Supplementary Materials

iRGD-mediated Transcytosis of an Irinotecan Silicasome Carrier in Pancreatic Cancer

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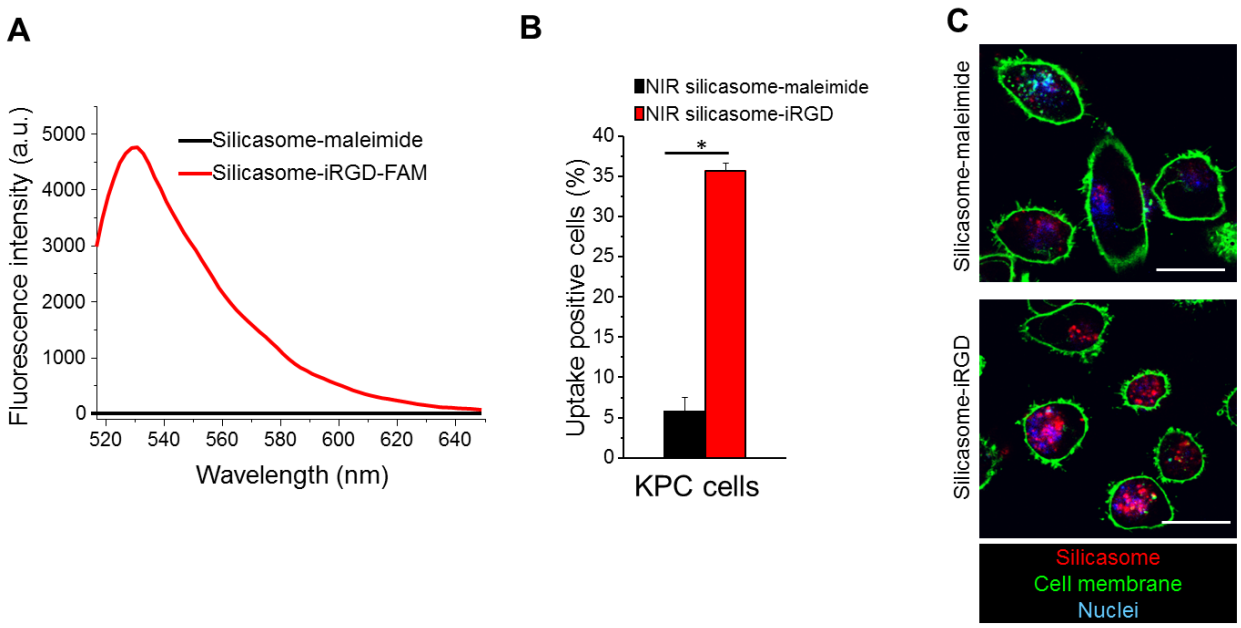
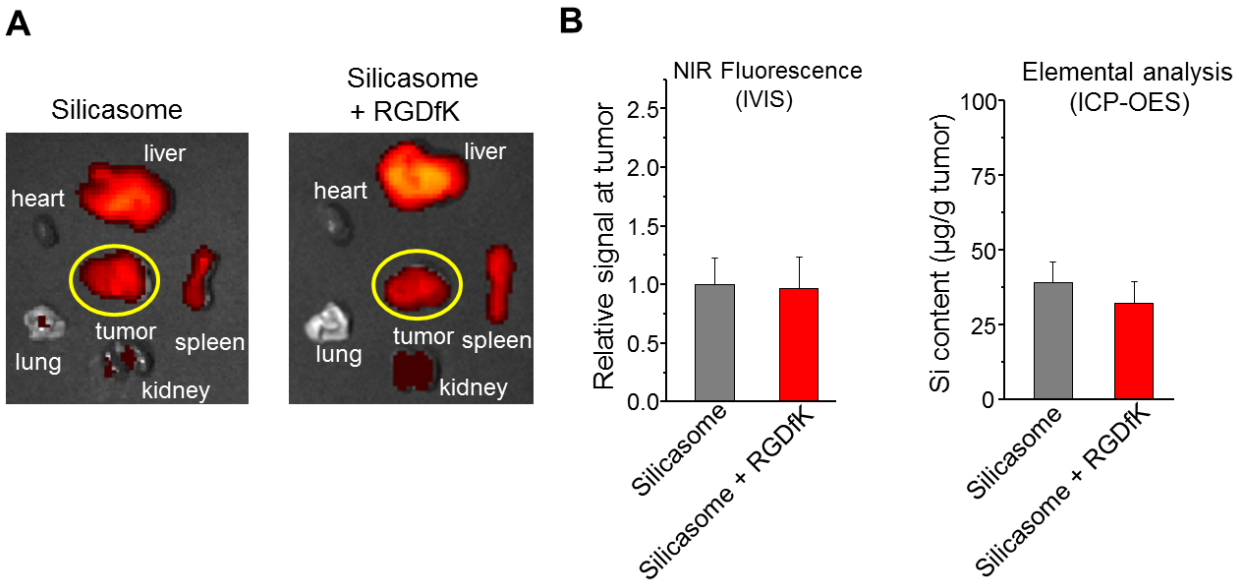
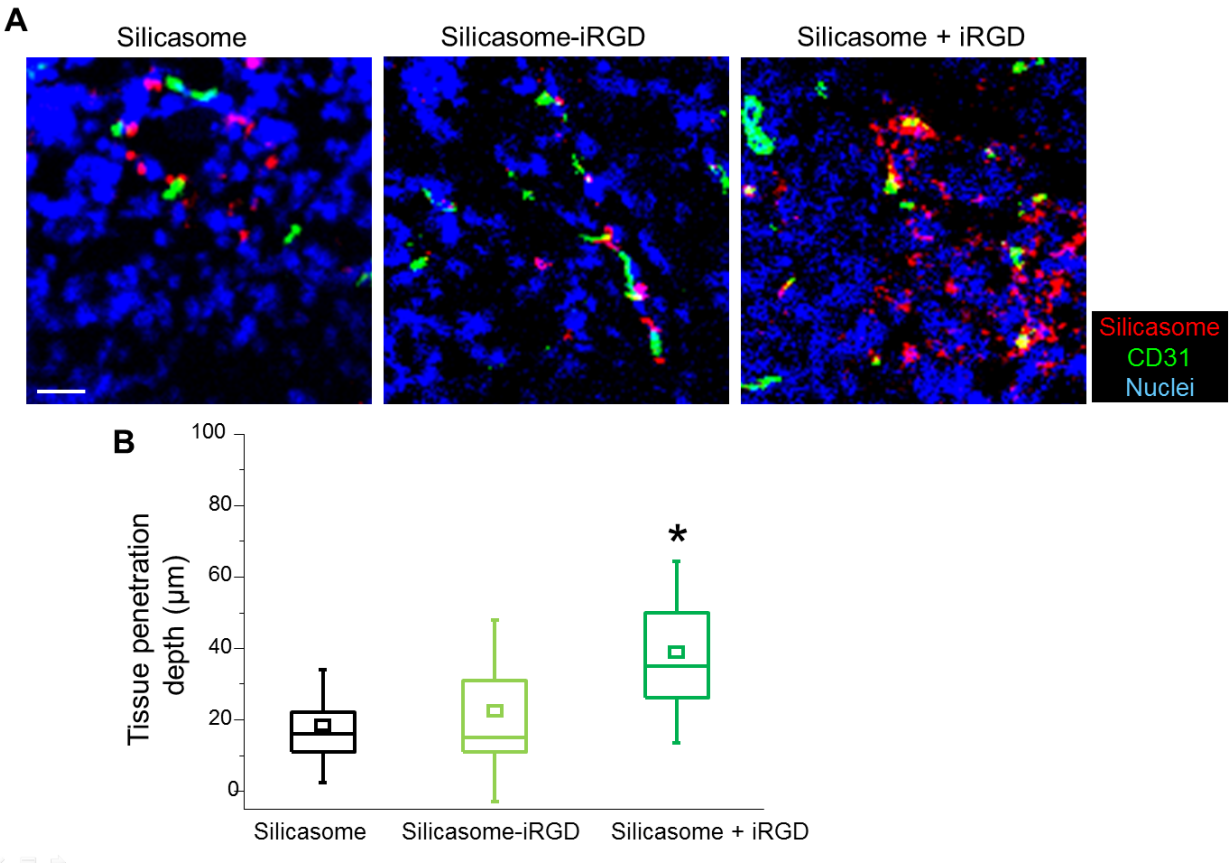


