Supporting Information

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SI Materials and Methods

Materials. Poly(2-ethyl-2-oxazoline) (50 kDa, 200 kDa, and 500 kDa), 37% hydrochloric acid, Hepes, sodium hydroxide, and other chemical reagents not specified were purchased from Sigma-Aldrich. High-potency linear polyethylenimine (PEI MW 4000; equivalent to ∼2.5 kDa in free base form) was purchased from Polysciences, and in vivo-jetPEI was purchased from Polyplus Transfection. *N*-hydroxylsuccinimide (NHS)-functionalized 5-kDa polyethylene glycol (PEG5-NHS) was purchased from NanoCS.

Polymer Characterization. Polymer analysis was performed by NMR spectroscopy (Brucker 400 MHz with D_2O solvent), gel permeation chromatography (Superose 6 10/300 GL; GE Healthcare) and PAGE coupled with visualization via Coomassie blue staining.

Synthesis of 88-kDa, 22-kDa and 220-kDa Linear PEI. For the synthesis of 88-kDa, 22-kDa and 220-kDa linear PEI (LPEI88, LPEI22, and LPEI220, respectively), 5 g of poly(2-ethyl-2-oxazoline) (50 kDa, 200 kDa, or 500 kDa) was refluxed with 10 mL of concentrated (37%) hydrochloric acid at 95–105 °C for 24 h. The reaction was cooled and left to precipitate overnight. The white solid was collected by filtering and promptly washed ith 10% hydrochloric acid. The product was dried under vacuum and yielded 2.1 g. ^{1}H NMR (D_2O): δ (ppm) 3.53 (s, $NHCH_2CH_2$). The LPEI88 was dissolved into double-distilled water (ddH_2O) at 150 mM as stock solution and kept frozen at −20 °C for long-term storage.

Synthesis of PEGylated 2.5-kDa Linear PEI. For the synthesis of PEGylated 2.5-kDa linear PEI (PEG5-LPEI2.5), 2 g of HCl salt of LPEI2.5 was dissolved into 1.5 M Hepes buffer (pH 8), and 1.06 g of PEG5-NHS was added, followed by vigorous stirring overnight. Then 1 N NaOH was added to the reaction solution to pH 11, with stirring for another 5 min. The white solid was filtered and washed with ddH_2O. The dried product was dissolved in 0.1 N HCl and lyophilized. ^{1}H NMR (D_2O): δ (ppm) 3.54 (s, $NHCH_2CH_2$, 4H), 3.68 (s, OCH_2CH_2, 4H). The PEG5-LPEI2.5 was dissolved into ddH_2O at 150 mM as stock solution and kept frozen at −20 °C for long-term storage.

Plasmids. pGL4.75 (Promega) containing the CMV-driven *Renilla reniformis* luciferase (RLuc) transgene was propagated in DH10B in the presence of ampicillin. pCpGfree-LacZ (Invivogen) containing the CMV-driven β-galactosidase transgene, an R6kγ origin of replication, and Zeocin resistance cassette was prepared by propagation in GT115 (Invivogen), a pir$^+$ strain of *Escherichia coli*, in the presence of Zeocin. The red fluorescent protein (RFP)-containing plasmid was constructed by synthesizing the tandem dimer Tomato (1) ORF fused on the 3′ end to three copies of the simian virus 40 large T-antigen nuclear localization signal (2) and cloning into the pCpGfree backbone. All plasmids were purified with the Qiagen Endotoxin-Free Giga Kit, followed by resuspension in ddH_2O and storage at −20 °C.

Preparation and PEGylation of Whole Nanoparticles. The required amount of in vivo-jetPEI or LPEI in H_2O was added into plasmid DNA in H_2O at the intended N:P ratio, followed by brief vortexing. The resulting nanoparticles were left at room temperature for 30 min and reconditioned to 1× Hepes-buffered glucose (HBG) [20 mM Hepes (pH 7.4) with 10% glucose] by mixing with an appropriate volume of 4× HBG. For nanoparticle PEGylation, PEG5-NHS was mixed with the foregoing nanoparticles at the intended PEG:LPEI weight ratio. For in vivo use, the particles were filtered through a 0.2-μm sterile syringe filter before injection.

