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Nanoparticle-mediated delivery of siRNA targeting Parp1 extends survival of mice bearing tumors derived from Brca1-deficient ovarian cancer cells

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Inhibition of the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) with small molecules has been shown to be an effective treatment for ovarian cancer with BRCA mutations. Here, we report the in vivo administration of siRNA to Parp1 in mouse models of ovarian cancer. A unique member of the lipid-like materials known as lipidoids is shown to deliver siRNA to disseminated murine ovarian carcinoma allograft tumors following intraperitoneal (i.p.) injection. siParp1 inhibits cell growth, primarily by induction of apoptosis, in Brca1-deficient cells both in vitro and in vivo. Additionally, the treatment extends the survival of mice bearing tumors derived from Brca1-deficient ovarian cancer cells but not from Brca1 wild-type cells, confirming the proposed mechanism of synthetic lethality. Because there are 17 members of the Parp family, the inherent complementarity of RNA affords a high level of specificity for therapeutically addressing Parp1 in the context of impaired homologous recombination.

The overall cure rate of ovarian cancer has remained low, greatly owing to the fact that few diagnoses are made while the tumor is limited to the ovaries (1). Because the disease is so heterogeneous, enhanced outcomes await translating insights at the molecular level into personalized treatment strategies (2). Germ-line mutations of BRCA1 confer a lifetime risk of ∼85% for breast cancer and ∼45% for ovarian cancer in families with multiple cases of such cancers (3). Mutations in the BRCA1 gene occur in ∼5% of ovarian cancer cases in the general population (4), and inheritance of DNA repair defects contributes to ∼10% of all ovarian cancers (5). Additionally, many ovarian cancers fail to express BRCA1 due to epigenetic silencing (6). There are a number of studies showing that breast cancer tumors that assay as defective in DNA repair are also sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors (7).

Cells lacking BRCA1 or BRCA2 exhibit defects in DNA repair (8). Specifically, the inability to correct double-strand breaks by homologous recombination in these mutants results in chromosomal rearrangements and genomic instability (9). PARP1 is involved in the detection of DNA damage, DNA repair, and the maintenance of genomic stability (10). Activated in response to DNA damage, PARP1 has been implicated in nonhomologous end-joining and base excision repair through both short-patch and long-patch pathways (11). It has been shown that BRCA-deficient cells exhibit profound sensitivity to inhibition of PARP1, resulting in cell cycle arrest, chromosome instability, and cell death (12, 13). In patients harboring BRCA mutations, an orally active PARP inhibitor has shown antitumor activity as a single agent (14). The mechanism underlying this biological outcome is known as synthetic lethality, wherein although the loss of either of two proteins alone is not lethal, the loss of both functions is catastrophic to the cell (15).

siRNA, which directs the sequence-specific degradation of target mRNA, represents a unique class of potential therapeutics for cancer (16), a disease often caused by aberrant overexpression of oncoproteins. Recently, this class of molecules was used to treat patients with melanoma upon systemic administration (17). For ovarian cancer, i.p. administration of chemotherapy has been shown to result in improved outcomes relative to i.v. administration (18). In view of this, we tested in a mouse model the effectiveness of delivery of nanoparticles containing siRNA targeting Parp1 for inhibition of ovarian cancer following i.p. injection.

Nanoparticles can facilitate the delivery of siRNA into cells. Previous work has demonstrated the potential of nanoparticles formed from lipid-like materials, termed lipidoids (19), in a variety of animal models, including following i.p. injection in ovarian tumor models (20). This class of materials is derived from an amine-containing backbone from which aliphatic chains extend, forming a hybrid structure that resembles dendrimers and lipids. The amines likely electrostatically interact with the phosphate backbone of siRNA and facilitate endosomal escape as the compartment becomes acidified, and the hydrophobic chains probably mediate interaction with the cell membrane.

