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SUPPLEMENTARY INFORMATION

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In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight

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7C1 Synthesis, characterization, and formulation

C_{15} lipids and PEI_{600} were combined and heated to 90˚C in 100% ethanol for 48-72 hours. Products were characterized with MALDI-TOF and ^{1}H-NMR (Supplementary Fig. 5a-b). To formulate particles, 7C1 was combined with $C_{14}PEG_{2000}$ and mixed with siRNA in a microfluidic device as previously described[35]. Particles were dialyzed against 1X PBS before sterile filtration through a 0.22 um membrane. Particle size and structure was analyzed by dynamic light scattering (DLS) (Zetapals, Brookhaven Instruments), TEM, or cryo-TEM. DLS samples were measured in PBS at an approximate siRNA concentration of 1.0-3.0 ug/mL. TEM samples were prepared on carbon film-coated grid to which 1% sodium phosphotungstate was added for negative staining before images were obtained (JEOL 200CX EM, 120 kV). Cryo-TEM specimens were prepared in the controlled environment vitrification system at 25 °C and ~ 100% relative humidity. Vitrified samples were examined with a Tecnai 12 G^2 TEM (FEI), and images were recorded on an UltraScan 1000 CCD camera (Gatan) at low dose conditions as previously described. siRNA concentration was measured using Quant-iT™ RiboGreen (Invitrogen), HPLC absorbance against a known standard.

TNS pKa assay

TNS, a molecule whose fluorescence is quenched in aqueous solution, was added to particles and buffer solution at variable pH. As pH decreased, 7C1 charge increased; this increased charge promoted interactions with TNS, thereby increasing its fluorescence. Fluorescence was measured in a black plate (excitation 322 nm, emission 431 nm).

siRNA synthesis and *in vitro* siRNA selection

siRNAs were synthesized and modified to at the 2' position to avoid off-target effects and immunostimulation (Alnylam Pharmaceuticals)[36]. siRNAs to the same gene target were screened *in vitro* to maximize potency (Supplementary Fig. 6a-b). siRNAs to CD45, Luciferase (siCntrol), and Factor 7, and Alexa 647-tagged-GFP have been previously described, while all others are listed in Supplementary Table 1[5,22]. Sequences were selected after measuring target mRNA expression in Bend.3 (ATCC) following Lipofectamine RNAiMAX transfection (Invitrogen).

Gene	Sense	Antisense
CD45	cuGGcuGAAuuucAGAGcATdT	UGCUCUGAAAUUcAGCcAGTdT
Dll4	uccuGuAuGGGAcAucuuudTsdT	AAAGAUGUCCcAuAcAGGAdTsdT
FVII	GGAUfCfAUfCfUfCfAAGUfCfUfUfACfdTsdT	GUfAAGACfUfUfGAGAUfGAUfCfCfdTsdT
Icam2	AGGAcGGucucAAcuuuucdTsdT	GAAAAGUuGAGACCGUCCUdTsdT
Luc	cuuAcGcuGAGuAcuucGAdTsdT	UCGAAGuACUcAGCGuAAGdTsdT
Tie1	GuGAGAAuGuGAcAuuAAudTsdT	AUuAAUGUcAcAUUCUcACdTsdT
Tie2	GAAGAuGcAGuGAuuuAcAdTsdT	UGuAAAUcACUGcAUCUUCdTsdT
VE-Cadherin	ccAAAAGAGAGAcuGGAuudTsdT	AAUCcAGUCUCUCUUUUGGdTsdT
Vegfr1	cAGAAGuucucGuuAGAGAdTsdT	UCUCuAACGAGAACUUCUGdTsdT
Vegfr2	cAAccAGAGAcccucGuuudTsdT	AAACGAGGGUCUCUGGUUGdTsdT
Vegfr3	GuGuuGAGAAGAAccGuuudTsdT	AAACGGUUCUUCUcAAcACdTsdT

Supplementary Table 1. siRNA sequences used in this publication. All sequences were optimized using the process outlined in Supplementary Fig. 6. Lowercase letter correspond to nucleotides modified with 2-O-Methyl modifications, which decrease immunostimulation and promote antisense strand selection in the RISC complex.