Analysis of PEGylation on Whole Particles. Gel permeation chromatography (Sephacryl 200; GE Healthcare) was used to separate the core from the shell in a LPEI88-DNA nanoparticle at N:P 4.5 (3). LPEI in the purified core was further separated from DNA by precipitation at pH 11–12. The precipitated LPEI was dissolved into pH 2 HCl, after which the pH was changed to pH 7 by Hepes buffer before gel loading. PEGylation of whole nanoparticles or LPEI88 controls was done at a PEG5-NHS:LPEI88 weight ratio of 40:1 in 20 mM Hepes buffer (pH 7). Samples were subjected to gel retardation analysis via PAGE and Coomassie blue staining.

Preparation of Core/PEGylated-Shell Nanoparticles. To prepare core PEGylated-shell (CPS) nanoparticles, 6 mg of plasmid DNA (0.015 mM phosphate) in 120 mL of water was titrated into 0.0375 mM LPEI88 (N/P 2.5) in 120 mL of ddH_2O with vigorous stirring. The resultant nanoparticles were kept at 4 °C overnight and then concentrated to ∼6 mL in a Vivaspin 20-mL centrifugal concentrator (Vivaproducts). DNA concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific), with values normalized to samples with known DNA and LPEI concentrations. The concentrated core could be kept at 4 °C for at least 3 mo.

CPS nanoparticles were produced by adding PEG5-LPEI2.5 at specified N:P ratios to the core, followed by brief vortexing and reconditioning to 1× HBG. For in vivo use, the particles were filtered through a 0.2-μm sterile syringe filter before injection.

Dynamic Light Scattering Analysis of Particles. Average nanoparticle size, polydispersion index, and average zeta potentials (with SDs) were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS90 particle analyzer (Malvern Instruments). Measurements were performed in water unless specified otherwise.

Negative-Staining Transmission Electron Microscopy. For negative-staining analysis, 400-mesh copper grids coated with formvar/carbon were floated on 10-μL drops of nanoparticle preparations for 5 min, washed through six puddles of ddH_2O, and then placed onto 2% aqueous uranyl acetate (UA). Excess UA was removed from the grids by blotting, and the grids were allowed to air-dry. Samples were observed and images recorded with an FEI Tecnai 12 transmission electron microscope equipped with an Olympus Soft Imaging Solutions MegaView III camera and AnalySIS software. Figures were assembled in Adobe Photoshop with only linear adjustments to brightness and contrast.

Calculation of Plasmids per Particle. A Nanosight NS500 was used to characterize the nanoparticles by NTA. CPS nanoparticle suspensions were diluted in ddH_2O to concentrations of 10^7–10^9 particles/mL and loaded into the sample chamber connected to a 405-nm laser source to illuminate the particles via oblique angle light scattering on the microscope stage. A 90-s movie was recorded using the NTA software (version 2.3) and subsequently processed using manufacturer-recommended settings with manual adjustment of gain, blur, and brightness to count the number of particles per milliliter. Known plasmid concentration in the CPS suspension was then divided by the total number of particles in the suspension to calculate the average number of plasmids per particle.

Cell Lines. The colon cancer cell lines HCT116, HepG2, A549, DLD1, SW480, HT29, Neuro2A (American Type Culture Collection), and HCT116-Luc2 (Caliper Life Sciences) were cultured in McCoy's 5A growth medium (Gibco) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Gibco) at 37 °C with 5% CO_2. HCT116-EGFP was generated by stable transfection of HCT116 with pEGFP-N1, followed by neomycin selection and clonal expansion.

In Vitro Transfection. Cells were plated into 96-well Costar plates (Corning) and transfected when confluency reached 70–85%. For transfection, culture medium was removed and 80 μL of fresh McCoy's 5A medium with 10% FBS were added to each well, followed by 50 μL of nanoparticles in HBG. Cells were then incubated for 24 h at 37 °C before measurement of luciferase activity according to standard protocols with the Promega Dual Luciferase Assay System. Luciferase light units were measured in 96-well plate format with a Promega GloMax 96-well luminometer.

