SUPPLEMENTARY METHODS

CELL LINES

HepG2 hepatocytes (courtesy of Yale Liver Center) were maintained in Eagle’s minimum essential medium (E-MEM, ATCC) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and 1% (v/v) penicillin/streptomycin (Sigma). NIH/3T3 fibroblasts were maintained in Dulbecco’s Modified Eagle’s Medium with high glucose (DMEM/High) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. HeLa cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. A HEK293-T cell line stably expressing EGFP was obtained as a gift from the laboratory of Dr. Edward M. Uchio (Department of Surgery, Yale University). The clone were established by transfecting HEK293-T cells (ATCC) with the EGFP-Luc vector (Clontech), which contains the coding region for the EGFP gene (nt 613-1329) fused in-frame with the firefly luciferase gene. After selection with G418 containing growth media, cells were sorted with FACS to identify high intensity GFP-expressing clones. The 293T-EGFPLuc cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, and 400 μg/mL G418.

PREPARATION OF SIRNA NANOPARTICLES

PLGA nanoparticles loaded with naked siRNA (naked) or siRNA/polyamines were formulated by a double-emulsion solvent evaporation technique. In brief, siRNA were reconstituted in deionized water in the absence or presence of complexing agents. Polyamine complexes with siRNA were formed at room temperature for 15 min on a rotary shaker. The siRNA (25 – 200 nmoles) was combined with the polyamine at a molar ratio of the polyamine nitrogen to the polynucleotide phosphate (N/P ratio) of 3:1, 8:1 or 15:1 according to published methods. A molecular weight per nitrogen of 85 g/mole and 44 g/mole was used for spermidine and putrescine, respectively. This aqueous solution was then added dropwise to a polymer solution of PLGA (200 mg, 50:50, Polysciences and Birmingham) dissolved in dichloromethane (2 mL) to form the first emulsion. This mixture was then added dropwise to 4 mL of 5% polyvinyl alcohol (PVA) and sonicated to form the double emulsion. The final emulsion was poured into a beaker containing aqueous 0.3% (v/v) PVA and stirred for 3 h to allow the dichloromethane to evaporate and the particles to harden. The concentration of PVA used to form the emulsion, and the sonication time and amplitude were optimized for formulating particles with a diameter of 0.1 to 300 μm. Particles were collected by centrifugation, washed, rapidly frozen, and lyophilized.
CHARACTERIZATION OF NANOPARTICLE SIZE, SIRNA LOADING, AND ENCAPSULATION EFFICIENCY

To determine loading and encapsulation efficiency, 10-20 mg of siRNA nanoparticles were dissolved in 0.5 mL of dichloromethane at room temperature for 30 min. The siRNA was extracted from the organic phase using two volumes each of 0.5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The TE buffer was added to the organic phase and vortexed vigorously for 1 min, and then centrifuged at 12,000 rpm for 5 min at 4°C. The combined 1 mL aqueous fraction was analyzed for double-stranded RNA content using the QuantIT™ PicoGreen™ assay according the manufacturer’s instructions (Invitrogen). A standard curve correlating fluorescence and siRNA concentration was used to determine the amount of siRNA loaded into the PLGA particles. The efficiency of the extraction procedure was assessed by extracting a known amount of siRNA. Following fluorescence quantification, the siRNA were precipitated from the aqueous fraction by addition of 0.3 M sodium acetate and 70% (v/v) ethanol. This solution was incubated at -20°C for 30 min, and then centrifuged at 12,000 rpm and 4°C for 30 min to collect the precipitate. The pellet was dried and analyzed on a 3% (w/v) agarose gel.

Particle size and surface charge were determined by dynamic light scattering (DLS) using a ZetaPals (Brookhaven Instruments) ζ-potential and particle size analyzer. Particle formulations were analyzed in triplicate at a concentration of 10 μg/mL in a total volume of 2 mL in various solvents. The average values from a total of 5-10 runs of 30 s each were used to determine both values. Particle size was also analyzed by image analysis of micrographs obtained by scanning electron microscopy (SEM). The samples were coated with 25 nm-thick gold using a quick carbon coater. The particle diameter and size distribution of the microspheres were determined by image analysis of >1,000 particles using image analysis software (ImageJ, National Institute of Health). These micrographs were also used to assess particle morphology.

