

siRNA Lipid Nanoparticle Potently Silences Clusterin and Delays Progression When Combined with Androgen Receptor Cotargeting in Enzalutamide-Resistant Prostate Cancer

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Abstract

Purpose: Lipid nanoparticle (LNP) formulations facilitate tumor uptake and intracellular processing through an enhanced permeation and retention effect (EPR), and currently multiple products are undergoing clinical evaluation. Clusterin (CLU) is a cytoprotective chaperone induced by androgen receptor (AR) pathway inhibition to facilitate adaptive survival pathway signaling and treatment resistance. In our study, we investigated the efficacy of siRNA tumor delivery using LNP systems in an enzalutamide-resistant (ENZ-R) castration-resistant prostate cancer (CRPC) model.

Experimental Design: Gene silencing of a luciferase reporter gene in the PC-3M-luc stable cell line was first assessed in subcutaneous and metastatic PC-3 xenograft tumors. Upon validation, the effect of LNP siRNA targeting CLU in combination with AR antisense oligonucleotides (ASO) was assessed in ENZ-R CRPC LNCaP *in vitro* and *in vivo* models.

Results: LNP LUC-siRNA silenced luciferase expression in PC-3M-luc subcutaneous xenograft and metastatic models. LNP CLU-siRNA potently suppressed CLU and AR ASO-induced CLU and AKT and ERK phosphorylation in ENZ-R LNCaP cells *in vitro*, more potently inhibiting ENZ-R cell growth rates and increased apoptosis when compared with AR-ASO monotherapy. In subcutaneous ENZ-R LNCaP xenografts, combinatory treatment of LNP CLU-siRNA plus AR-ASO significantly suppressed tumor growth and serum PSA levels compared with LNP LUC-siRNA (control) and AR-ASO.

Conclusions: LNP siRNA can silence target genes *in vivo* and enable inhibition of traditionally non-druggable genes like CLU and other promising cotargeting approaches in ENZ-R CRPC therapeutics. *Clin Cancer Res*; 21(21): 4845–55. ©2015 AACR.

Introduction

Prostate cancer is the most prevalent cancer in men in the Western world and the second leading cause of cancer deaths among males in Western countries (1). Androgen receptor (AR) signaling remains the key driver of castration-resistant prostate cancer (CRPC). Potent AR pathway inhibitors like enzalutamide (ENZ) inhibit AR nuclear translocation and transcriptional activ-

ity (2, 3), but despite significant activity, progression to ENZ-resistant (ENZ-R) CRPC frequently occurs. This progression occurs with rising serum PSA levels, thereby implicating AR importance in disease progression. Activation of adaptive survival pathways that support AR signaling is an important mechanism of treatment resistance. The molecular chaperone clusterin (CLU) is induced by AR pathway inhibition and highly expressed in ENZ-R CRPC (4), and as a mediator of the stress response confers treatment resistance when overexpressed (5–7). CLU inhibits stress-induced apoptosis by suppressing p53-activating stress signals (8), and conformationally altered Bax (6, 8) in addition to enhancing AKT phosphorylation (9) and transactivation of NF- κ B (10) and autophagy (11). In keeping with these cytoprotective mechanisms, CLU inhibition potentiates activity of anticancer therapy in many preclinical models (12), and is a promising target for novel therapeutics.

Although small-interfering RNA (siRNA) offers the promise for potent and specific gene silencing, poor accumulation at sites of disease and intracellular translocation coupled with poor stability, sensitivity to nucleases, immune stimulation, and rapid clearance have made its therapeutic application difficult. A delivery system is crucial to the efficient delivery to target tissue in order to overcome these shortcomings. Encapsulation of siRNA using a lipid nanoparticle-based delivery system has shown to be crucial in protecting the nucleic acid-based drug from nucleases as well as

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Translational Relevance

Suppression of androgen receptor (AR) signaling remains a therapeutic goal for castration-resistant prostate cancer (CRPC). Despite newer potent AR-pathway inhibitors, resistance frequently occurs. Although cotargeting the AR with adaptive survival pathways is a rational goal, many biologically relevant genes are undruggable with small-molecule inhibitors. Gene silencing of non-druggable targets using small-interfering RNA (siRNA) is a promising approach but *in vivo* delivery remains problematic without a delivery system. We developed a lipid nanoparticle (LNP) system demonstrating silencing of luciferase reporter gene using LNP-LUC-siRNA in both subcutaneous and metastatic PC3-Luc-xenograft models. LNP-CLU-siRNA inhibited AR-antisense-induced upregulation of clusterin (CLU) *in vitro* and *in vivo*, and significantly suppressed tumor growth and serum PSA levels in enzalutamide-resistant (ENZ-R) LNCaP xenografts compared with AR-antisense monotherapy. These data provide novel proof-of-principle that LNP-siRNA can target genes *in vivo* enabling inhibition of traditionally non-druggable genes like CLU and other promising cotargeting approaches in ENZ-R CRPC therapeutics.

prolonging circulation, reducing immune stimulation, and improving intracellular uptake (13). The most clinically advanced delivery of siRNA uses LNP and currently there are six different siRNAs encapsulated in LNP drugs undergoing clinical studies. The most promising study looks at the treatment of transthyretin-induced amyloidosis using a second-generation cationic lipid (DLin-MC3-DMA) that is well tolerated and potently silences transthyretin (14). Two siRNA-LNP studies focus in the treatment of hepatic cancers and early indications suggest safe and active treatments (15). Propensity of LNP systems to accumulate in the liver has become a major obstacle in the extrahepatic delivery of siRNA-LNP systems. Although preclinical studies in prostate cancer (16) and immune cells (17) are promising, distant tumor delivery has not been tested in the clinic. In order to mitigate this higher Peg-lipid content is used to improve the pharmacodynamic and biodistribution of LNP systems to the tumor site (18–20).

Antisense oligonucleotides (ASO) offer another approach to selectively target genes. Although ASOs are primarily used to inhibit "undruggable" targets (21, 22), they may also be of use against drug-resistant targets like that AR in ENZ-resistance (ENZ-R) (23). Although AR extinction approaches using ASOs (24) or shRNA (25) can reduce AR levels and inhibit tumor growth in CRPC models, they have neither been studied in the context of ENZ-R disease nor in combination with siRNA-mediated cotargeting strategies. In this study, we first investigated the efficacy of LNP siRNA tumor delivery in AR-negative PC-3 and AR-positive ENZ-R LNCaP prostate cancer. Gene silencing was first validated using LNP LUC-siRNA to silence PC3 stably expressing firefly luciferase (PC3-M-luc) *in vitro* followed by successful gene silencing in subcutaneous and metastatic xenograft models. Once *in vivo* silencing of LUC was demonstrated, we then evaluated combinatory gene silencing of CLU (using LNP siRNA) and AR (using an AR-ASO) in an

ENZ-R LNCaP model and demonstrated enhanced apoptosis and growth inhibition *in vitro* and *in vivo*.