Animal Models. All animal experiments were designed in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals* and were approved by Johns Hopkins University's Institutional Animal Care and Use Committee. Metastatic animal models were generated as described previously (4). NOD.Cg-Prkdc^{scid} Il2rgt^{m1Wjl}/SzJ (NOG) mice were purchased from Jackson Laboratories.

Surgery was performed on anesthetized mice to expose the spleen, and 10^5 cells in 50 μL of media without serum were injected intrasplenically. The wound was closed and the mice recovered quickly after the procedure. All other intrasplenic models were created in NOG or other mouse backgrounds (A/J and SCID-1 mice from the National Cancer Institute and BALB/c and BALB/c nude mice from Jackson Laboratories) according to the foregoing protocol but with varying numbers of injected cells as follows: HepG2 (1×10^5), DLD1 (3×10^6), SW480 (1×10^5), HT29 (1×10^5), Neuro2A (1×10^6), and CT26 (1×10^5). Subcutaneous tumors were established by hind-flank injection of the indicated number of cells in 100 μL of PBS.

Nanoparticle formulations were administered via tail vein injection at 2–4 wk after surgery. After 24 h, body weight gain/loss was measured, and mice were euthanized by CO_2 inhalation followed by tissue collection and homogenization and luciferase measurement. For toxicity studies, animals were monitored closely after i.v. injection. The maximum tolerated dose (MTD) was defined as 0.5 mg/kg below the dose at which any animal died within 10 h of i.v. injection.

Dual Luciferase Reporter Assays. NOG mice bearing HCT116-Luc2 were treated i.v. at 1.5 wk after tumor inoculation with nanoparticles bearing pGL4.75. The mice were euthanized at 24–48 h after transfection. The lungs, liver, and spleen were dissected, washed in PBS, homogenized in Passive Lysis Buffer (Promega), and centrifuged at $5{,}000 \times g$ for 10 min. The supernatant was then assayed for luciferase activity using Luciferase Assay Reagent II (Promega) and Stop-and-Glo buffer containing coelenterazine (Promega) in a GloMax 96 luminometer (Promega).

β-Galactosidase Staining and Histology. β-galactosidase staining was performed with whole organs or tissue sections. For whole-mount staining, mice were anesthetized with Avertin and perfused via cardiac puncture with 10 mL of ice-cold PBS supplemented with 2 mM $MgCl_2$ (Mg-PBS). The organs were dissected and washed at 4 °C in ice-cold Mg-PBS for 5 min. Tissues were then fixed in 2% paraformaldehyde Mg-PBS for 2 h at 4 °C, followed by for 15-min washes with Mg-PBS. Staining was performed in PBS containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 at 37 °C for 10–16 h. After two washes with PBS, images were obtained under a dissecting microscope with bright-field optics. For long-term preservation, the stained tissues were kept in 70% ethanol at 4 °C. For preparation of tissue sections, β-galactosidase–stained organs were embedded in paraffin, cut into 5-μm sections, mounted onto glass slides, and stained with H&E or nuclear fast red.

Counting Transfected Cells. Transfected and control mice were euthanized by CO_2 inhalation and their organs dissected. The collected organs were washed with cold PBS. Weighed tissues were then minced with surgical scalpels and shaken at 180 rpm at 37 °C for 30 min in the presence of Liberase (Roche) in 5 mL DMEM:F12 per 200 mg of organ. The Liberase concentration was10 μg/mL for liver and spleen and 20 μg/mL for lung. Tissue samples were pipetted 20 times, followed by another 10 min of shaking. The digested tissues were strained through 100-μm nylon mesh, and cells were collected by spinning at $2{,}000 \times g$ for 5 min at 4 °C. The pellet was resuspended in 1 mL ACK Lysis Buffer (Invitrogen) for 5 min at room temperature and swirled occasionally. Then 5 mL DMEM:F12 containing 10% FBS was added to the suspension, after which the cells were again spun down at $2{,}000 \times g$ for 5 min at 4 °C and resuspended at approximately 5×10^5 cells/mL before being strained through 40-μm nylon mesh. Cell suspensions (30 μL) were scanned on a Molecular Devices ImageXpress Ultra confocal scanner running on MetaXpress software, and images were processed in CellProfiler (Broad Institute).