Results

NC100 Effectively Delivers siRNA to Ovarian Cancer Cells. Cells lines and tumors with defined genetic alterations provide ideal systems in which to test the molecular mechanisms of tumor sensitivity to pathway- and/or siRNA-mediated therapy (21). We have previously developed a system in which multiple defined genetic lesions can be introduced into mouse ovarian surface epithelial cells, a cell type that is believed to be the precursor for ovarian carcinoma. This system is based on avian RCAS virus transduction of cells that are programmed to express the avian TVA receptor. Briefly, to generate ovarian surface epithelial cell lines with defined genetic alterations, we introduced coding sequences for human c-myc, mouse K-rasG12D, and HA-tagged mouse myristoylated Akt1 oncosomes into ovaries isolated from K5-TVA/p53−/− mice or Cre recombinase in association with oncosomes into ovaries isolated from K5-TVA/Brca1Δbocox/p53Δbocox mice (21, 22). Some of the resulting cell lines generate ovarian cancer when injected into normal and immune-deficient mice.

To identify lipidoids that can be used as carriers for siRNA delivery into mouse ovarian carcinoma, 22 top-performing members of a lipidoid library were screened for the ability to transfect mT2K-Luc, a firefly luciferase-expressing murine ovarian cancer cell line.

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The lipidoid NC100 effectively back... A second siRNA targeting a distinct sequence within the Parp1 gene, siParp1-1, was used to confirm that the observed inhibition of cell growth was the consequence of knockdown of Parp1 and not specific to the sequence of siParp1 (Fig. S1). Consistent with the dose–response observed during screening, whereas a very robust phenotype is observed at higher siRNA concentrations in vitro, the transfection efficiency of BR5FVB1 by NC100 was observed to be only ∼70% at the concentrations used for these biological assays, limiting the degree of knockdown and growth inhibition observed. Transfection efficiency was measured using dye-modified siRNA, which probably yields an overestimate of uptake, as not all siRNAs bound to cells are ultimately loaded into RISC. BR5FVB1 cells were found to be much more difficult to transfect than mT2K, the cell line in which the original screen was performed. We have observed that differential transfection efficiency across cell lines is not uncommon for lipidoid reagents, as they seem to have inherent specificity to particular cells.

Having established that NC100-mediated delivery of siParp1 yields target knockdown and inhibition of proliferation by induction of apoptosis, we confirmed that the material is well tolerated by mice upon i.p. injection (Fig. S2). Escalating doses of formulated and crudely mixed lipidoid-siRNA nanoparticles were administered to mice, and body weight was monitored at 0, 24, and 72 h. Notable weight loss was observed at the highest doses examined, but the transient decrease was not significant at 5 mg/kg, the dose used in all of the in vivo studies herein described. Next, to test whether NC100 can mediate delivery to ovarian carcinoma nodules disseminated in the i.p. cavity, Brca1 wild-type T22H cells were injected into the i.p. cavity of nude mice and allowed to grow for 2 wk. Fluorescently labeled siRNA was then formulated with NC100 and injected into the i.p. cavities of these mice at a dose of 0.5 mg/kg of labeled siRNA in 4.5 mg/kg of unlabeled siRNA. The mice were visualized 24 h later, and NC100 was found to confer greater delivery than the positive control ND98 (20) (Fig. S4). Naked siRNA yielded a similar signal to the PBS negative control, confirming the importance of delivery reagents not only to protect against nucleases but also to facilitate the transport of these large, charged molecules into tissues. In...
addition to performing whole-mouse imaging, we extracted the tumor nodules and visualized the fluorescently labeled siRNA, confirming the efficacy of NC100 as a delivery agent (Fig. 3B).