Materials and Methods

Cell lines and reagents

LNCaP were kindly provided by Dr. L.W.K. Chung (1992, MD Anderson Cancer Center, Houston, TX) and ENZ-R MR49F cell lines were generated, and maintained as previously described (4, 26). PC-3M-luc (C6) cells, stably expressing firefly luciferase protein, were obtained from Caliper Life Sciences and were cultured in DMEM (Invitrogen) with 5% FBS and 2 mmol/L L-glutamine. Supplementary Table S1 shows source and authentication of cell lines. Permanent stocks of cells of authenticated or purchased were prepared and were stored in liquid nitrogen until use. Cells were used for experiments within 6 months. Enzalutamide was purchased from Haoyuan Chemexpress Co., Limited.

siRNA and AR antisense oligonucleotide

All siRNA were purchased from Thermo Scientific or Integrated DNA Technologies. The lower case letters indicates 2'-O-methyl modification, while upper case letter represents unmodified residue and "s" indicates phosphorothioate modification.

Clusterin. Sense: 5'-AuGAuGAAGACuCuGCuGCdTdT-3'
Antisense: 5'-GCAGCAGAGuCuCAuCAuGC-3'

Luciferase. Sense: 5'-cuuAcGcuGAGuAuuuGAdTsdT-3'
Antisense: 5'-UCGAAGuACUcAGCGuAAGdTsdT-3'

GFP. Sense: 5'-AcAuGAAGcAGcACGACuUdTsdT-3'
Antisense: 5'-AAGUCGUGCUGCUUCAUGUdTsdT-3'

AR and scrambled (SCR) antisense was supplied by Isis Pharmaceuticals as previously described (4). The AR-ASO targeting exon-1 and scrambled (SCR) control sequences were 5'-GCGACTACTACAACCTT-3' and 5'-CAGCGCTGACAACTTTCAT-3', respectively. Prostate cells were treated with the indicated oligonucleotides, using protocols described previously (23, 27, 28).

Lipid nanoparticle encapsulation of siRNA

The ionizable cationic lipids O-(Z,Z,Z,Z-heptatriacont-6,9,26,29-tetraen-19-yl)-4-(N,N-dimethylamino)butanoate (DLin-MC3-DMA) and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and PEG lipids PEG-DMG and PEG-DSG were purchased from Biofine International Inc. and have been previously described (29–31). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were obtained from Avanti and Sigma-Aldrich Co., respectively. The lipid composition of all LNPs containing siRNA (LNP-siRNA) was cationic lipid/DSPC/cholesterol/PEG-DMG (50/10/38.5/1.5; mol%) for *in vitro*, while cationic lipid/DSPC/cholesterol/PEG-DSG (50/10/37.5/2.5; mol%) or (50/10/35/5; mol%) for *in vivo*. LNP-siRNAs were prepared using a microfluidic mixing apparatus as previously described (30, 32).

Bioluminescence imaging

PC-3M-luc cells and tumors in mice were imaged using an IVIS200 camera (Caliper Life Sciences) as previously described (33). Data were acquired and analyzed using Living Image software version 3.0 (Caliper Life Sciences).

Cell proliferation assays

PC-3M-luc cells were seeded at a density of 5×10^3 in 96-well plates, and ENZ-R MR49F and parental LNCaP were seeded at a density of 1.25×10^4 in 48-well plates. Cell viability in PC-3M-luc cells and cell growth in MR49F and LNCaP cells were measured by crystal violet assay as previously described (34). Each assay was done in triplicate three times.

Western blotting analysis

Total proteins were extracted using RIPA buffer (50 mmol/L Tris, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mmol/L NaCl, Roche complete protease inhibitor cocktail) and subjected to Western blot analysis as described previously (28). Primary antibodies are shown in Supplementary Materials and Methods.

Quantitative reverse transcription-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Inc.) as previously reported (27). Primers (described in Supplementary Table S2) were normalized to β -actin levels as an internal standard, and the comparative cycle threshold (C_t) method was used to calculate relative quantification of target mRNAs. Each assay was conducted in triplicate.

In vivo PC-3M-luc subcutaneous tumor

Male athymic mice were inoculated subcutaneously with 2×10^6 PC-3M-luc cells. Once tumor bioluminescence signals reached approximately 1×10^8 photons/second, they were randomly assigned for treatment. LNP containing indicated doses of LUC-siRNA or GFP-siRNA as control was administered intravenously (i.v.) through the lateral tail vein. To reduce possible toxicity due to daily dosing, the dosing regimen was modified from previous studies performed by Lee and colleagues where repeat doses administered at 10 mg/kg for 3 consecutive days followed by administration at days 7, 9, and 11 (16) were modified to daily dosing of 7 mg/kg for 5 days. To evaluate LUC expression, mice were then imaged on days 0 (before treatment), 3, 5, and 8 using an IVIS200 Imaging System and then sacrificed on day 8. LUC expression (photons/second) measured at days 3, 5, and 8 was normalized to corresponding animal at day 0 and expressed as relative increase (%). All animal procedures used in this manuscript were approved and carried out according to the guidelines of the Canadian Council on Animal Care and appropriate institutional certification.

In vivo PC-3M-luc metastasis model

PC-3M-luc cells (2×10^6) were injected intravenously into the tail vein of male athymic mice as described previously (33). Once metastatic bioluminescence signals reached approximately 1×10^5 photons/second, they were randomly assigned to either 5 mg/kg LNP LUC-siRNA or GFP-siRNA as control and i.v. injected through the lateral tail vein daily for 5 days. To evaluate LUC expression, mice were then imaged on days 0 (before treatment), 3, 5, and 8 using an IVIS200 Imaging System and then sacrificed on day 8. LUC expression measured at days 3, 5, and 8 were normalized to corresponding animal at day 0 and expressed as relative increase (%).