Exogenous Agents. Exogenous agents were administered to the mice 30 min before CPS nanoparticle injection except where specified otherwise. The tumor-homing peptide iRGD was injected i.v. at 20 μmol/kg (5, 6); chloroquine, i.p. at 100 mg/kg (7), paclitaxel, i.p. at 20 mg/kg (8); TNF-α, i.v. at 25 μg/kg (9); enalapril, i.p. at 20 mg/kg (10); and beraprost, i.v. at 7 or 60 μg/kg (11). In addition, 2% nitroglycerin ointment was applied abdominally at 150 mg/mouse (12). Vorinostat (100 mg/kg, i.p.) was given to mice in three doses at 26 h, 14 h, and 3 h before euthanasia (13). Dexamethasone 5 mg/kg/day was injected i.p. on the day of, 1 d before, and 2 d before CPS injection (14). Losartan was injected i.p. at 60 mg/kg/day for 12 d before CPS injection (15). Albumin was directly mixed with CPS at an albumin:DNA weight ratio of 10:1 (16). Angiotensin II (6 μg/kg/min) and relaxin (0.35 mg/kg/day) were started at 14 d before CPS injection through s.c.-implanted ALZET osmetic pumps (17, 18). Liposomes (hydrogenated egg PC 38.1 mg/mL, cholesterol 17.4 mg/mL, and PEG(2000)DSPE 14.0 mg/mL) and lipofectamine (N/P ratio 15) were mixed directly with CPS before injection (19, 20).

Shell Substitutes. LPEI2.5-PEG5-PDP was obtained by reacting LPEI with PDP-PEG5-NHS- (succinimidyl PEG5 orthopyridyldisulfide). Peptide conjugates were then synthesized by conjugating the cysteine containing peptides with LPEI2.5-PEG5-PDP. Transferrin-LPEI conjugate and β-cyclodextrin LPEI conjugates were synthesized as follows. Transferrin was oxidized by sodium periodate, coupled with LPEI, and then reduced with sodium cyanoborohydride (21). β-cyclodextrin mono-6-O-(p-toluenesulfonyl)-β-cyclodextrin was used to react with free base LPEI in DMSO (22). Cholesterol conjugates and lipid conjugates were synthesized according to a similar protocol for CD-LPEI synthesis, except with cholesteryl chloride and 1-iodooctadecane as reactants (23–25). Quality control was performed by UV absorption for PDP, protein, and peptides, as well as PAGE and Coomassie blue staining.

Statistical Analyses. *P* values were calculated using the two-tailed Student *t* test.

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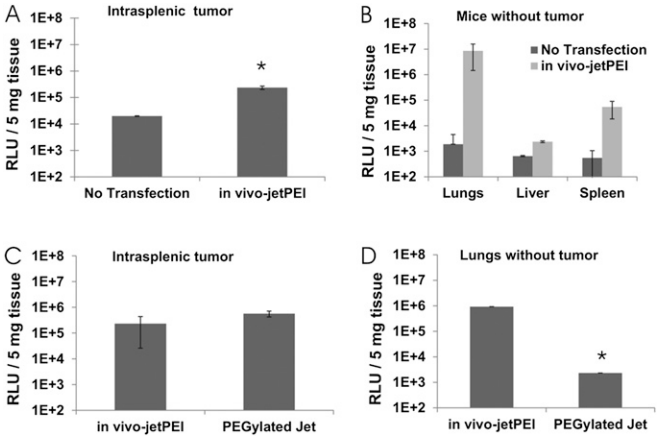

Fig. S1. Benchmark in vivo transfection in an internal tumor model. (*A*) RLuc RLUs in intrasplenic tumors from nontreated and in vivo-jetPEI–treated mice. (*B*) RLuc RLUs in lung, liver, and spleen of nontreated and in vivo-jetPEI–treated mice without tumors. (*C*) RLuc RLUs in intrasplenic tumors after transfection with in vivo-JetPEI or in vivo-jetPEI after PEGylation (PEGylated Jet). (*D*) RLuc RLUs in lungs without tumors after transfection with in vivo-JetPEI or PEGylated in vivo-jetPEI (PEGylated Jet). Data are mean ± SD of values collected from at least two mice per group. $*P < 0.05$.