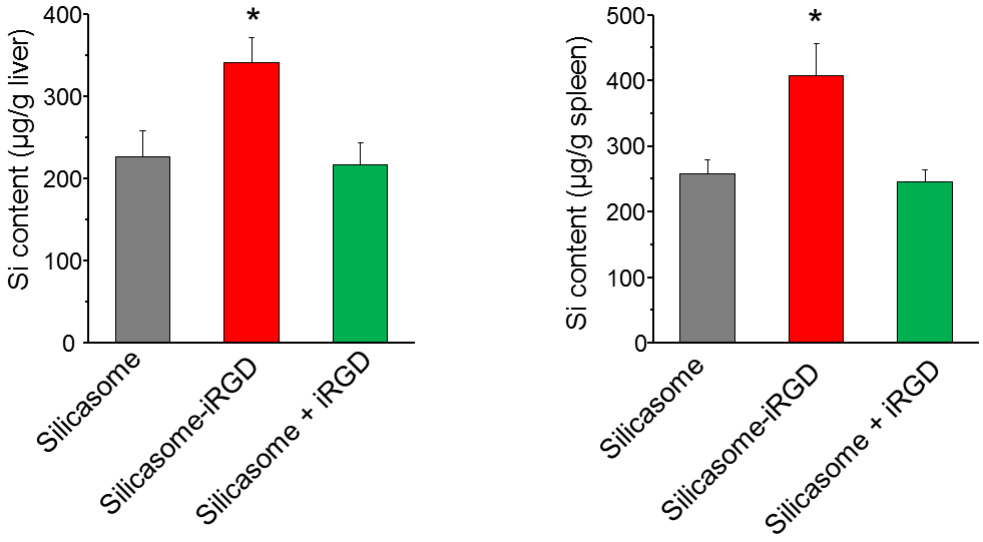
Supplemental Figure S1. The use of a thiol-maleimide reaction for successful covalent conjugation of iRGD to the lipid bilayer on silicasomes. To validate the success of covalent conjugation, a reactive fluorescein (FAM) labeled iRGD peptide, provided by Dr. Ruoslahti (*Cancer Cell* 2009, 16: 510), was used for binding to DSPE-PEG-maleimide. The detailed method is described in the methods section. **(A)** Fluorescence spectra of pristine and FAM-iRGD-silicasomes, suspended at 100 µg/mL, were obtained in a microplate reader (Molecular Device M5e) at excitation wavelength of 488 nm., the significant retentions of the fluorescence signal on the washed and purified FAM-iRGD-silicasomes confirms a successful conjugation reaction (*Cancer Cell* 2009, 16:510). **(B, C)** The effect of iRGD conjugation to the silicasome was tested for its impact on cellular uptake. A batch of the silicasome-iRGD was synthesized with NIR-labeling (DyLight 680) of the MSNP framework, as described in the methods section. KPC cells were treated at a particle concentration of 100 µg/mL at 37 °C for 2 h and uptake was determined by flow cytometry (B), and confocal microscopy (C). An iRGD-free NIR-silicasome with similar labeling efficiency was used as control. Data represent mean ± SD (n=3), *p<0.05 (2-tailed Student's t test). Confocal microscopy confirmed the results (cell membranes were green stained with wheat germ agglutinin and nuclei blue stained with Hoechst 33342). Bars = 20 µm.