In vivo ENZ-R MR49F treatment

Male athymic mice were castrated and inoculated subcutaneously with 2×10^6 ENZ-R MR49F cells; mice were treated with

ENZ at 10 mg/kg/each orally daily for maintenance of ENZ resistance. Once tumors reached 100 mm^3 , mice were randomly assigned to 10 mg/kg SCRB or AR-ASO (administered intraperitoneally, i.p., once daily for 5 days and then three times per week thereafter) plus either 5 mg/kg LNP siCLU or siLUC siRNA as control with 2.5 or 5% PEG (i.v. through the lateral tail vein once daily for 4 days and then 3 times per week thereafter). Tumor volume and serum PSA was measured as previously described (27). Mice were sacrificed on day 21 and tumors were harvested for evaluation by Western blot analyses, mRNA expression by real-time monitoring of qPCR, and IHC. Treatment was extended over a 3-week period, with four daily injections in the first week and three daily injections during the second and third weeks.

IHC

IHC was performed as previously described previously (27). All comparisons of staining intensities were made at $\times 200$ magnifications.

Statistical analysis

All *in vitro* data were assessed using the Student *t* test. All *in vivo* data were compared using the Kruskal-Wallis test (JMP version 8). Levels of statistical significance were set at $P < 0.05$.

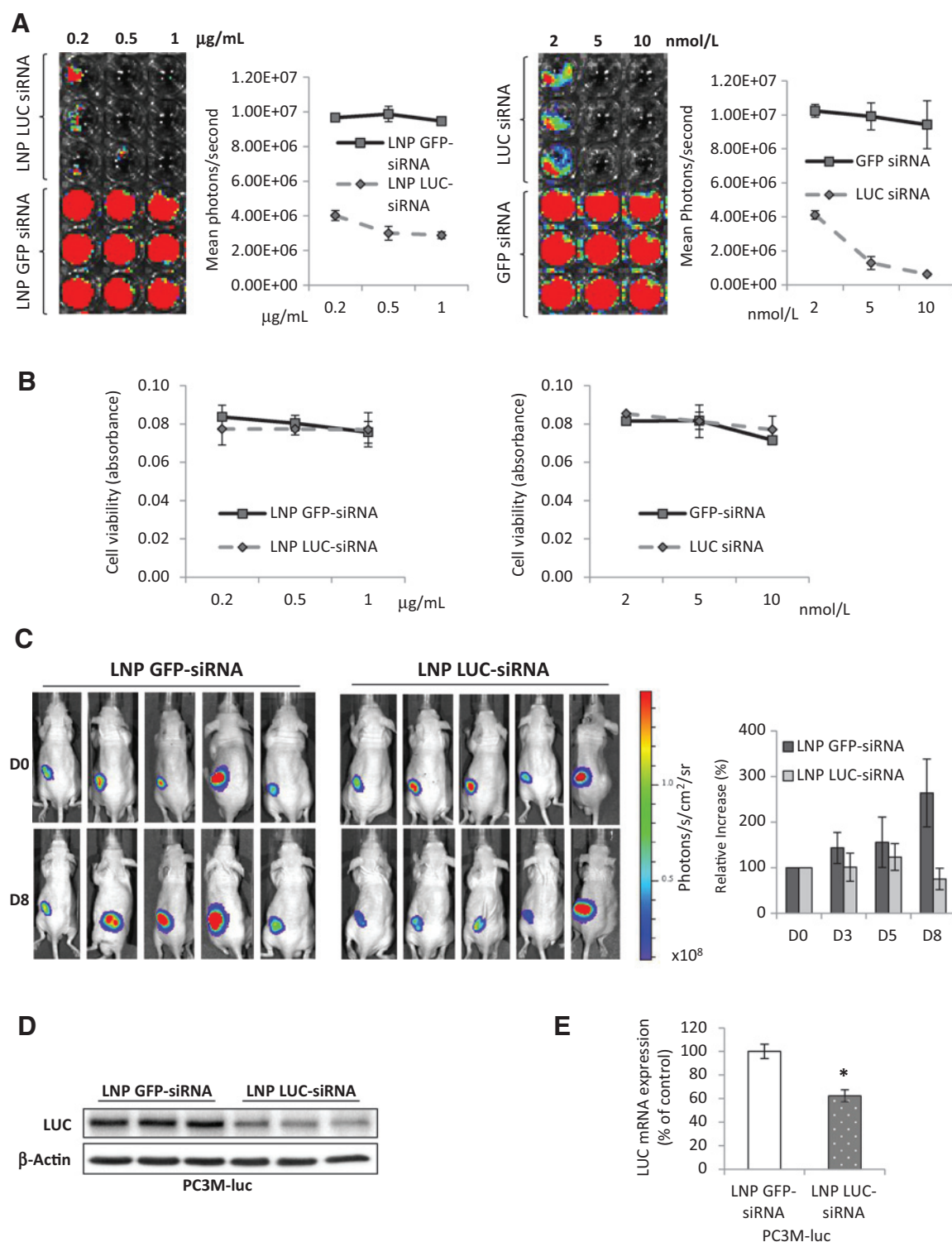
Results

LNP LUC-siRNA decreases LUC expression in PC-3M-luc subcutaneous xenografts

To assess the effects of LNP siRNA tumor delivery *in vivo*, the expression of luciferase in PC-3M-luc stably transfected cells treated with LNP LUC-siRNA containing DLin-KC2-DMA (16, 31) was examined by the IVIS imaging system. The ionizable cationic lipid, DLin-KC2-DMA, is highly active in the liver (31) and also previously shown to silence AR and reduce PSA levels upon intravenous administration in LNCaP tumor model (16). PC-3M-luc cells showed significant correlation between mean bioluminescence and both the total numbers of these cells *in vitro* (Supplementary Fig. S1) and mean subcutaneous tumor volume *in vivo* (Supplementary Fig. S2). LUC siRNA transfected with Lipofectamine 2000 or delivered in LNP systems decreased the expression of LUC in a dose-dependent manner in PC-3M-luc *in vitro* (Fig. 1A) without changing the cell numbers, as evaluated by crystal violet assay (Fig. 1B).