IN VITRO CONTROLLED RELEASE

Nanoparticles (10-15 mg) were suspended in 1.0 mL of phosphate buffer (pH 7.4), and incubated at 37º with gentle shaking (70 rpm). Release of siRNA was monitored at several time intervals over 30 days. At each sampling time, the nanoparticle suspension was centrifuged for 5 min at 14,000 rpm. The supernatant (0.5 mL) was removed for quantification of fluorescence and an equivolume of PBS was replaced for continued monitoring of siRNA release. Citrate buffer (180 mM, pH 5.0) was used to mimic the lower pH conditions of the cervicovaginal tract (pH 5.0) while maintaining a similar ionic strength to PBS. The amount of residual siRNA in
the nanoparticles was determined using the same analytical method of encapsulation efficiency.

NANOPARTICLE DELIVERY TO CULTURED CELLS

Cell viability was determined using a CellTiter-Blue® Cell Viability Assay (Promega). Cells were plated at 1X10^5 cells per well in a 96-well tissue culture treated plate and left to proliferate. After 48 hours in culture, cells were at ~80% confluence. Particle treatment groups and controls were diluted in the cell type-specific media at concentrations of 10, 5, 2, 1, 0.5, and 0.1 mg/mL for particles and 0.5, 0.25, 0.1, 0.05, 0.025, 0.01 mg/mL for free SPE. Treatment groups included Blank/SPE and SPE/Mimic nanoparticles, and free SPE. Negative controls were completed with blank media, while positive controls were completed in media with 10% sodium azide for cell death. Control wells with no cells were examined with both blank media and 10mg/mL particles. Treatment groups and controls were placed on the cells in 100uL volumes and left for 24 hours at 37C and 5% CO2. At the end of the incubation, 20uL of cell titer blue reagent was added to each well. Plates were shaken at 37C for 1 minute and then removed to an incubator. At 1, 2, 3, and 4 hour time points, the plates were removed from the incubator, shaken at 37 ºC for 10 sec, and then measured for fluorescence (Ex = 560 nm, Em = 590 nm).

We evaluated nanoparticles encapsulating siRNA against control nanoparticles encapsulating only the polyamine or dsDNA/polyamine complexes in cultured HepG2 hepatocytes, NIH/3T3 fibroblasts, and HeLa cells. Nanoparticles were re-suspended in the appropriate cell media at 0.5 mg/mL (10 nM siRNA) or 1 mg/mL (20 nM siRNA) polymer. Cells were seeded in T25 flasks and treated with the nanoparticle preparations when the cells reached 30-50% confluency. Cells were analyzed for gene expression 3 d after nanoparticle treatment. For the time course experiments, HeLa cells were seeded at different levels of confluency depending on when the cells were being analyzed for gene expression: 1 d = 80%, 3 d = 50%, 5-14 d = 10%. For the longer time points (5-14 d), cells were passaged when they became 90% confluent and then resuspended in fresh media containing the nanoparticle suspension that was collected by centrifugation. For the dosing experiments, 293T-EGFPLuc cells were seeded at 50% confluency and dosed with lipoplexes or nanoparticles loaded with siLUC. Cells were analyzed for luciferase gene expression after 3 d.

For confocal microscopy, OK cells were grown to confluency and incubated for 1 h with 0.5 mg/mL nanoparticles. Cells were washed, fixed, and permeabilized before labeling the cell cytoskeleton with Texas-Red-X phalloidin (F-actin). Images were captured along the z-axis and the middle panel from the z-stack
(z=4.2 μm) was taken to determine intracellular and perinuclear localization of nanoparticles. Cells were visualized in the red channel and fluorescent nanoparticles were visualized in the green channel. Images were obtained with a Zeiss LSM510Meta confocal scanning microscope using a 63x objective.

QUANTITATIVE RT-PCR

Total RNA (1 μg) was isolated using an RNeasy® Mini Kit (Qiagen) according to the manufacturer’s protocol. Purified total mRNA was reverse transcribed using the iScript™ cDNA Synthesis kit (Biorad) to generate cDNA. Real-time PCR was performed on 2 μL of cDNA combined with the iQ SyBr™ Green (Biorad) reagents for fluorescent detection of PCR products. All reactions were performed in a 20 μL volume in duplicate.