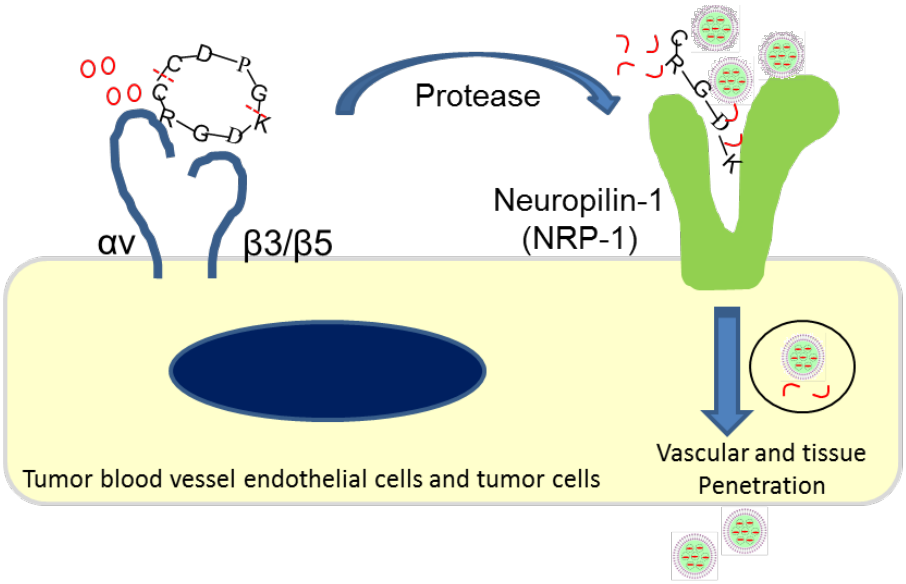
Supplemental Figure S2. Assessment of the effect of a non-functional peptide (lacking the CendR motif) on IV silicasome biodistribution. **(A)** We used a non-CendR peptide, cyclo (RGDfK), to repeat the biodistribution experiment in orthotopic model shown in Figure 2. Briefly, tumor-bearing animals received IV injection of 50 mg/kg NIR-labeled silicasome co-administrated with PBS or 8 µmol/kg free cyclo (RGDfK), followed by animal sacrifice at 24 h (n=3). Representative *ex vivo* organ NIR fluorescence images were obtain to show nanoparticle biodistribution. **(B)** NIR fluorescence intensity analysis (by IVIS software) and determination of Si content (by ICP-OES) showed that non-CendR peptide is incapable of improving silicasome uptake at the tumor site. Data represent mean ± SD.