The *in vivo* activity of LNP LUC-siRNA containing DLin-KC2-DMA was first evaluated using PC-3M-luc subcutaneous xenografts. After tumor bioluminescence signals reached approximately 1×10^8 photons/second, mice were randomly assigned for treatment with 7 mg/kg LNP LUC-siRNA (5 mice) or LNP GFP-siRNA (5 mice) as control. At baseline, mean bioluminescence signals were similar in LNP LUC and GFP-siRNA groups (1.22 and 1.17×10^9 photons/second, respectively). LNP LUC-siRNA exhibited LUC silencing effects in PC-3M-luc xenografts as early as day 3 after initial administration by maintaining LUC expression at baseline levels, whereas LNP GFP-siRNA treated showed a 143% increase when normalized to day 0 (Fig. 1C). Significant silencing was achieved at day 8 as the LUC expression was below baseline at 75%, whereas LNP GFP-siRNA treatment showed a 263% increase in LUC expression. The difference between LNP LUC-siRNA and control LNP GFP-siRNA corresponded to approximately 3-fold decrease in LUC expression (Fig. 1C). As expected, LUC protein in tumors collected from representative PC-3M-luc xenografts ($n = 3$

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**Figure 1.**

DLin-KC2-DMA LNP LUC-siRNA decreases LUC expression in PC-3M-luc subcutaneous xenograft model *in vitro* and *in vivo*. A, PC-3M-luc cells were seeded in 96-well plates and treated for 48 hours with the indicated doses of LUC or GFP siRNA with or without LNP. To evaluate LUC emission, PC-3M-luc cells were imaged using the IVIS200 Imaging System and quantitated below corresponding images. Bars, SD. B, PC-3M-luc cell numbers were determined by crystal violet assay. Bars, SD. C, mice were inoculated subcutaneously with PC-3M-luc cells. Once tumor bioluminescence signals reached approximately 1×10^8 photons/second, they were randomized into two groups receiving 7 mg/kg LNP LUC-siRNA or LNP GFP-siRNA as control through daily i.v. injection for 5 days. To evaluate LUC emission (photons/second), mice were imaged using the IVIS200 Imaging System on days 0 (before treatment), 3, 5, and 8 and emission values were normalized to corresponding baseline (day 0) and expressed as relative increase (%) represented in a bar graph below images. Bars, SEM. D, total proteins were extracted on day 8 from three representative xenograft tumors from each group and LUC and β -actin were analyzed by Western blotting. E, mRNAs were extracted from five xenograft tumors from each groups and LUC mRNA were analyzed by quantitative real-time PCR. Bars, SEM. *, $P < 0.05$.

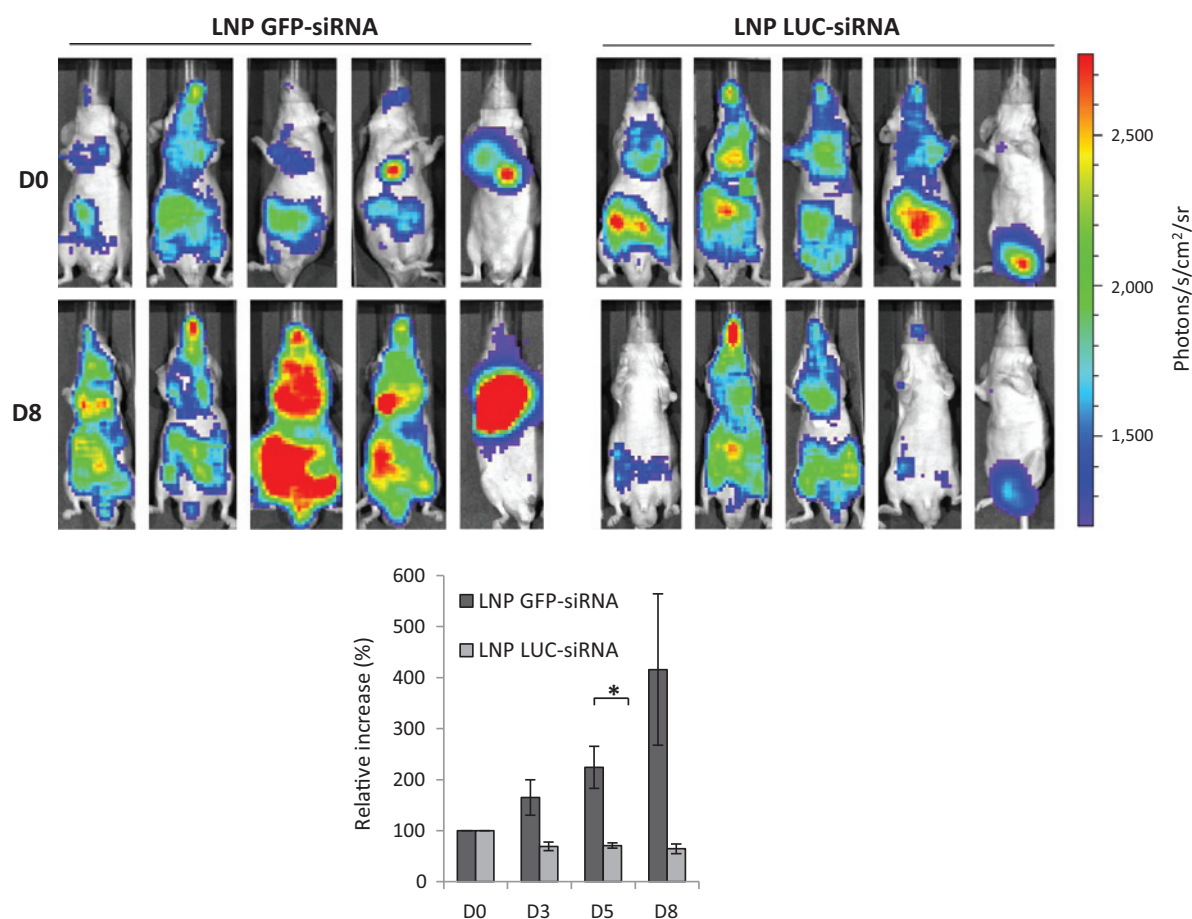


Figure 2.

DLin-KC2-DMA LNP LUC-siRNA decreases LUC expression in PC-3M-luc metastatic model *in vivo*. PC-3M-luc cells were injected into the tail vein of mice. Once metastatic bioluminescent signals reached approximately 1×10^5 photons/second, they were randomly assigned to either 5 mg/kg LNP LUC-siRNA or GFP-siRNA as control and given daily i.v. injections for 5 days. Evaluation of mice LUC emission (photons/second) was done using the IVIS200 Imaging System on days 0 (before treatment), 3, 5, and 8 and emission values were normalized to corresponding baseline (day 0) and expressed as relative increase (%). Bars, SEM. *, $P < 0.05$.

per group) decreased after treatment with LNP LUC-siRNA as opposed to LNP GFP-siRNA treatment (Fig. 1C and D) and transcript levels decreased by 40% as assessed by quantitative real-time PCR (Fig. 1E). The reduction of luciferase detection was not due to toxicity induced by LNP siRNA treatment as no significant body weight loss was observed (Supplementary Fig. S3A), and more importantly, tumor volume increased regardless of the treatment (LNP GFP or LUC-siRNA; Supplementary Fig. S3B) while showing significant reduction in LUC detection when treated with LNP LUC-siRNA (Supplementary Fig. S3C–S3E).