Primers used for RT-PCR are:

- Hu-erk2 forward: 5´-CCGTGACCTCAAGCCTTC-3´
- Hu-erk2 reverse: 5´-GCCAGGCCAAAGTCACAG-3´
- Mu-erk2 forward: 5´-CGTTCTGCACCGTGACCT-3´
- Mu-erk2 reverse: 5´-ATCTGCAACACGGGCAAG-3´
- GAPDH forward: 5´-TTCACCACCATGGAGAAGGC-3´
- GAPDH reverse: 5´-GGCATGGACTGTGGTCATGA-3´

PCR parameters consisted of 5 min activation of the DNA polymerase at 95ºC, followed by 40 cycles of 95ºC x 20 s, 60ºC x 30 s, and 72ºC x 20s. Messenger RNA expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer specificity was verified by melt curve analysis and agarose gel electrophoresis.
Table S1. Properties of PLGA nanoparticles loaded with siRNA

| Formulation               | Size (nm)  | ζ-potential (mV) | Loading (pmoles/mg PLGA) | Encapsulation Efficiency (%) | siRNA molecules per particle |
|---------------------------|------------|------------------|--------------------------|----------------------------|-----------------------------|
| **Initial loading = 25 nmoles** |            |                  |                          |                            |                             |
| siERK2/Spe                | 198 ± 54   | -34.1 ± 1.4      | 65 ± 12                  | 56 ± 10                    | 225                         |
| siEGFP/Spe                | 183 ± 61   | -29.4 ± 0.7      | 27 ± 2                   | 21 ± 1                     | 81                          |
| **Initial loading = 200 nmoles** |          |                  |                          |                            |                             |
| siEGFP/Prot               |            |                  |                          |                            |                             |
| siEGFP/Prot               | 189 ± 83   | -23.4 ± 1.6      | n.d.                     | 12 ± 0                     | 340                         |
| siEGFP/Prot               |            |                  |                          |                            |                             |
| siLUC/Spe                 | 174 ± 64   | n.d.             | 418 ± 27                 | 42 ± 3                     | 1165                        |

*a* Sequences for siRNA used for formulations: siERK2, sense 5’-UGCUGACUCCAAGCUCU/GdTdT-3’ and antisense 5’-CAGAGCUUUUGGAGUCAGCAGdTdT-3’; siEGFP, sense 5’-GCUACGCUAGGAGCACCdtdTdT-3’ and antisense 5’-UGCGUCUGGAGCGUAGCCU/UtdTdT-3’, and siLUC, sense 5’-CUUACGCUAGAGCUAGCUA/AGUUU-3’, and antisense 5’-AUCGAAGUACUCGAGUAGUU-3’.

*b* Mean particle diameter was determined by ImageJ (NIH) analysis of SEM micrographs. For the lower initial loading formulations, the mean diameter determined by static light scattering for siERK2/Spe and siEGFP/Spe nanoparticles was 359 nm and 348 nm, respectively (ZetaPals, Brookhaven Instruments). For the higher initial loading formulations, the mean diameter determined by static light scattering for siEGFP/Spe and siEGFP/Prot was 357 nm and 287 nm, respectively.

*c* Nanoparticles were analyzed in triplicate at a concentration of 10 µg/mL in a total volume of 2 mL. Conductance of deionized (DI) water is 3.8 ± 1.0 µS.

*d* Loading and encapsulation efficiency could not be determined due to the inability of quantitative dyes to intercalate nucleic acids that were complexed with protamine (Prot).