**Delivery of siParp1 to Brca1-Deficient Ovarian Tumors Induces Apoptosis and Increases Survival.** The BR5FVB1 cell line lacks Brca1 and overexpresses c-myc. Having confirmed that we had an siRNA sequence that knocked down c-myc protein ~85% (Fig. S3), we thought that siMyc would enhance the likelihood of observing a reduction in tumor growth when combined with siParp1. Indeed, in vitro data demonstrated that BR5FVB1 cells treated with siMyc experienced a proliferation defect (Fig. 2B). Accordingly, mice bearing BR5FVB1-derived tumors were treated i.p. with siControl (siCon; siRNA targeting firefly luciferase for in vivo studies) or a combination of siParp1 + siMyc at a total dose of 5 mg/kg for three doses. Five animals in each group were challenged with BR5FVB1 tumor cells and, after establishment of disseminated nodules, formulated siRNAs were injected on days 14, 17, and 20. The mice were subsequently monitored, and the development of ascites in the peritoneal cavity was used as a measure of disease burden. When fluid accumulation became severe, the mice were killed. Treatment with siParp1 + siMyc reduced ascites formation and significantly (P < 0.05) extended survival relative to siCon (Fig. 4A). To test if the observed phenotype was caused by apoptotic induction, as observed in vitro by FACS analysis, TUNEL staining was performed on tumor tissue that was extracted from the mice at the time of euthanization. The number of apoptotic cells in the treated tumors was more than twice that observed in the control-treated tumors (Fig. S4).

To elucidate the contribution of c-myc oncogene knockdown relative to Parp1 knockdown, a follow-up study was performed in which siMyc and siParp1 were administered i.p. individually as well as in combination at a total dose of 5 mg/kg for six doses. Mice treated with siMyc had the same survival rate as those treated with either siCon or PBS. This suggests that the degree of growth inhibition observed with siMyc is not adequate to impact on tumor growth in vivo. In contrast, animals treated with either siParp1 alone or siParp1 + siMyc survived significantly longer (P < 0.01). The results indicated that the observed survival extension can be attributed solely to the targeting of Parp1 (Fig. 4B). Importantly, the survival effect is specific to the Brca1-deficient genotype, as it was not observed in tumors derived from Brca1 wild-type T22H cells (Fig. 4C), supporting the proposed mechanism of synthetic lethality. Though T22H cells experience a proliferation defect in vitro upon knockdown of c-myc, the inhibition of Parp1 does not affect their growth or induce apoptosis (Fig. S5), and the in vivo results are consistent with these observations. Immunoblotting of lysates from tumors derived from Brca1-deficient cells treated with siParp1 confirmed target knockdown in vivo (Fig. 4D). Notably, a lower band denoting Parp1 cleavage, a signature of apoptosis (23), was observed after two doses in the siParp1 treatment group. An examination of the Parp1 levels after a single dose of siCon vs. siParp1 confirms a greater than twofold knockdown of Parp1 by the latter. This comparison suggests that the decrease in the upper band of intact Parp1 after two doses is due not only to apoptosis-related cleavage but also to RNAi-mediated targeting of the protein.

**Immunostimulation Is Not Responsible for the Observed Effect.** It has been reported that some effects attributed to RNAi may actually be caused by an innate immune response to foreign nucleic acids (24). To address this possibility, we examined the immunostimulatory potential of siParp1, siMyc, and the control siRNA that were used for the in vivo experiments described previously. Whole blood was collected from mice, and a marked induction of IFN-α in serum was detected by ELISA after 6 h upon administration of NC100-formulated siParp1 compared with PBS solution or siCon; siMyc induced a moderate response (Fig. 5A). This response is not unusual, as it has been observed by other groups at this time-point (20). The strong immunostimulatory activity evoked by siParp1 diminished to basal level over 24 h, suggestive of a transient innate immune recognition mechanism. Still, we wanted to confirm that the observed survival extension was not due to an innate immune response.

**Fig. 2.** Knockdown of Parp1 by NC100-delivered siRNA arrests proliferation and induces apoptosis in Brca1-deficient mouse ovarian cancer cells. (A) Delivery of siParp1 confers target knockdown at the mRNA level, as determined by qPCR. BR5FVB1 cells were transfected with 5 nM siRNA, and total RNA was collected after 24 h. (B) Proliferation of BR5FVB1, a Brca1-deficient mouse ovarian cancer cell line that overexpresses c-myc, is inhibited by treatment with siParp1 or siMyc. Cells were transfected with 20 nM siRNA and counted 72 h after treatment. (C) Treatment with siParp1 causes BR5FVB1 cells to undergo apoptosis. Annexin V-PE apoptosis analysis was performed 48 h after treatment with 100 nM siRNA. Annexin V-PE positive and 7-AAD negative cells were defined as apoptotic cells by flow cytometry. NT, no treatment. siCon, siRNA targeting factor VII.