LNP LUC siRNA decreases LUC expression in PC-3M-luc *in vivo* metastatic model

Next, the *in vivo* activity of LNP LUC-siRNA containing DLin-KC2-DMA was evaluated in the PC-3M-luc metastatic model. To establish an *in vivo* metastatic cancer model, highly metastatic PC-3M-luc cells stably expressing luciferase were injected into the tail vein of male nude mice. After metastatic bioluminescent signals reached approximately 1×10^5 photons/second, mice were randomly assigned for treatment with 5 mg/kg LNP LUC-siRNA (5 mice) or LNP GFP-siRNA (5 mice) as control. At day 3, 69% of

LUC expression was detected when treated with LNP LUC-siRNA when normalized to day 0 in comparison with 165% when treated with LNP GFP-siRNA (Fig. 2). Significant LUC silencing was observed in day 5 reaching >3-fold difference with 71% LUC activity when treated with LNP LUC-siRNA in comparison with 224% when administered with LNP GFP-siRNA treatment (Fig. 2). At day 8, LUC expression was maintained below baseline levels at 70% when treated with LNP LUC-siRNA, while increased LUC expression at 416% when treated with LNP GFP-siRNA corresponding to a substantial 6.5-fold difference in LUC activity (Fig. 2).

DLin-MC3-DMA-based LNP CLU-siRNA systems enhance AR-ASO-induced apoptosis in parental and ENZ-R LNCaP cells *in vitro*

We previously reported that CLU inhibition represses ENZ-induced activation of AKT and MAPK pathways, and that combined ENZ plus OGX-011 (CLU ASO) synergistically delays CRPC LNCaP tumor growth *in vivo* (4). Since cotargeting the AR and CLU in CRPC is a promising synergistic strategy, we therefore used the most clinically advanced ionizable cationic lipid DLin-MC3-DMA

(14) to deliver CLU-siRNA and knockdown CLU in *in vivo* ENZ-resistant MR49F cells. Our *in vitro* data indicate that LNP CLU-siRNA reduces CLU and PSA protein levels, as well as the phosphorylation of AKT and extracellular signal-regulated kinase (35) in ENZ-R MR49F and parental LNCaP cells (Fig. 3A and B). LNP CLU-siRNA reduces mRNA expression of CLU dose dependently (Fig. 3C). Furthermore, LNP CLU-siRNA significantly enhanced ENZ activity and reduced cell viability in a dose-dependent manner (Supplementary Fig. S4), similar to reports using OGX-011 (4). We next confirmed this synergistic effect of LNP CLU-siRNA with AR knockdown using AR-ASO. LNP CLU-siRNA significantly enhanced AR-ASO activity and reduced cell viability in a dose-dependent manner in ENZ-R MR49F (Fig. 3D) and parental LNCaP cells (Fig. 3E). In addition, combination of LNP CLU-siRNA with AR-ASO increased caspase-dependent apoptosis, as shown by cleaved PARP and caspase-3 activity in ENZ-resistant MR49F (Fig. 3F) and parental LNCaP cells (Fig. 3G). Collectively, these data indicate that LNP CLU-siRNA enhances AR-ASO-induced apoptosis *in vitro*.

LNP CLU-siRNA enhances AR-ASO-induced inhibition of ENZ-R LNCaP cell growth *in vivo*

We previously reported that cotargeting the AR (with ENZ) and CLU (with OGX-011) delayed CRPC LNCaP tumor progression *in vivo* (4). Because the AR commonly remains active in ENZ-resistant disease, the *in vivo* anticancer activity of LNP CLU-siRNA containing Dlin-MC3-DMA was evaluated using a cotargeting strategy with AR-ASO in ENZ-R MR49F LNCaP xenografts. After MR49F tumors exceeded 100 mm³, mice were randomly assigned for treatment with AR-ASO plus either 5 mg/kg LNP CLU-siRNA with 2.5% or 5% PEG or LUC-siRNA as control with 2.5% or 5% PEG. Different PEG molar% was tested as PEG-lipid is a well-characterized tool to improve pharmacokinetics as well as divert distribution to extrahepatic sites (20). At baseline, mean tumor volume and serum PSA levels were similar in both groups. Of note, 2.5% and 5% PEG LNP CLU-siRNA significantly reduced mean tumor volume from 1,451 mm³ and 1,444 mm³ to 656 mm³ and 826 mm³ by 3 weeks (***, $P < 0.001$ and **, $P < 0.01$, respectively), compared with LNP LUC-siRNA (Fig. 4A); serum PSA levels were also significantly lower (**, $P < 0.01$ and *, $P < 0.05$, respectively; Fig. 4B). Waterfall plots of the best tumor volume and serum PSA decline per mouse at any time in 2.5% and 5% PEG are shown in Fig. 4C–F, respectively. LNP CLU-siRNA formulated with 2.5% PEG trended to better tumor volume and serum PSA decline compared with 5% PEG. CLU protein expression in tumors collected from representative MR49F xenografts ($n = 3$ per group) decreased after treatment with LNP CLU-siRNA (Fig. 5A) compared with LNP LUC-siRNA (control). CLU mRNA levels were significantly decreased after treatment with 2.5% or 5% PEG LNP CLU-siRNA systems compared with LNP LUC-siRNA (**, $P < 0.01$ and *, $P < 0.05$, respectively; Fig. 5B). LNP formulated with 2.5% PEG showed enhanced silencing as indicated by reduced levels of CLU protein and mRNA compared with 5% PEG *in vivo*. As expected, AR silencing is observed in all mice treated with AR ASO in comparison with the Scramble control (Fig. 5C). IHC analysis revealed significantly decreased CLU expression after treatment with 2.5% PEG LNP CLU-siRNA LNP compared with 2.5% PEG LNP LUC-siRNA as control (***, $P < 0.001$; Fig. 5D and E). In addition, tumors treated with 2.5% PEG LNP CLU-siRNA had significantly higher apoptosis rates than 2.5% PEG LNP LUC-siRNA as shown by increased TUNEL stain-

