified (44). Methods to select aptamers have become streamlined in recent
years by combining deep sequencing and bioinformatics to more rapidly identify the features of active sequences (45). AsiCs are ideal for personalized therapy that can be altered to suit the molecular characteristics of an individual tumor or tumor subtype. The AsiC does not appear to activate the receptor, presumably because it does not crosslink it (15). AsiCs are a single chemical entity that is stable in the blood, simple to manufacture and not likely to be toxic. We did not see any evidence of toxicity or weight loss in treated mice, but we have not done formal testing. AsiCs, unlike liposomes and nanoparticles, do not get trapped in the liver and other filtering organs and should be able to readily penetrate tissues as well as small molecule drugs (46, 47). EpCAM-AsiCs did not stimulate an innate immune response when injected in vivo and tested in the most sensitive immune cells (splenocytes) and at the time of a peak immune response with sensitive qRT-PCR assays. RNAs on their own do not induce antibodies, although the absence of AsiC antibodies needs to be formally tested. An aptamer drug, pegatanib (Macugen) is approved for macular degeneration and at least 8 aptamer drugs are in clinical trials, so aptamer RNAs on their own are well tolerated (48, 49).

The small size of the EpCAM aptamer used here is ideal for an AsiC drug, since RNAs <60 nt can be efficiently synthesized. Despite their promise, there is still considerable room to improve AsiCs - to optimize circulating T_{1/2}, cellular uptake, and endosomal release and to reduce the needed dose (although it is currently acceptable: ~1-5 mg/kg) (11, 13, 15, 18). Recent studies have shown that the efficiency of GalNac-conjugated siRNAs in nonhuman primates can be improved by as much as 50-fold by optimizing the chemical modifications of the active strand to enhance stability and activity within
the RNA-induced silencing complex (M. Manoharan, pers. comm.). Experiments to optimize the EpCAM-AsiCs are planned.

One potential source of toxicity of EpCAM-AsiCs could be targeting of epithelial tissue stem cells, especially in the gut. Because the EpCAM aptamer does not target mouse EpCAM, mouse experiments cannot adequately assess this possible toxicity. We will therefore need to use human tissues (or possibly primates) to look for EpCAM-AsiC toxicity to human tissue stem cells. An alternate drug development strategy would be to select for an EpCAM aptamer that cross-reacts with mouse and human EpCAM.

In addition to their potential therapeutic use, EpCAM-AsiCs could also be a powerful in vivo research tool for identifying the dependency genes of tumors and T-ICs to define novel drug targets. In principle, aptamer chimeras could be designed to deliver not only siRNAs but also miRNA mimics or antagonirs, antisense oligonucleotides that function by other mechanisms besides RNAi, or even longer mRNAs or noncoding RNAs (50, 51). They could also be designed to incorporate more than one aptamer, multiple siRNAs, or even toxins or small molecule anticancer drugs.
References

Table 1. EpCAM protein expression on breast cancer cells, immortalized normal breast
epithelial cells and normal human fibroblasts, assessed by flow cytometry (MFI, mean
fluorescence intensity)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subtype</th>
<th>EpCAM MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPE</td>
<td>immortalized normal epithelium</td>
<td>2</td>
</tr>
<tr>
<td>BPLER</td>
<td>basal-A TNBC</td>
<td>109</td>
</tr>
<tr>
<td>HMLER</td>
<td>unclassified TNBC (myoepithelial)</td>
<td>72</td>
</tr>
<tr>
<td>HCC1143</td>
<td>basal-A TNBC</td>
<td>1068</td>
</tr>
<tr>
<td>HCC1937</td>
<td>basal-A TNBC</td>
<td>806</td>
</tr>
<tr>
<td>HCC1187</td>
<td>basal-A TNBC</td>
<td>289</td>
</tr>
<tr>
<td>HCC1806</td>
<td>basal-A TNBC</td>
<td>558</td>
</tr>
<tr>
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</tr>
<tr>
<td>MB468</td>
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<tr>
<td>MCF7</td>
<td>luminal</td>
<td>583</td>
</tr>
<tr>
<td>T47D</td>
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</tr>
<tr>
<td>MB436</td>
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<td>4</td>
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<tr>
<td>Human fibroblast</td>
<td>Normal tissue</td>
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</tr>
</tbody>
</table>
Figure Legends