**Fig. 3.** Lipidoids mediate efficient siRNA delivery to murine ovarian carcinoma allograft tumors. Fluorescently labeled siRNA delivered by NC100 or ND98 is taken up by tumors in vivo following i.p. administration at a dose of 0.5 mg/kg of labeled siRNA. (A) Representative images of mice that were injected with PBS, Vivo-Tag 750-labeled siRNA delivered by NC100 or ND98 lipidoid nanoparticles, or naked Vivo-Tag 750-labeled siRNA. Images were taken 24 h after injection. (B) Detection of fluorescently labeled siRNA in the tumor nodules themselves.
Importantly, 2′-OMe-modified siParp1 (designated siParp1* herein) was nonimmunostimulatory (Fig. 5A), enabling us to investigate the specific causality of the knockdown of Parp1 on survival. BR5FVB1 cells were transfected with 5 nM modified or unmodified siRNA, and total RNA was collected after 48 h. Both siParp1 and siParp1* were shown to impart robust silencing, as determined by qPCR (Fig. 5B). After confirming that the modifications did not affect the siRNA’s ability to impart mRNA knockdown, the therapeutic efficacy of siParp1* was examined by repeating the in vivo survival challenge experiment. Again, a significant survival extension was observed in the siParp1* treatment group (P < 0.05) compared with the control siRNA treatment group (Fig. 5C), suggesting that the observed immunostimulatory activity evoked by unmodified siParp1 6 h post-injection was not critical to the enhanced observed survival and that knockdown of the target gene in the context of Brca1 deficiency confers synthetic lethality.

Discussion

We have shown that siRNA targeted against Parp1 confers synthetic lethality in vivo, extending the survival of mice bearing tumors derived from Brca1-deficient cells. These data agree with the effects observed in Brca1-deficient cells when Parp1 is drugged by a small molecule (12). Here, we show that the response is retained in a murine model of ovarian cancer. The model involves i.p. administration of materials to disseminated ovarian carcinoma allograft tumors. Delivery of lipidoid-formulated nanoparticles containing siRNA to these tumors was shown to be effective and consistent with results for a similar ovarian tumor model using a related lipidoid with siRNA targeting claudin-3 (20). These studies suggest that nanoparticle delivery to tumor cells in the i.p. cavity can be effective in the treatment of ovarian cancer. This local delivery avoids the uptake of systemically delivered nanoparticles by the liver that makes targeting to other organs difficult. In fact, there have been few reports of systemic siRNA administration to organs other than the liver (17).

Through three separate experiments in vivo, we confirm that the mechanism of action is synthetic lethality. siParp1 was administered in combination with siMyc or alone. It was administered as an unmodified or a chemically modified siRNA. The results consistently demonstrated that treatment with siParp1 affords extension of survival. This effect was shown to be specific to a Brca1-deficient genetic background, as the enhanced survival was not observed in a Brca1 wild-type background. The knockdown of target protein in recovered tumors was confirmed by repeating the in vivo survival challenge experiment. Again, a significant survival extension was observed in the siParp1* treatment group (P < 0.05) compared with the control siRNA treatment group (Fig. 5C), suggesting that the observed immunostimulatory activity evoked by unmodified siParp1 6 h post-injection was not critical to the enhanced observed survival and that knockdown of the target gene in the context of Brca1 deficiency confers synthetic lethality.
affords another type of specificity beyond small molecules for therapeutically targeting PARP1 in the context of impaired homologous recombination. The sequence used herein, siParp1, specifically targets Parp1 but not any of the other 16 isoforms, siParp1-1 similarly targets a unique sequence in the distinctive 5' end of the target mRNA transcript. This is important because Parp2 is the only other PARP family member that has been implicated in DNA repair, and the functions of the other isoforms are very important to the cell, including cell cycle control (PARP-3), intracellular transport (PARP-4), regulation of telomere length (PARP-5a) and chromatin structure (PARP-10), and antiviral responses (PARP-13) (25).