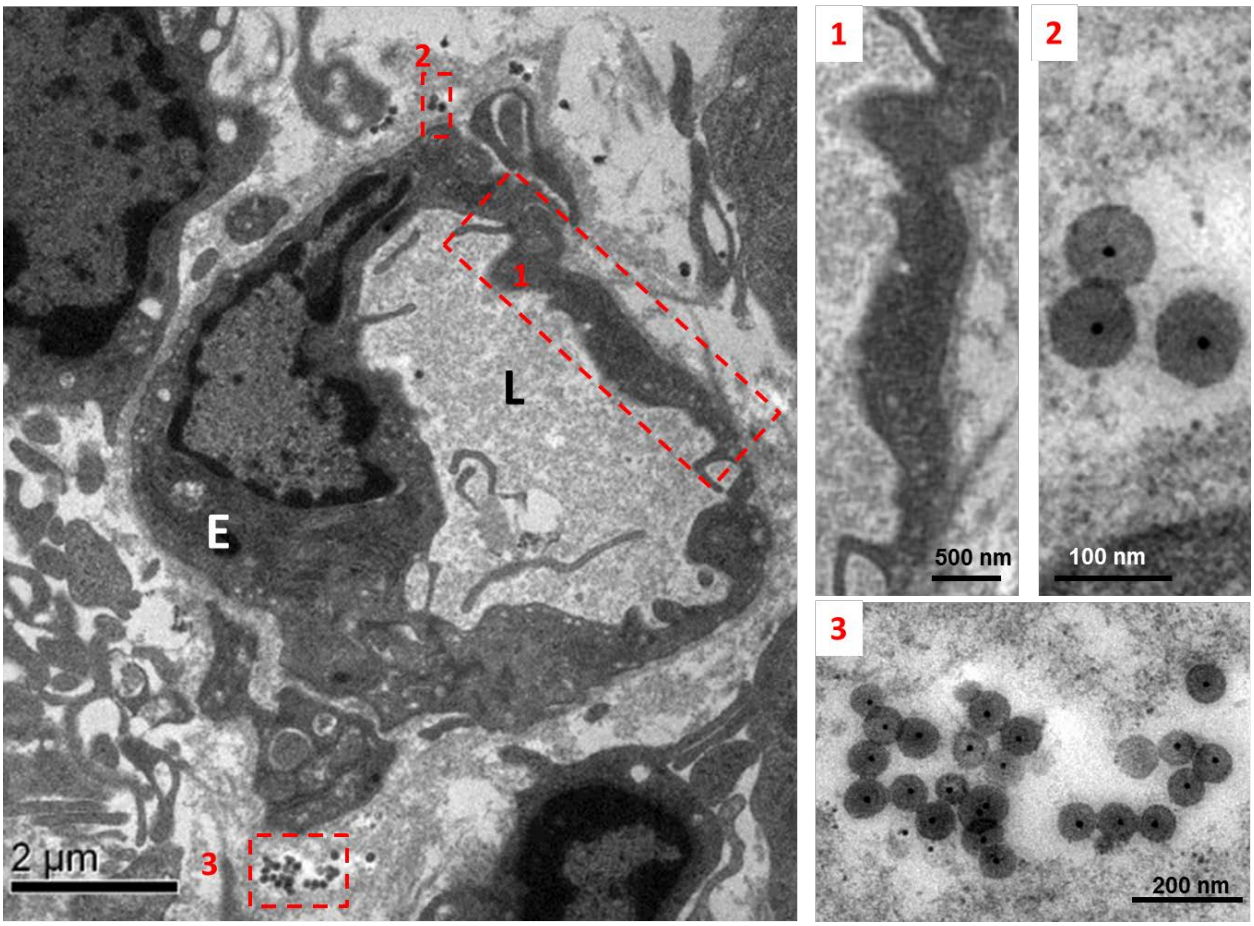
Supplemental Figure S3. Assessment of silicasome transport in the tumor by confocal microscopy. **(A)** Tumor sections were obtained from experiment in Figure 2. Representative confocal images were obtained to assess the intratumoral abundance of the NIR labeled silicasome abundance under a SP2-1P-FCS, Leica microscope, using a 633 nm laser. IHC staining of the same section to review the presence of blood vessels (CD31 in green) and the presence of nuclei (DAPI staining in blue), allowed us to determine the presence and migration of nanoparticles in the tumor tissue, using the methodology in *Science* (2010, 328:1031). Bar = 20 µm. **(B)** Calculation of the penetration distance silicasomes from the closest tumor blood vessel was estimated for ~15 blood vessels, using Image J software. Box-and-whisker plots (Origin software) were developed to show median (horizontal line), 25^{th}-75^{th} percentiles (box), mean (open square) and SD (whiskers). *, $p<0.05$ (1-way ANOVA followed by a Tukey's test) compared with silicasome alone or silicasome-iRGD.



Supplemental Figure S4. Si contents in the liver (left) and spleen (right), using organs obtained from the animals used in Figure 2, were determined by ICP-OES analysis. Data represent mean ± SD, **p*<0.05 (1-way ANOVA followed by a Tukey's test) compared with silicasome or "silicasome + iRGD" groups.