Figure 1 – EpCAM-AsiCs knockdown gene expression specifically in EpCAM+ epithelial breast cancer cells

a, EpCAM mRNA expression in epithelial basal-A TNBC (red) and luminal (blue) breast cancer cell lines versus mesenchymal basal-B TNBC (green) cell lines. Data from the Cancer Cell Line Encyclopedia(40). b, Design of PLK1 EpCAM-AsiC, containing an EpCAM aptamer linked to the sense strand of a PLK1 siRNA, which is annealed to the antisense strand of the siRNA. The AsiC long strand was chemically synthesized with 2’-fluoropyrimidines. c, Fluorescence microscopy comparison of uptake of Cy3-labeled EpCAM aptamer (red) by the basal-A TNBC cell line MDA-MB-468 and the immortalized epithelial cell line BPE(41). Plasma membrane is counter stained (blue). d, EpCAM expression (top), uptake of Alexa-647-labeled EpCAM-AsiC (middle) and eGFP knockdown by eGFP EpCAM-AsiC or transfected eGFP siRNA (lower) in basal-A TNBC cell line BPLER and immortalized breast epithelial cell line BPE. Uptake was assessed 24 h post incubation and knockdown was assessed 3 d post incubation. Data are representative of 3 independent experiments. Grey histograms in upper and middle panels are unstained and mock treated cells, respectively. e-h, AKT1 mRNA, assessed by qRT-PCR relative to GAPDH mRNA (e,f), and protein, assessed by flow cytometry (h,i), measured 3 d after indicated incubation with AsiCs or with lipid-complexed siRNAs. Controls were mock and transfection lipid-treated cells. The cancer cell line subtypes are color coded in the labels as in (a). (e, g) show mean ± SEM of 3 independent experiments. (*, p<0.05; **, p<0.01, 2-tailed Student’s t-test). (f,h) show
correlation between gene knockdown and EpCAM mean fluorescence intensity (MFI) after treatment with \textit{AKT1} EpCAM-AsiC (left) or \textit{AKT1} siRNA lipoplexes (right).

Figure 2 - EpCAM-AsiCs targeting PLK1 specifically kill EpCAM+ breast cancer cells

\textbf{a,b}, Breast cancer cell lines (luminal (blue), basal-A TNBC (red), basal-B TNBC (green)) were incubated with EpCAM-AsiCs targeting \textit{eGFP} or \textit{PLK1} or with lipoplexed \textit{PLK1} siRNAs (or with medium or transfection lipid as controls). Cell viability was assessed by Cell-TiterGlo 1 d later. Mean ± SEM of 3 independent experiments is shown in (\textbf{a}) and correlation between cell viability after incubation with \textit{PLK1} EpCAM-AsiCs (left) or \textit{PLK1} siRNAs (right) and EpCAM mean fluorescence intensity (MFI) is shown in (\textbf{b}).

\textbf{c}, Breast cancer cell lines were incubated with medium (mock), EpCAM aptamer or \textit{PLK1} EpCAM-AsiC and cell viability was assessed 1 d later. Mean ± SEM of 3 independent experiments is shown. In (\textbf{a}) and (\textbf{c}), *, p<0.05; **, p<0.01, relative to mock control by 2-tailed Student’s t-test.)

\textbf{d,e}, An equal mixture of GFP+ TNBC cells (red, basal-A; green, basal-B) and immortalized GFP+ normal breast epithelial cells (BPE) were untreated or treated with \textit{PLK1} EpCAM-AsiCs and analyzed for survival by flow cytometry 3 d later. Shown are representative flow cytometry plots (\textbf{d}) and survival (\textbf{e}) of the TNBC cell line relative to BPE (mean ± SEM of 4 independent experiments; *, p<0.05; **, p<0.01, relative to the MB231 basal-B cell line by 2-tailed Student’s t-test).

Figure 3 - Human breast cancer tissues specifically take up Cy3-labeled EpCAM-AsiCs
a, Single cell suspensions of a human basal-A TNBC tumor biopsy and adjacent normal tissue were analyzed by flow cytometry for EpCAM expression. b-d, Two luminal and one basal-A TNBC human biopsy and adjacent normal tissue were incubated with eGFP Cy3-EpCAM-AsiCs, Alexa647-labeled siRNAs or chol-siRNAs for 24 h, photographed (b) and digested to a single cell suspension and analyzed by flow cytometry for RNA uptake (c,d). Cy3 is visible to the naked eye. (b,c) show results for the basal-A TNBC sample and (d) shows the mean ± SEM uptake of 3 replicates of 3 patient samples. In (c), gray histograms and numbers (MFI) represent untreated samples; red histograms and numbers, fluorescence after indicated treatment. (*, p<0.05, Student’s t-test of tumor versus normal tissue).