The inhibition of PARP1 might also be used to address sporadic cancers that exhibit “BRCAness,” the functional traits of BRCA mutation in the absence of actual mutations (26). Though not detected in normal tissue, aberrant methylation of the BRCA1 promoter and silencing of expression are found in 11–14% of breast cancers and 5–31% of ovarian cancers (6, 27). This epigenetic pattern is typically restricted to these two cancers, consistent with the tumorigenicity of BRCA mutations. Notably, promoter methylation of Fanconi anemia complementation group F (FANC), which inactivates the FANC-BRCA pathway, has been observed in several other types of sporadic cancer (28, 29). In addition to epigenetic alterations at the genomic level, functional inactivation of BRCA at the protein level is also possible (30). ESMY, which has been reported to bind to the activation domain of PARP1 and inhibiting its activity is amplified in sporadic breast cancer (13%) and higher-grade ovarian cancer (17%) (26). Thus, the range of cancers that might specifically respond to siParp1 could be much larger than just those with mutations in BRCA genes.

Our findings validate the possibility of assessing ovarian cancer in its native context, the i.p. setting of immunocompetent animals, for the effect of inhibition of specific genes on tumorigenesis. For example, nanoparticle-mediated delivery of siRNA targeting ErbB3 (31) could be used to investigate the dependence of ovarian cancer on downstream signaling pathways. Here, we show that nanoparticle-mediated i.p. delivery of siRNA targeting Parp1 to disseminated tumors, which resemble the presentation in humans, can induce apoptosis and extend survival specifically in a Brca1-deficient genetic background. These findings raise the possibility of creating synthetic lethal phenotypes through the delivery of siRNA combinations, because multiple siRNAs can be delivered simultaneously in nanoparticles (19).

Materials and Methods

Cell Lines and Mouse Models. We have previously generated genetically defined murine ovarian cancer cell lines based on an RCAS-TVA gene delivery system (21, 22). To establish mT2K cells, T2 cells (p53−/−, Akt, myc) were infected in vitro with RCAS-K-rasG12D virus. To establish T2H cells, T2 cells (an independently derived cell line with p53−/−, Akt, myc background) were infected in vitro with p eighty-puro-H-rasG12D and subsequently selected for 10 d in media containing 2.0 μg/mL of puromycin. T2H cells were able to grow in both immunodeficient nude mice and immunocompetent FVB mice. Tumor nodules from immunocompetent FVB mice injected with the Brca1-deficient cell line BRS were used to generate the BRSFV1 tumor cell line, which was able to grow in recipient FVB mice. The stably luciferase-expressing cells mT2K-Luc and BRSFV1-Luc were generated by infecting mT2K cells and BRSFV1 cells with pM5SV-puro-Firefly luciferase viral supernatant and selecting the cells in a medium containing 2.0 μg/mL of puromycin. To generate allografts, 1 × 106 murine ovarian cancer cells in 150 μL of Opti-MEM media (Invitrogen) were injected i.p. into 5- to 6-wk-old female athymic nude mice or female FVB/NJ mice (Jackson Laboratory). Tumors and/or visible ascites generally developed within 4–7 wk of injection, depending on the distinct genetic composition of the cell lines. All studies on mice were approved by the Massachusetts Institute of Technology Committee on Animal Care.