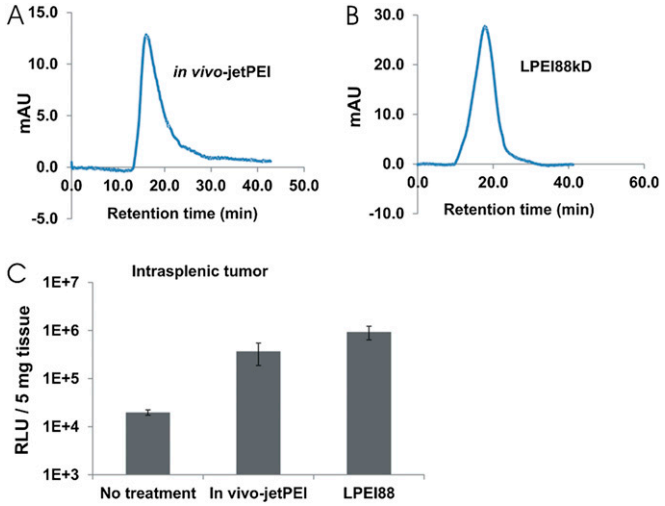

Fig. S2. Characterization of LPEI88. (*A* and *B*) Gel permeation chromatography analysis of in vivo-jetPEI (*A*) and LPEI88 (*B*). (*C*) RLuc RLUs in intrasplenic tumors after transfection with PEGylated in vivo-jetPEI or PEGylated LPEI88. Data are mean ± SD of values collected from at least two mice per group.