7C1 *in vitro* delivery

7C1 was selected from an *in vitro* screen of 500 compounds. 500 structurally diverse PEI analogs were synthesized by conjugating small polyamines to alkyl tails. Amines reacted with epoxide-terminated alkyl tails in 100% EtOH at 90˚C for 48-72 hours (Supplementary Fig. 1a). 7C1-mediated mRNA silencing in HeLa, murine endothelioma cells (bEnd.3, ATCC), and pooled human dermal microvascular endothelial (HMVECs, Clonetics, Lonza) was measured (Fig. 1b). HeLa cells (cultured with 10% heat inactivated FBS) expressing Firefly and Renilla Luciferase were transfected with 7C1 complexed to siRNA targeting firefly (siFire) at a dose of 30 nM[5]. HeLa cells were seeded in a 96 well plate at a density of 15,000 cells/well. 24 hours later, cells were treated with 30 nM of siRNA before luminescence was measured 24 hours after transfection.

bEnd.3 and HMVEC were transfected with siTie2. Endothelial cells were seeded at density of 15,000 cells/well in a 96 well plate. 24 hours after seeding, 7C1 complexed with siTie2 was added before cells were analyzed 24 hours later. bEnd.3 cells were cultured under their suggested conditions (10% heat inactivated FBS). HMVEC cells were cultured under their suggested conditions (EGM-2 MV) and used within the first six passages.

7C1 endothelial cell uptake

The role of clathrin- and caveolae-mediated endocytosis in the uptake of 7C1 by HMVEC cells was studied. HMVEC cells were incubated with 7C1 complexed to fluorescent siRNA. Cells were seeded at 15,000 cells/well in black 96-well plates (Greiner Bio-one) and pre-incubated with inhibitors of endocytosis: chlorpromazine 10μM (clathrin mediated endocytosis), Fillipin 5μM (caveolae mediated endocytosis) and dynasore 100μM (clathrin and caveolae mediated endocytosis) for 30 minutes. These non-toxic concentrations did not affect cell viability, confirmed by constant cell number (data not shown). Cells were transfected with 50nM of 7C1 encapsulated with Alexa 647 labeled siRNA for 60 min in the presence or absence of the endocytic inhibitors. Cells were washed, fixed and counterstained in PBS containing Hoechst (2 μg/ml) for nuclei identification. The cells were imaged using an automated spinning disk confocal microscope (OPERA, Perkin Elmer) with a 40X objective. The same defined pattern of 20 fields from each well was acquired to eliminate bias and provide a statistically significant number of cells for analysis. After identification of cell location and perimeter, intracellular siRNA signal intensity over single field was calculated using Acapella software. Data represents intracellular intensity from 20 different fields. All experiments were done in triplicates and errors are reported as the Standard Error Mean (S.E.M).

Mouse experiments
All animal procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with local, state, and federal regulations. All experiments were tested in 6 to 8 week-old C57BL/6 female mice (Charles River Laboratories). All experiments used 4-5 mice per group unless noted otherwise.

7C1 serum concentration and biodistribution
BL\6 mice were injected with 1.5 mg/kg 7C1 formulated with Cy5.5-tagged siRNA. Mice were immediately bled following injection to obtain an individual baseline serum Cy5.5 value. Mice were then bled at the appropriate time point. Blood was centrifuged at 13,000 rpm for 10 minutes to retrieve serum. 12.5 uL of serum was placed in a black 96 well plate and diluted with 37.5 uL 1X PBS before Cy5.5 fluorescence (excitation 695 nm, emission 720 nm) was quantified against a standard curve.

Biodistribution in tissues associated with clearance (liver, spleen, and kidney) as well as lung and heart were measured 4 and 24 hours after injection with 1.5 mg/kg 7C1 formulated with Cy5.5-tagged siRNA. Cy5.5 fluorescence was quantified (IVIS imaging) and normalized by individual tissue weight.