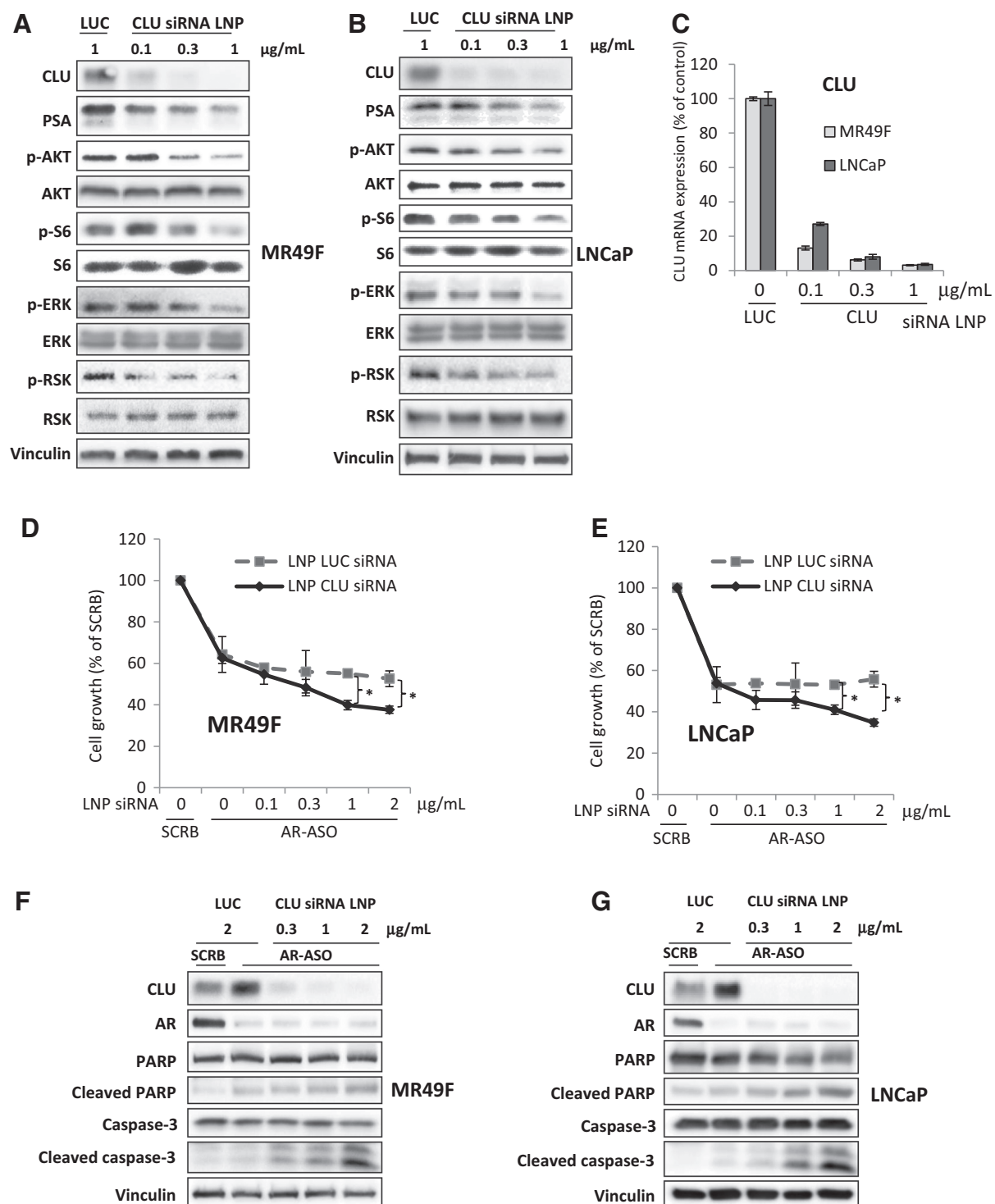
ing (*, $P < 0.05$; Fig. 5F). Collectively, these results suggest that LNP siRNA delivery systems potently silence CLU and induce apoptosis in ENZ-R prostate cancer *in vivo*.

Discussion

Potent AR pathway inhibitors like abiraterone and ENZ prolong survival in men with CRPC but resistance develops in many initial responders, frequently heralded by rising PSA levels indicative of continued AR signaling (3). Therefore, reactivation of AR activity remains a central driver of post-AR pathway inhibitor CRPC progression (25), and can result from AR gene amplification, promiscuous AR mutants, splice variants, androgen biosynthesis, and activation by oncogenic signaling pathways. ASO offers an approach to selectively inhibit "undruggable" or drug-resistant targets (21, 22), which includes nuclear AR in the context of ENZ-resistance. Although AR ASOs (24) or shRNA (25) can inhibit CRPC xenograft growth, they have not been studied in the context of ENZ-R disease. In this study, we used a next-generation constrained-ethyl (cEt) modified AR-ASO (Gen 2.5) to suppress AR levels *in vivo* (23, 36, 37). The improved resistance against nuclease-mediated metabolism afforded by Gen 2.5 chemistry results in a significantly improved tissue half-life *in vivo* and a longer duration of action with a more intermittent dosing schedule. We show that systemic administration of this AR-ASO potently suppressed levels of AR and delayed progression *in vivo* compared with controls.

In addition to directly targeting the AR in post-AR pathway inhibitor CRPC, defining interactions between the AR and adaptive survival pathways will define mechanisms supporting treatment-resistant CRPC and guide new combinatorial strategies that delay progression. As a key mediator of the stress response, CLU is induced by AR pathway inhibition and its overexpression confers treatment resistance in prostate cancer (4–7). CLU has been targeted in prostate cancer where the CLU inhibitor OGX-011 (custirsen, OncoGenex Pharmaceuticals) potentiates anticancer therapies in many preclinical models (12), including prostate cancer (4, 27). CLU suppression also inhibits epithelial-to-mesenchymal transition and suppresses prostate cancer metastatic progression (33). Previous reports show that CLU inhibition abrogates cross-talk activation of AKT and MAPK pathways following AR blockade, further reducing AR transcriptional activity (4). Our results are consistent with previous reports, where LNP CLU-siRNA reduces expression of PSA as well as phosphorylation of AKT/S6 and (ERK)/RSK signaling pathways. These data provide the basis to investigate the efficacy of LNP siRNA tumor delivery and LNP CLU-siRNA sensitized AR-ASO AR knockdown activity in ENZ-R prostate cancer in *in vitro* and *in vivo* models.