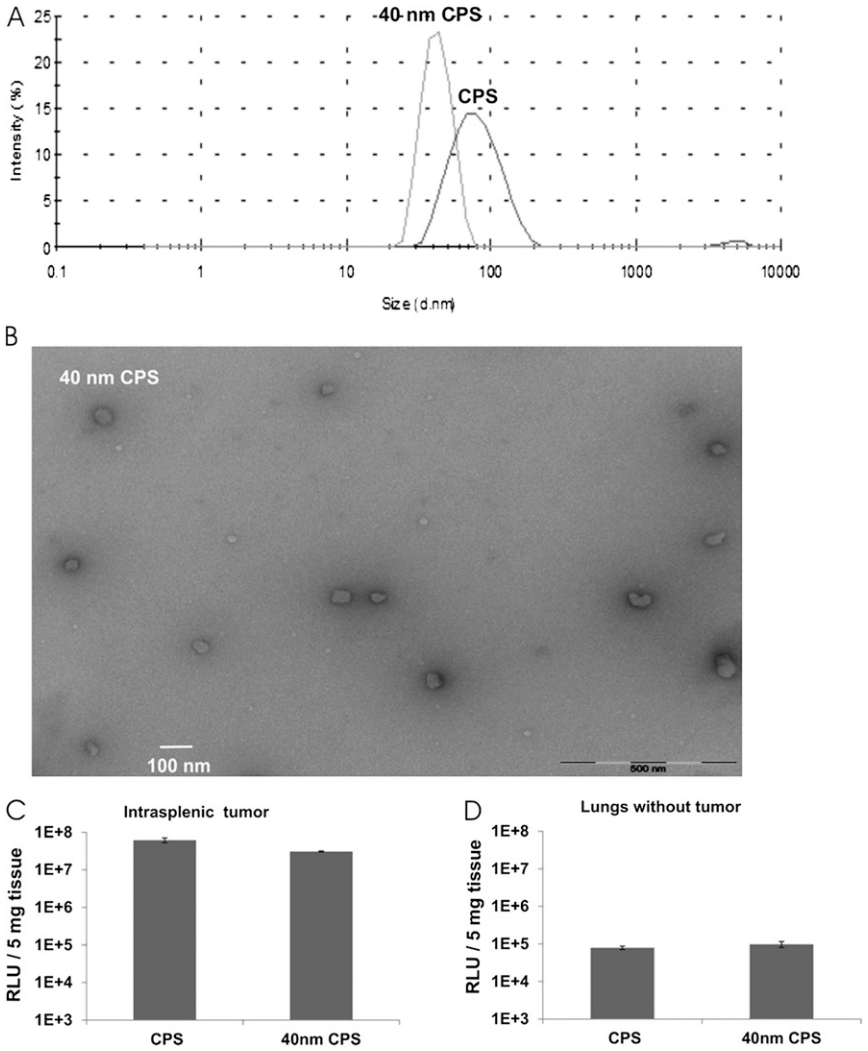

Fig. S3. Characterization of CPS and 40-nm CPS nanoparticles. (*A*) DLS measurement of CPS nanoparticles and the 40-nm CPS nanoparticles made from centrifugation-purified core. (*B*) Transmission electron microscopy of 40-nm CPS nanoparticles. (*C*) RLuc activity in intrasplenic tumors from HCT116-Luc2–bearing NOG mice treated with CPS or 40-nm CPS and processed for dual luciferase assays. (*D*) RLuc RLUs in normal lungs and liver from the same mice as in *C*. Data are mean ± SD of values collected from at least two mice per group.