7C1 purification
Since PEI_{600} is not monodisperse (M_n=600, PDI = 0.33), 7C1 was purified using high performance liquid chromatography (HPLC). More specifically, 7C1 ran on a silica column with DCM. Over 45 minutes, MeOH and NH_4OH were added to the solvent, decreasing its polarity. In this way, the HPLC separated 7C1 into fractions related to the hydrophobic C_{15}: hydrophilic PEI ratio. The mixture was split into five fractions, which were tested for their ability to reduce gene expression *in vivo.* Fractions that were less potent were discarded, leaving the most potent fraction (Supplementary Fig. 7a).

Confocal imaging
One hour after intravenous injection with Alexa647-tagged siRNA formulated with 7C1, mice were sacrificed. Ear or omentum tissues were harvested and fixed with PLP solution. Whole mount tissues were stained with anti-CD31 (clone 390) and anti-ICAM2 (clone 3C4).

Flow cytometry and fluorescent activated cell sorting analysis of pulmonary tissue
Mice were sacrificed, and perfused with sterile 1X PBS. Lungs were digested with DNAse, collagenase XI, and collagenase I for 30 minutes at 37˚C. Endothelial cells ($CD31^+CD45^-$), hematopoietic cells ($CD31^-CD45^+$), epithelial cells ($CD31^-CD45^-CD326^+$), T cells ($CD31^-CD45^+TCR\beta^+$), and B cells ($CD31^-CD45^+CD19^+$) were isolated using the following antibodies: CD31 (clone 390), CD45 (clone 104), CD326 (clone G8.8), TCRß (clone H57-597), and CD19 (clone 6D5).

Flow cytometry in skin, lymph node, and adipose tissue
Mice were injected with 0.6 mg/kg si-ICAM2, siCntrol, or PBS on day 1 and 4. Three days after the final injection, mice were sacrificed and $CD31^+CD45^-CD11b^-Ter119^-$ endothelial cells were isolated from skin, lymph node and adipose tissue (Supplementary Fig. 2b). Data is representative of two experiments (n = 4 mice / group / experiment).

7C1 biophysical optimization
We characterized gene silencing in thirteen separate formulations; six made by extrusion, and seven by microfluidic mixing (N=4 to 5 mice per group, per formulation). Particles made with microfluidic devices

were more potent and consistent (Supplementary Fig. 7b). Target gene silencing after varying 7C1: siRNA ratio and size was measured. Particle size varied from 135 nm to 40 nm while the formulation process was optimized (Supplementary Fig. 2g). All formulations had a mass ratio of 15:1 and a 7C1: Cholesterol: PEG molar ratio equal to 62: 22: 16 or 80: 0: 20, two ratios with equal potency (Supplementary Fig. 7c). Smaller particles were obtained with $C_{14}PEG_{2000}$, while larger particles were formulated with $C_{16}PEG_{2000}$.

Pulmonary, renal, and cardiovascular Tie2 expression was also measured after formulating nanoparticles with 7C1: siRNA mass ratio between 15:1 and 3:1 and injecting intravenously with a dose of 0.15 mg/kg (Supplementary Fig. 2h).

***In vivo* mRNA and protein measurements**

All endothelial gene silencing (siTie2, siTie1, siICAM2, siVE-Cadherin, siVEGFR-2) experiments were conducted 48-72 hours after injection, unless otherwise specified. Hepatocyte silencing (Factor 7) was examined 24 hours after injection as previously described[5]. Blood was collected from mice and centrifuged at 13,000 rpm for 10 minutes to isolate serum before F7 concentration was measured according to manufacturer instructions (Aniara). CD45 median fluorescent intensity was measured 72 hours after injection[22]. Cells from the peritoneal cavity were harvested using PBS and stained following red blood-cell lysis. The following clones were used: CD45 (clone 30-F11), CD11b (clone M1/70), TCRβ (clone H57-597); CD19 (clone 1D3), CD11c (clone N418); and propidium iodide (Sigma-Aldrich). Cells were analyzed with flow cytometry (LSR-Fortessa, BD Biosciences, and FloJo).

mRNA silencing was measured as target gene/GAPDH levels. The target gene/GapDH ratio was measured in mice treated with 7C1, and compared to the ratio in mice treated with siCntrol or PBS. For multi-gene silencing, five endothelial-specific siRNAs were simultaneously formulated in the same particle. The concentrations for each siRNA was based on its potency so that 1 mg of total siRNA contained 0.075 mg of siVEcad, 0.125 mg of siICAM2, 0.3 mg of siTie1, 0.25 mg of siTie2, and 0.25 mg of siVEGFR-2.