Supplemental Figure S5. Schematic to demonstrate the mechanism by which iRGD initiates nanoparticle transcytosis (picture modified from Fig. S4 in *Science* 2010, 328:1031)**.**



Supplemental Figure S6. TEM images of a KPC-derived tumor from an animal that was prior (24 h) injected with 50 mg/kg Au-embedded silicasomes without iRGD co-administration is shown. We re-used the low magnification part of Fig. 5A here to show the nanoparticle extravasation in the same tumor tissue section. The images demonstrate the absence of transcytosis vesicles, with sparse particle deposition on the adluminal side. E = endothelial cell, L = lumen.

S7. Supplemental Materials and Methods

Materials

Tetraethylorthosicate (TEOS), triethanolamine, cetyltrimethylammonium chloride solution (CTAC, 25 wt% in water), (3-aminopropyl)triethoxysilane (APTES), triethylamine (TEA), gold (III) chloride hydrate, trisodium citrate dehydrate, Dowex 50WX8 resin, and Sepharose CL-4B were purchased from Sigma-Aldrich, USA. 1, 2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG_{2000}-maleimide) and cholesterol (Chol) were purchased from Avanti Polar Lipids, USA. Sucrose octasulfate (SOS) sodium salt was purchased from Toronto Research Chemicals, Canada. Irinotecan hydrochloride trihydrate was purchased from LC Laboratories, USA. Penicillin, streptomycin, and Dulbecco's modified Eagle medium (DMEM) were obtained from Invitrogen. Fetal bovine serum (FBS) was purchased from Gemini Bio Products, USA. iRGD (CRGDKGPDC) was purchased from Biomatik, USA. Reactive iRGD modified with cysteine residue and blocking anti-NPR-1 antibody (against recombinant b1b2 domain of NRP-1) were kindly provided as gifts by Dr. Ruoslahti's lab (Cancer Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA). Cyclo (-RGDfK) was purchased from ApexBio Technology, USA. Anti-CD31 antibody (catalog# 553708) was purchased from BD Pharmingen™, USA. Anti-NPR-1 antibody (catalog# ab81321) was purchased from Abcam, USA. Control normal goat IgG (catalog# sc-2028) was purchased from Santa Cruz Biotechnology, USA. Alexa Fluor® 488 conjugated goat anti-rabbit IgG (H+L) secondary antibody (catalog# A11008), Alexa Fluor® 594 conjugated goat anti-rat IgG (H+L) secondary antibody (catalog# A11007) and DyLight 680

NHS ester were purchased from Thermo Fisher Scientific Inc, USA. Matrigel™ Matrix Basement Membrane was purchased from BD Bioscience, USA. All chemicals were directly used without further purification.

NIR labeling of MSNPs using DyLight 680

The NIR fluorescent dye, DyLight 680 NHS ester was used for MSNP labeling. First, the MSNP surface was functionalized with NH_2 groups for conjugation of the NHS ester. Briefly, 10 mg MSNPs were suspended in 1 mL of ethanol and mixed with 1 µL of APTES. The reaction took place under an inert N_2 atmosphere, while stirring at 80 °C overnight. Subsequently, the mixture was centrifuged and washed 3 times with ethanol. The NH_2-conjugated MSNPs were suspended in 1 mL of DMF, mixed with 0.01 mg of DyLight 680 NHS ester, and stirred at room temperature for 2 h. The labeled MSNPs were washed with ethanol and deionized water. NIR-labeled MSNPs were used to make the silicasomes.

Synthesis of ~10 nm gold nanoparticle

Gold nanoparticles of ~10 nm were made by adding 5 mL $HAuCl_4$ (10 mM) and 45 mL Milli-Q water to a 100 mL round-bottom flask equipped with a condenser. After reaching boiling temperature, while being stirred vigorously, 5.8 mL of sodium citrate (38.8 mM) was added into the boiling solution. This was accompanied by in a color change from pale yellow to burgundy. The boiling solution was stirred for 10 min at 160 °C and then stirred for an additional 15 min without heating. The gold particles were used as a core for synthesis of silicasomes, as described in the methods section of the manuscript.