Although monotargeting of AR or CLU with ASO or siRNA has been reported previously (16, 24, 35), and combinatorial cotargeting the AR and CLU in ENZ-R CRPC with nucleotide-based therapeutics is biologically rational, combining two antisense drugs *in vivo* is precluded by toxicity induced by doubling the dose of the oligonucleotide backbone. To circumvent this backbone toxicity limitation, we combined the AR-ASO with a CLU LNP-siRNA formulation. We first show that LNP-siRNA could silence luciferase gene in both subcutaneous and metastatic PC3-Luc xenograft models. The LNP systems tested *in vivo* incorporated 5% PEG-lipid with C-18 acyl chains which improves circulation time (20) thus improving the accumulation at extrahepatic sites.

**Figure 3.**

DLin-MC3-DMA LNP CLU-siRNA enhances AR-ASO-induced apoptosis in ENZ-R and parental LNCaP *in vitro*. MR49F (A) and LNCaP (B) cells were treated with LNP CLU or LUC-siRNA (control) with the indicated concentrations for 48 hours. Western blot analyses were conducted for CLU, PSA, pAKT/AKT, pS6/S6, pERK/ERK, and pRSK/RSK, using vinculin as a loading control. C, quantitative reverse transcription PCR was used to evaluate levels of CLU mRNA. MR49F (D) and LNCaP (E) cells were treated with indicated concentrations of LNP CLU or LUC-siRNA. The next day, cells were treated with 50 nmol/L AR-ASO followed by indicated concentrations of LNP CLU or LUC-siRNA for 96 hours. Cell growth were determined by crystal violet assay and compared with LNP LUC-siRNA. Bars, SD. *, $P < 0.05$. MR49F (F) and LNCaP (G) cells were treated with indicated concentrations of LNP CLU or LUC-siRNA. The next day, cells were treated with 50 nmol/L AR-ASO followed by indicated concentrations of LNP CLU or LUC-siRNA for 96 hours and CLU, AR, PARP, caspase-3, and vinculin were analyzed by Western blotting.

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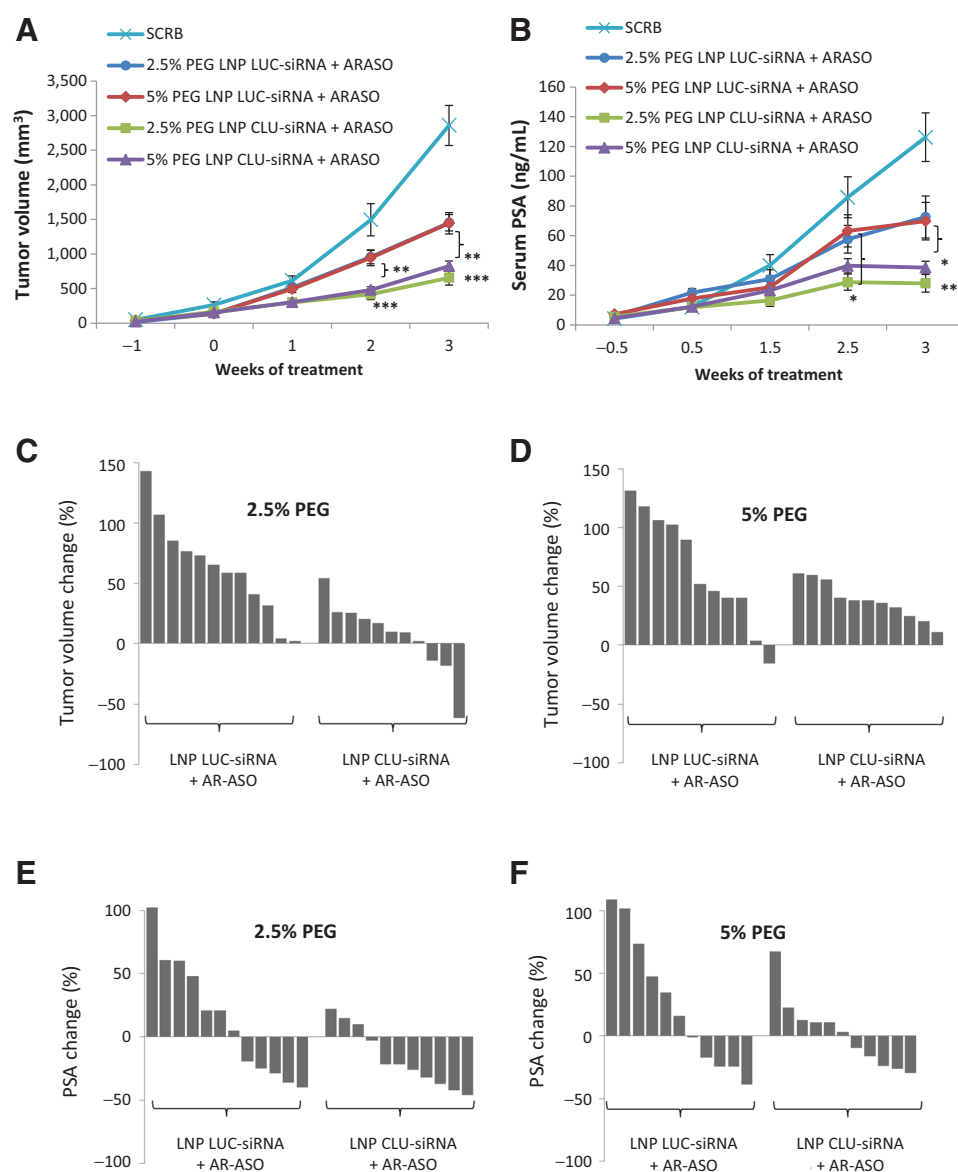
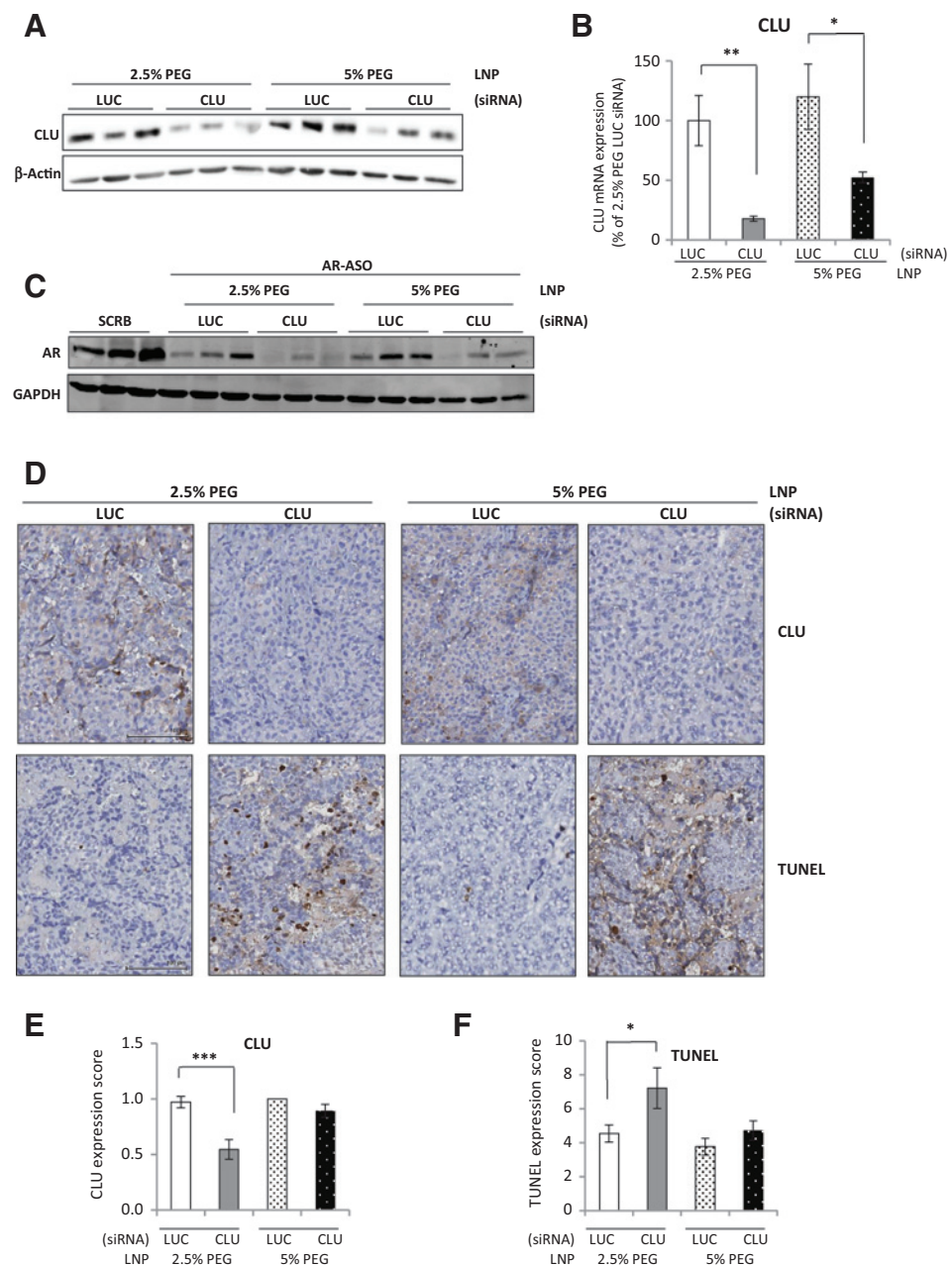