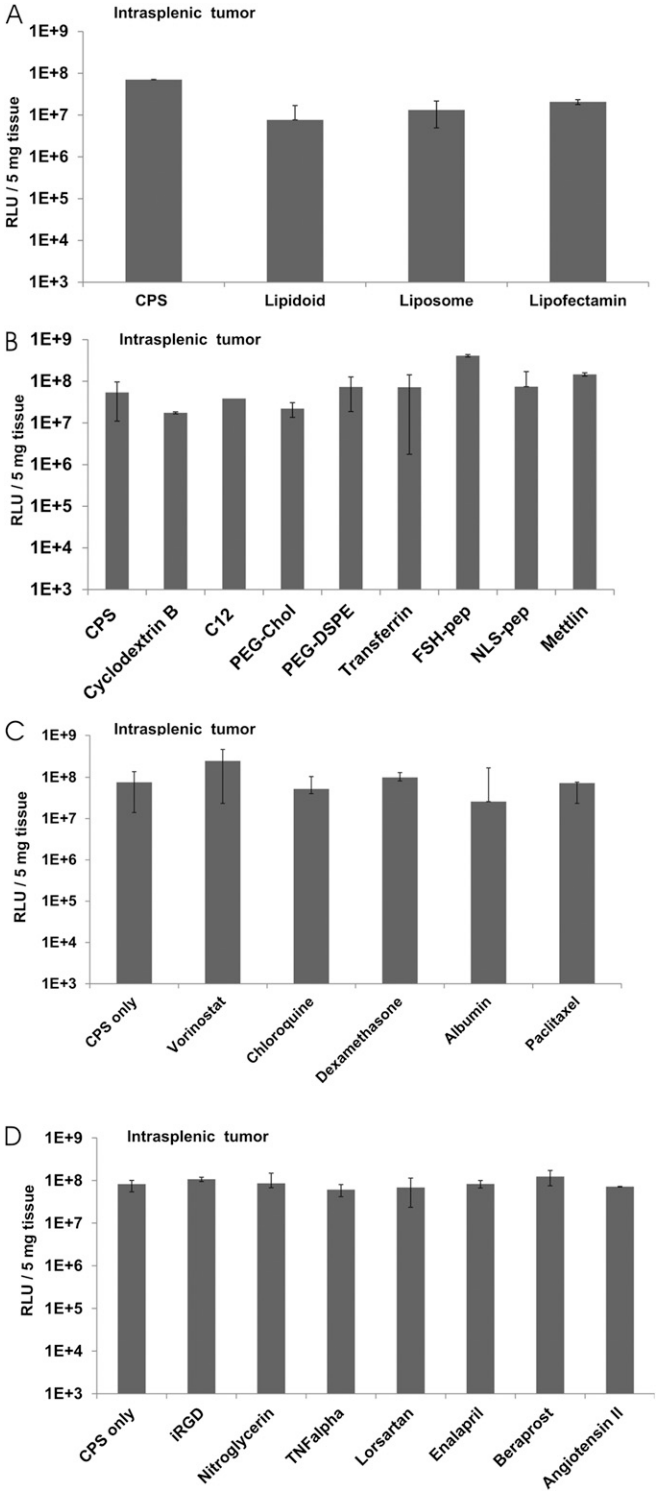

Fig. S4. Evaluation of shell substitutes and exogenous agents. RLuc RLUs are shown in intrasplenic tumors from HCT116-luc2–bearing NOG mice treated with CPS or modified CPS nanoparticles and processed for dual luciferase assays. Data from animals transfected with CPS nanoparticles were used for normalization across different experiments. (*A*) Anionic and cationic lipids as shells. (*B*) Lipids, peptides, and other functional groups conjugated to LPEI as shells. (*C*) Nanoparticle transfection enhancers. (*D*) Nanoparticle delivery enhancers. Data are mean ± SD of values collected from at least two mice per group.

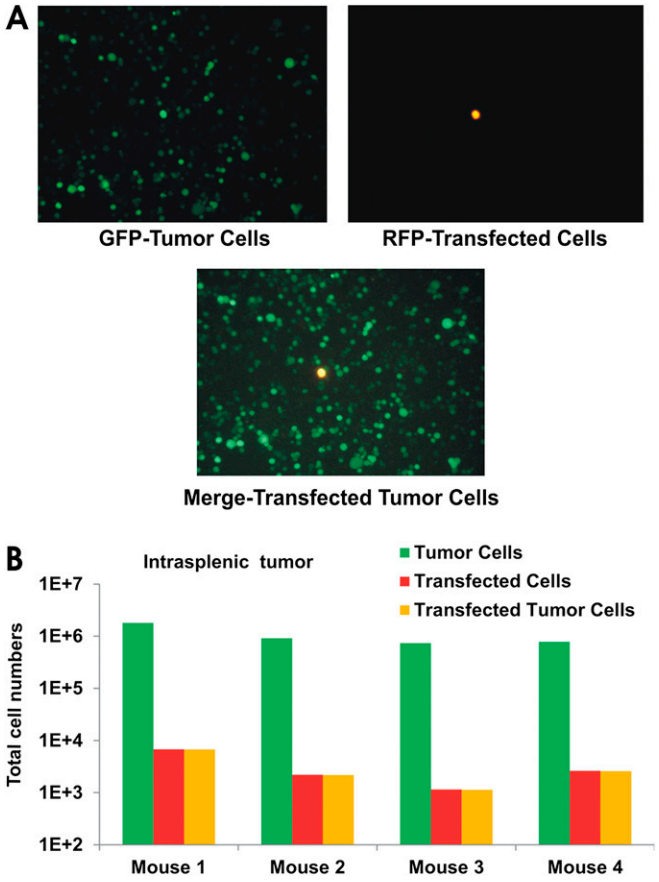

Fig. S5. Counting transfected intrasplenic tumor cells. NOG mice bearing HCT116-EGFP tumors were transfected with CPS containing RFP plasmids. (*A*) Representative fluorescent signals from tumor cells (EGFP, green), transfected cells (RFP, orange), and transfected tumor cells (merged green and orange images) under 200× magnification. (*B*) Number of tumor cells, transfected cells, and transfected tumor cells in the spleens of four individual mice as determined by confocal scanning of dissociated cells.