Cell line

An immortalized cell line was derived from a spontaneously primary tumor in a transgenic $Kras^{LSL\text{-}G12D/+}$; $Trp53^{LSL\text{-}R172H/+}$; Pdx-1-Cre mouse. To allow bioluminescence tumor imaging of the growing tumors after orthotopic implantation, the cells were permanently transfected with a luciferase-based lentiviral vector in the UCLA vector core facility. A representative cell clone was obtained after use of a limiting dilution protocol.

Preparation of orthotopic tumors in immune competent mice, using the KPC-derived cell line

All animal experiments were performed with protocols approved by the UCLA Animal Research Committee. Female B6/129 mice (~8 weeks) were purchased from The Jackson Laboratory. To grow orthotopic xenografts, the mice were anesthetized with isoflurane, followed by IP injection of 50 mg/kg ketamine and 10 mg/kg xylazine. The surgical site was shaved to leave a margin of 1 cm around the incision site and sterilized by scrubbing with betadine and 70% ethanol. The mice were positioned for surgery on a heating pad, and the incision site in the left flank was draped with sterile gauze. A surgical incision of ~0.7 cm was made to expose the injection site, followed by an injection of 50 μL of DMEM/Matrigel (1:1 v/v) containing 2×10^6 KPC-luc cells into the pancreatic tail through a 27 G needle. The fascial layers were closed with absorbable sutures (PDS II, Ethicon) and the skin with nonabsorbable sutures (PROLENE, Ethicon). The mice were kept on the warming pads until full recovery from the anesthesia, and then transferred to clean cages. Artificial tear ointment was used to protect the mouse eyes during the surgery.

Use of anti-NRP-1 blocking antibody to interfere in the iRGD effect

50 μg of a blocking anti-NRP-1 antibody or a control IgG was injected 15 min before the KPC-derived orthotopic tumor-bearing mice received IV injection of 50 mg/kg NIR-labeled

silicasome plus 8 μmol/kg free iRGD. Animals receiving the silicasome alone were used as controls. Animals were sacrificed at 24 h post-injection, and *ex vivo* NIR imaging was used to study the biodistribution of NIR labeled silicasomes. The *ex vivo* imaging data was quantified by NIR intensity analysis using IVIS software, followed by Si content analysis using ICP-OES.

HPLC analysis

For HPLC analysis of irinotecan in tissues, the harvested tumor and organ samples were weighed and homogenized on ice. Following the extraction of 0.1 mL tissue homogenate with 0.4 mL of an acidic solution (0.1 mol/L phosphoric acid/methanol, 1:4 v/v), the extracts were vortexed twice for 10s and centrifuged at 13,000 rpm for 10 min. The irinotecan-containing supernatants were filtered through 0.22 μm filters for HPLC analysis in a system containing a Knauer Smartline pneumatic pump, C18 column, K-2600 spectrophotometer, and Gina data acquisition software. The mobile phase, delivered at a flow rate of 1.0 mL/min, was comprised of a 3% triethylammonium acetate aqueous buffer (pH=5.5) and acetonitrile (73:27 v/v). Twenty microliters of an irinotecan-containing sample was injected to measure the drug absorption at 254 nm, typically eluted in ~4.4 min. An irinotecan standard curve was generated over the concentration range 0.05-100 μg/mL.

Immunofluorescence staining

Dual color immunofluorescence staining was used to determine the NPR-1 positive blood vessels in KPC tumor tissue. The tumor tissues were cryo-embedded, using the OCT reagent, and used to prepare tumor sections. The sections were first treated with anti-NRP1 monoclonal antibody (1:250) at 4 °C overnight. After removal of the primary antibody and washing in PBS 3 times, the Alexa Fluor® 488 secondary antibody (1:500) was added and incubated for 1 h at room

temperature. The same sections were also stained with anti-CD31 antibody, followed by Alexa Fluor® 594-conjugated secondary antibody treatment to identify CD31 expression. DAPI was used to localize the cellular nuclei. The stained slides were examined under a fluorescence microscope (Observer D1, Zeiss). The colocalization ratio of NRP-1^{+}/$CD31^{+}$ blood vessels were determined by Imaging J software.