*e* N:P = 3:1

n.d. = not determined.
**Fig. S1.** Natural polyamines improve the actual loading and encapsulation efficiency of a siRNA mimic. Higher initial loading enhances (a) actual loading but decreases (b) encapsulation efficiency of the siRNA mimic, deoMMC. Natural polyamines such as spermidine (Spe) and putrescine (Put) were combined with deoMMC at a N:P ratio of 8 and then encapsulated into PLGA nanoparticles. The combined effect of higher initial loading (25 nmoles = open bars, 200 nmoles = hatched bars) and pre-complexing with spermidine led to >40x increase in encapsulation compared to loading of the naked siRNA mimic (c). Values represent the mean ± s.d. of three independent formulations (* = p ≤ 0.05).
Fig. S2. Size and morphology of siRNA nanoparticles are independent of siRNA target sequence, polyamine, or loading. Scanning electron micrographs show siRNA nanoparticles prepared using siERK2 (a) and siEGFP (b) using spermidine as a counterion and an initial loading of 25 nmoles. Nanoparticles encapsulating siEGFP were also prepared at a higher initial loading of 200 nmoles with spermidine (c) or protamine (d) as counterions. Bar = 1 µm.
Fig. S3. Physicochemical and functional properties of siRNA is preserved upon encapsulation in nanoparticles. (a) Gel electrophoresis identified that the charge and molecular weight of siERK2 extracted from PLGA nanoparticles was similar to the control stock siRNA reagent. DNA molecular weight ladder (lane 1); running buffer (lane 2), naked siRNA (lane 3), extracted Spe nanoparticles (lane 4), and extracted siRNA/Spe nanoparticles (lane 5). (b) Protein immunoblotting shows that siMAPK1 extracted (*) from PLGA nanoparticles silences gene expression in cultured HepG2 hepatocytes and HeLa cells (lane 2 and 4). Gene silencing does not occur in the absence of a delivery vehicle (lane 3).
Fig. S4. Nanoparticles delivering siERK2 are internalized and cause sustained gene silencing in cultured cells. MAPK1 expression was reduced in cultured cells administered with 0.5 mg/mL nanoparticles encapsulating an siRNA targeted against erk2 but not with empty nanoparticle controls. HepG2 hepatocytes (open bars) and 3T3NIH fibroblasts (hatched bars). Bars represent the mean ± s.d. (n = 3, *= p ≤ 0.05).
Fig. S5. Gene silencing with nanoparticles delivering siERK2 is dose-dependent. Cultured HeLa cells were administered 0.5 mg/mL (open bars) or 1.0 mg/mL (hatched bars) nanoparticles encapsulating a siRNA targeted against erk2. Reduction of MAPK1 expression was higher in groups treated with a higher concentration of nanoparticles. Bars represent the mean ± s.d. (n = 3; * = p ≤ 0.05).
Fig. S6. In vitro release of siERK2 from PLGA nanoparticles is sustained for over 30 d. Cumulative release of siERK2/Spe from nanoparticles was measured for ~30 d. Burst release of 20% of the total encapsulated siRNA occurred within the first day, followed by a sustained linear release of ~0.4 pmoles/mg•day. Over 50% of siMAPK1 was still available for release after 30 d. Values represent the mean ± s.d.
Fig. S7. Nanoparticles delivering siMAPK1 are internalized and cause sustained gene silencing in cultured cells. Gene silencing is sustained for at least 14 d in HepG2 cells cultured with siMAPK1 nanoparticles (closed circles). Total recovery of MAPK1 expression was seen after 7 d when using a commercial transfection agent to deliver siERK2 (open circles).
Figure S8. Interaction of fluorescent nanoparticles with OK epithelial cells. Confocal microscopy was used to visualize the interaction and internalization of coumarin-6 PLGA nanoparticles with cultured OK epithelial cells. OK cells were grown to confluency and incubated for 1 h with 0.5 mg/mL nanoparticles. Cells were washed, fixed, and permeabilized before labeling the cell cytoskeleton with Texas-Red-X phalloidin (F-actin). Images were captured along the z-axis and the middle panel from the z-stack (z=4.2 µm) is shown here. Cells were visualized in the red channel (a) and fluorescent nanoparticles were visualized in the green channel (b). Merge image is shown in (c). Nucleus is outlined by a dotted line. Images were obtained with a Zeiss LSM510Meta confocal scanning microscope using a 63x objective.
Fig S9. Intravaginal delivery of siRNA using biodegradable nanoparticles causes gene silencing throughout the reproductive tract of transgenic GFP mice. A siRNA targeted against EGFP was delivered using (a) cationic lipids or PLGA nanoparticles with the siEGFP complexed with (b) spermidine or (c) protamine. Reduction in EGFP expression was seen the vagina, cervix, and uterine horns in GFP transgenic mice 10 days after topical administration. Images were obtained at 10x magnification and fluorescence images were obtained at 1 s exposure times.