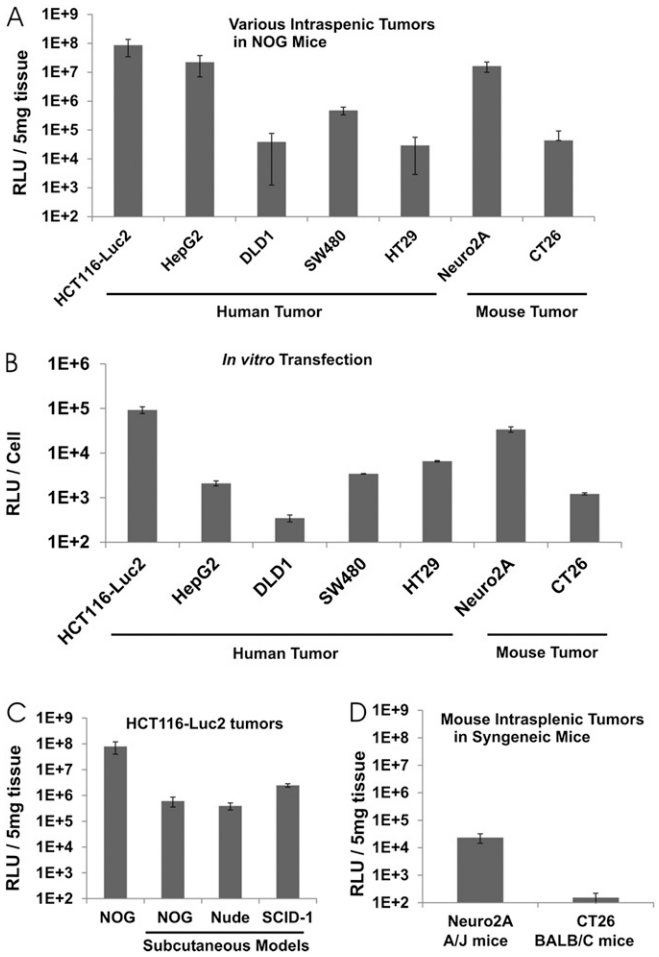

Fig. S6. CPS nanoparticle transfection in various tumor models. RLuc RLUs are shown in tissues from the indicated tumor-bearing mice treated with CPS and processed for dual luciferase assays. (*A*) Transfection of intrasplenic tumors in NOG mice generated from human and mouse cell lines. (*B*) In vitro transfection of human and mouse tumor cell lines. (*C*) Transfection of either intrasplenic or s.c. HCT116-Luc2 tumors in NOG, nude, or SCID-1 mice. (*D*) Transfection of intrasplenic Neuro2A and CT26 tumors in A/J and BALB/c mice, their syngeneic immunocompetent hosts. Data are mean ± SD of values collected from at least two mice per group.

Table S1. Toxicity of LPEIs after tail vein injection

LPEI	MTD,* mg LPEI/kg
in vivo-jetPEI	2.7
LPEI2.5	8.4
LPEI22	2.4
LPEI88	2.4
LPEI220	2.8

*The MTD is 0.5 mg/kg below the dose at which any animal died within 10 h of i.v. injection. At least two animals were tested at each dose.

Table S2. Nanoparticle components at MTDs of various formulations

N:P ratio of formulation	Total LPEI at MTD,* mg LPEI88/kg	DNA at MTD,* mg DNA/kg	Shell LPEI at MTD,* mg LPEI88/kg
2.5	9.9	30.0	1.4
3.6	4.8	10.0	2.2
7.2	2.4	2.5	2.0

*The MTD is 0.5 mg/kg below the dose at which any animal died within 10 h of i.v. injection. At least two animals were tested at each dose.

Table S3. Characterization of core, CS, and CPS nanoparticles

Formulation	Size, nm (polydispersion index)			Zeta potential, mV
	In water 0 min	In PBS 0 min	In PBS 30 min	
Core	67.0 (0.211)	277 (0.107)	1,370 (0.461)	22.8 ± 7.02
CS	74.3 (0.108)	257.4 (0.120)	1,030 (0.223)	43.0 ± 4.96
CPS	70.8 (0.152)	80.33 (0.221)	89.4 (0.116)	35.2 ± 7.52

Core: LPEI88 complexed with DNA at an N:P ratio of 2.5; CS nanoparticle: a core and a shell of LPEI2.5 yielding a total N:P ratio of 20.5, non-PEGylated; CPS nanoparticle: a core and a shell of purified PEG5-LPEI2.5(1:1) yielding a total N:P ratio of 20.5.