siRNAs. siRNAs targeting luciferase (siLuc), GFP (siGFP), or factor VII (siFVII) and 2′-OMe-modified siRNA targeting murine Parp1 (siParp1−1) were synthesized by Alnylam Pharmaceuticals. Unmodified siRNAs targeting murine Parp1 (siParp1 and siParp1-1) or human Myc (siMyc) were synthesized by and purchased from Dharmacon. The sequences for the sense and antisense strands of siRNAs are as follows:

| siLuc sense: 5′- AACCGUUGGGCGUUAUCAAdTdT-3′ | antisense: 5′-UUGA-UUAAGGCGCCAGGGuUdTdT-3′ |
| siGFP sense: 5′-CCCAAUAGGACAGCACGAgCdTdT-3′ | antisense: 5′-GUCG-GUGCGUCUCAUGGgCdTdT-3′ |
| siFVII sense: 5′-GGGAUCUAUCUAAGGUAbCdTdT-3′ | antisense: 5′-GUAA-GAUCUGGAUGAUCdTdT-3′ |
| siParp1 sense: 5′-CCAAAGAUAUUCCGAAAAdCdTdT-3′ | antisense: 5′-UUU-GUGG-CAGCGGAUUGCudCdTdT-3′ |
| siMyc sense: 5′-GGCUAUCUUCUCGUCGCAAgCdTdT-3′ | antisense: 5′-CUUGG-CAGCGGAUUGCudCdTdT-3′ |

The sequence of the modified Parp1 siRNA (siParp1−2) is as follows:

| sense: 5′-ccAAAAGAUAUCGCAGGAAAdCdTdT-3′ | antisense: 5′-UUUCUCGGA-AUUCGdCdTdT-3′ |
| A, C, G, U are ribonucleotides; dT is deoxythymidine; and c and u are 2′-OMe-modified pyrimidines. |

Lipidoid Synthesis. Lipidoids were synthesized and purified as described previously (19).

Lipidoid Screening. A total of 8,000 cells/well of mT2K-Luc cells were plated into 96-well plates in DMEM with 10% FCS and 1% penicillin/streptomycin. After 2 d of growth in culture, the cells were transfected with lipidoid-siRNA complexes. Briefly, individual lipidoids were premixed with siGFP or siParp1 at a weight-to-weight ratio of 2.5:1 (optimized transfection conditions were determined empirically). The working concentration of siRNAs used for screening was 10 nM. Working solutions of each lipidoid were prepared in 25 μM sodium acetate buffer (pH 5.2). The transfection mixtures were incubated for 20 min to allow for complex formation, and 30 μL of each of the lipidoids/siRNA solutions was then added to 200 μL of medium in 96-well plates. Cells were allowed to grow at 37 °C with 5% CO2 and lysed 24 h after transfection. Firefly luciferase activity was determined using the Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Lipofectamine 2000 (Invitrogen) was used as a positive transfection control. Transfections were performed in triplicate.

Cell Proliferation Assay. To monitor cell proliferation, cells transfected with 20 nM siCon (siRNA targeting factor VII) or siParp1 were harvested 72 h after transfection, and cell proliferation was determined by measuring luciferase activity as described previously (32). The lipidoid-siRNA transfection protocol is described above.

In Vitro Apoptosis Assay. Brca1-deficient BR5FVB1 cells were transfected with 100 nm siCon (siRNA targeting factor VII) or siParp1 complexed with NC100. At 48 h after transfection, the cells were washed and resuspended at a concentration of 1 × 106 cells/mL. The apoptotic cells were determined by flow cytometry using Annexin V-PE Apoptosis Detection Kit I according to the manufacturer's instructions (BD Biosciences). Cells with positive Annexin V-PE and negative 7-AAD represented early apoptotic cells. The experiments were performed in triplicate.