Western blots lysates were prepared with RIPA Lysis and ExtractionBuffer, as well as Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology). Total protein concentration was determined by the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Each lane of the pre-cast Mini-PROTEAN TGX 4-15% polyacrylamide gradient gels was loaded with equal amounts of total protein. Equal total protein loading was confirmed by Ponceau S staining. Blots were probed with goat anti-mouse IgG polyclonal VE-cadherin (R&D Systems) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.) antibodies, and developed with Amersham ECL Prime Western blotting detection reagents (GE-Healthcare Life Sciences). Blots were imaged and densitometry was performed with a GE ImageQuant LAS 400 luminescent image analyzer and software (GE-Healthcare Life Sciences).

Batch-to-batch repeatability

We measured particle size and *in vivo* gene silencing with 7C1 synthesized with different PEI_{600} lots. Each batch was purified independently before being formulated with siRNA targeting ICAM-2 and injected intravenously at a dose of 0.15 mg/kg. There was no significant difference in particle size or ICAM-2 mRNA silencing across batches (Supplementary Fig. 8a-b).

Permeability Study

Mice were treated with 0.6 mg/kg of 7C1-siVEcad, 7C1-siCntrol, or PBS control (5-7 mice per group). After seven or fourteen days, mice were injected intravenously with an Evans Blue dye solution (200 uL of 0.5% in 0.9% NaCl, filtered). After 30 min, mice were sedated and perfused with PBS. Lungs were dried overnight at 55˚C. Evans blue dye was extracted in formamide (10 uL/mg of dried tissue, 55C, 24h), and 620 nm absorbance was measured.

Emphysema Model
7C1 was formulated with siVEGFR-2 or siCntrol. Mice (N=7 per group) were injected on days one and eight before lungs were removed on day 22 for analysis[23].

Lewis lung carcinoma Primary Tumour Study. C57BL/6 mice (N=7-10 per group) were injected subcutaneously with Lewis lung carcinoma cells as described[37]. Mice received 1.0 mg/kg doses of siCntrol, siVEGFR-1, or siDll4 ten, fifteen, and nineteen days after primary tumour cell injection before mice were sacrificed three days following the last injection. Tumour growth was measured by calipers[37]. At sacrifice, mice were perfused with 3.6% paraformaldehye (PFA) through the left ventricle. Tumour tissue was placed in 3.6% PFA at 4˚C for 8 hours. After rinsing with PBS, the sample was placed in 70% EtOH at 4˚C before fixation and CC3 staining (Rabbit anti-mouse 1˚ antibody, Cell Signaling).

Lewis lung carcinoma Metastasis Study
C57BL/6 mice (N=4-6 per group) were injected subcutaneously with Lewis lung carcinoma cells. The primary tumour was allowed to grow for twenty days before cells were resected as previously described[37]. Following resection, lung metastases grow aggressively. Two, six, ten, and twelve days after resection, mice were injected intravenously with 1 mg/kg siCntrol, siVEGFR-1, or siDll4. Three days after the final injection, mice were sacrificed and analysis of metastases was performed as previously described[37].

***In vitro* cell live dead study**
The effect of 7C1 on cell viability was assessed using the bEnd.3 endothelial cell line (ATCC). Cells were plated at 30,000/well in a 48 well plate, and cultured for 24 hours in DMEM containing 10%FBS and 1% penicillin/streptomycin/amphotericin-B. One day following treatment with 7C1, cells were assessed by live/dead viability staining (Invitrogen Life Technologies) and imaged using an EVOS fluorescent microscope (Advanced Microscopy Group). Calcein AM indicates live cells by green fluorescence of the cytosol and ethidium homodimer indicates dead cells by red fluorescence of the nucleus.

Statistics
Unless noted otherwise, all error bars are standard deviations. Statistical significance was measured by student T test.