Figure 4 – PLK1 EpCAM-AsiCs inhibit tumor initiation of EpCAM+ breast cancer cells

a-c, Human breast cancer cell lines (luminal (blue), basal-A TNBC (red), basal-B TNBC (green)) were untreated or treated with eGFP or PLK1 EpCAM-AsiCs or paclitaxel for 24 h and washed free of drug before assessing colony formation (a,b) and mammosphere assay (c). (a) shows representative colony forming assay and (b,c) depict mean ± SEM of 3 independent experiments (*, p<0.05; **, p<0.01, Student’s t-test versus untreated sample). d-f, MB468-luc cells, untreated or treated for 24 h with eGFP or PLK1 EpCAM-AsiCs, were injected s.c. in the flanks of nude mice (n=9). Mice were imaged every 5 days. (d) shows experimental scheme, (e) luciferase images of representative mice and (f) luminescence of all mice. AU, arbitrary units.
Figure 5 - Alexa750-EpCAM-AsiCs are taken up by EpCAM+ tumors

a, Experimental schema (n=7 or 8). b,c Luminescent and fluorescent images of representative treated mice (side views show indicated EpCAM+mCherry- basal-A tumor (red) and EpCAM-mCherry+ basal-B tumor (green)). (c) shows Alexa 750 uptake (mean ± SEM; *, p<0.05) in all treated mice. d, Images of harvested tumors removed 4 d after treatment (top) and mean ± SEM of Alexa 750 and mCherry signals in excised tumors (bottom). AU, arbitrary units (*, p<0.05; **, p<0.01; by 2-tailed Student’s t-test comparing EpCAM+ to EpCAM- tumors).

Figure 6 – PLK1 EpCAM AsiCs inhibit growth of basal-A TNBC tumors

a, Experimental schema. b, Representative luciferase images and change in tumor luminescence of individual tumors (bottom). AU, arbitrary units.
Figure 1

Background: Triple-negative breast cancer (TNBC) has the worst breast cancer prognosis.1-4 There is no targeted therapy, ...
Figure 2

(a) Cell viability relative to mock.

(b) Relative cell viability vs EpCAM MFI for PLK1 EpCAM-AsiC and PLK1 siRNA.

(c) Cell viability relative to mock for different cell lines.

(d) Flow cytometry analysis for GFP and EpCAM.

(e) Tumor cell vs BPE survival.
**Figure 3**

(a) Tumor and normal tissue normalized counts of EpCAM MFI.

(b) Images of NL tissue and tumor.

(c) Normalized counts of RNA uptake in NL tissue and tumor for siRNA, Chol-siRNA, and EpCAM-AsiC.

(d) Graph showing RNA uptake levels for NL tissue and tumor for siRNA, Chol-siRNA, and EpCAM-AsiC.
Figure 4

EpCAM+ Basal-A TNBC
EpCAM+ Luminal
EpCAM- Basal-B TNBC
Mock GFP
EpCAM-AsiC Paclitaxel
MCF7
HCC1954
HCC1806
HCC1937
PLK1
EpCAM-AsiC

Mammospheres, %

Day 5
Day 10
Day 15

Luminescent imaging

Treat MB468-Luc cells ex vivo
Implant cells sc
5, 10, 15 d
Luminescent imaging

Mock
eGFP
EpCAM-AsiC

PLK1
EpCAM-AsiC

Tumor luminescence, AU

Days

Luminescent counts

1.5 50 x10^3 50
Implant EpCAM+ MB468-Luc cells in the left flank
Implant EpCAM- MB231-Luc-mCherry cells in the right flank

Inject 0.5 mg/kg Alexa750-EpCAM-AsiCs sc

Luminescent and fluorescent live animal imaging

0, 2, 4 d

Figure 5

c

Alexa750 uptake, AU

Days post injection

0

2

4

a

5 d

b

15 min post injection

Luciferase

Alexa750

mCherry

MB468

MB231

Injection site

Luciferase

Alexa750

mCherry

MB468

MB231

Injection site

Luminescence (counts x10^3)

Epifluorescence (counts x10^7)

**

*

Figure 5
Implant EpCAM+ MB468-Luc cells in the left flank

Implant EpCAM- MB231-Luc-mCherry cells in the right flank

5 d PLK1 or eGFP EpCAM-AsiCs (5 mg/kg sc q 3d)

N=5-6/gp

5 d 13 d Live animal imaging

Day 2 Day 5 Day 9 Day 13

EpCAM+ MB468

PLK1 AsiC

eGFP AsiC

Mock

EpCAM+ MB468

PLK1 AsiC

eGFP AsiC

Mock

EpCAM- MB231

PLK1 AsiC

eGFP AsiC

Mock

Figure 6
Molecular Cancer Therapeutics

Gene knockdown by EpCAM aptamer-siRNA chimeras suppresses epithelial breast cancers and their tumor-initiating cells

Adi Gillboa-Geffen, Peter Hamar, Minh Le, et al.

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