Lipidoid-siRNA Nanoparticle Formulation and Treatment in Vivo. siRNAs targeting Parp1 or Luciferase were formulated with NC100 lipidoid, PEG ceramide, and cholesterol as described previously for ND98 (19). The formulated lipidoid-siRNA nanoparticles were determined to be ~75 nm by dynamic light scattering using a Zetasizer Nano ZS (Malvern). To assess in vivo efficacy, female nude or FVB mice were injected i.p. with 1 × 106 cells in Opti-MEM media. For the Brca1 wild-type cell line T2H, 7 d after the initial injection of cells, the mice were treated with 250 μL of NC100-siRNA nanoparticle solution (5 mg/kg i.p.) every 3 d for three doses. For the Brca1-deficient cell line BRSFV1, 14 d after the initial injection of cancer cells, mice were treated with 250 μL of nanoparticle solution (5 mg/kg i.p.) every 3 d for three doses, skipping one dose to mimic cyclic chemotherapeutic dosing, and then an additional three doses every 3 d. Mice were euthanized if they exhibited visible tumors or significant ascites. Mean survival times were calculated using the Log-rank test. Kaplan–Meier survival curves were drawn using GraphPad Prism software. For the lipidoid-siRNA nanoparticle tolerance assay, mice were injected with PBS, sodium acetate, and escalating
In Vivo Imaging. Brca1 wild-type T22H mouse ovarian cancer cells were injected i.p. into nude mice and allowed to grow for 14 d. The mice were then injected i.p. with PBS, naked fluorescent-labeled siRNA, or fluorescent-labeled siRNA formulated with NC100 or ND98 nanoparticles. Fluorochrome absorption was visualized using an IVIS Spectrum bioluminescent and fluorescent imaging system (Caliper Life Sciences).

Quantitative PCR. Total RNA was extracted from tumors using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was transcribed for reverse transcription using the ImProm-ll Reverse Transcription System Kit (Promega). The quantitative PCR was performed using SYBR Green PCR Master Mix reagent (Applied Biosystems). The average threshold cycle for each gene was determined from triplicate reactions, and the expression level was normalized to GAPDH. The primers used for detecting the expression levels of murine Parp1 and GAPDH were as follows: Parp1-F: 5′-CCA TGG ACG TCA AGG AGC-3′; Parp1-R: 5′-GGT CCG GTC AGC ATG GT3′. GAPDH-F: 5′-CAT GCC CTT CCG TGT TCC TA-3′; GAPDH-R: 5′-CCT GTC TCA CCA CCT TCT TGA T-3′.

Western Blotting. Frozen tumor tissues from mice injected with Brca1-deficient BRSFV1 cells and treated with either siLuc or siParp1 were minced and homogenized in cold modified RIPA buffer (Sigma) with a protease inhibitor mix (Roche). Equal amounts of total protein lysate were denatured in Laemmli buffer at 70 °C for 5 min, separated by a 4–12% SDS/PAGE NuPage gel (Invitrogen) and transferred to PVDF membranes. Anti-murine Parp1 (Cell Signaling) was used at a 1:1,000 dilution in 5% milk. Anti-human c-myc (Santa Cruz Biotechnologies) was used at a 1:500 dilution in 5% milk. Anti-β-tubulin (Sigma) was used as a loading control at a 1:10,000 dilution in 5% milk. The proteins were detected using an ECL detection system (Perkin-Elmer) according to the manufacturer’s instructions.

TUNEL Staining. Fresh tumor tissues from mice injected with Brca1-deficient BRSFV1 cells and treated with either siLuc or siParp1 were biopsied, immediately snap frozen, embedded in Optimal Cutting Temperature (OCT) reagent (Sakura Finetek), and stored at −80 °C. Frozen tissue sections (5 μm) were cut with a cryostat and fixed with 4% paraformaldehyde in PBS (pH 7.4). The apoptotic cells were detected using the In Situ Cell Death Detection Kit, TMR red, according to the manufacturer’s instructions (Roche).

Cytokine ELISA. Six and 24 h following i.p. injection of NC100-formulated siRNA (5 mg/kg), blood serum was collected and used for ELISA of mouse iFNa-1 using the VeriKine Mouse Interferon Alpha (Mu-iFNa-α) ELISA Kit according to the manufacturer’s instructions (PBL Biomedical).

Statistical Analysis. Statistical analysis was carried out using a two-tailed Student’s t-test. A value of P < 0.05 was considered to be statistically significant.

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