Figure 4. LNP CLU-siRNA enhances AR-ASO activity in ENZ-R LNCaP xenografts. A, castrated mice were inoculated subcutaneously with ENZ-R MR49F cells. Once tumors reached 100 mm³, mice were randomly assigned to 10 mg/kg SCRB (12 mice), AR-ASO plus either 5 mg/kg LNP CLU-siRNA with 2.5% PEG (11 mice) or 5% PEG (11 mice) or LUC-siRNA as control with 2.5% PEG (12 mice) or 5% PEG (11 mice). Injections were administered i.v. once daily for 4 days and then three times per week thereafter. The mean tumor volume (A) and the serum PSA level (B) were compared between the five groups $\pm$ SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Waterfall plots showed the greatest percent decline in tumor volume by LNP formulated in 2.5% PEG (C) and 5% PEG (D) from baseline at any time. Waterfall plots of greatest percent decline in PSA by LNP formulated in 2.5% PEG (E) and 5% PEG (F) from baseline at any time.

As shown by Lee and colleagues, the LNP platform using the cationic lipid DLin-KC2-DMA has successfully silenced AR in LNCaP xenograft tumor model (16). This was further improved with the use of a more potent cationic lipid DMAP-BLP to which doses as low as 1 mg/kg contributed >50% gene silencing (Supplementary Fig. S3). Although DMAP-BLP has been shown to be potent and safe in the brain (30), full toxicity profile of this lipid has yet to be characterized. However, the most potent lipid in the clinic, DLin-MC3-DMA, has shown to be tolerable and potent (14) and tested. As shown in Figs 4 and 5, combinatory treatment of AR and CLU silencing decreased PSA levels as well as tumor volume. Interestingly, LNP formulated with 2.5% PEG was more effective compared with 5% PEG *in vivo*. This can be attributed to PEG interfering with release of siRNA to the cytosol. Currently, less than 3% of siRNA of total siRNA delivered is detected in the cytosol using LNP systems with C14 Peg-lipid (38). With addi-

tional PEG coating on the LNP surface, the PEG can also interfere with intracellular uptake of particles. Although 5% PEG extends circulation time, the activity of the LNP system would be compromised, this can be overcome by the incorporation of targeting moieties (such as DIPA or folate) conjugated to PEG-lipids (39). Targeting siRNA comprising of a targeting moiety conjugated directly to the siRNA has been tested in the clinic as Gal-Nac conjugated siRNA accumulates and provide function in the liver (40). However, in order to reach extrahepatic sites, such an approach requires long circulation time, which can only be achieved by employing a delivery system. Alternative means to improve treatment also include coencapsulation of AR and CLU siRNA in the same LNP as this would ensure the same cells would have reagents to silence both AR and CLU. Such approach has been tested showing that up to five different targets silence key players within similar or divergent biologic pathways (41). In



combination with other improvements to the LNP such as developing more potent cationic lipids or using biocompatible LNP systems would allow for improved therapeutic index (42).

Our results indicate that LNP-LUC siRNA silences its target gene in both subcutaneous and metastatic PC3-Luc xenograft models, while LNP CLU-siRNA containing DLin-MC3-DMA demonstrates anticancer activity *in vivo* by significantly enhancing AR-ASO induced suppression of ENZ-R LNCaP tumor growth and serum PSA levels compared with control siRNA. These results demonstrate that LNP siRNA can silence target genes *in vivo*, enabling inhibition of traditionally non-druggable genes like CLU and other promising cotargeting approaches in ENZ-R CRPC therapeutics.

Disclosure of Potential Conflicts of Interest

P.R. Cullis reports receiving a commercial research grant from and has ownership interest (including patents) in Precision NanoSystems. No potential conflicts of interest were disclosed by the other authors.

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