iRGD-guided tumor penetrating nanocomplexes for therapeutic siRNA delivery to pancreatic cancer

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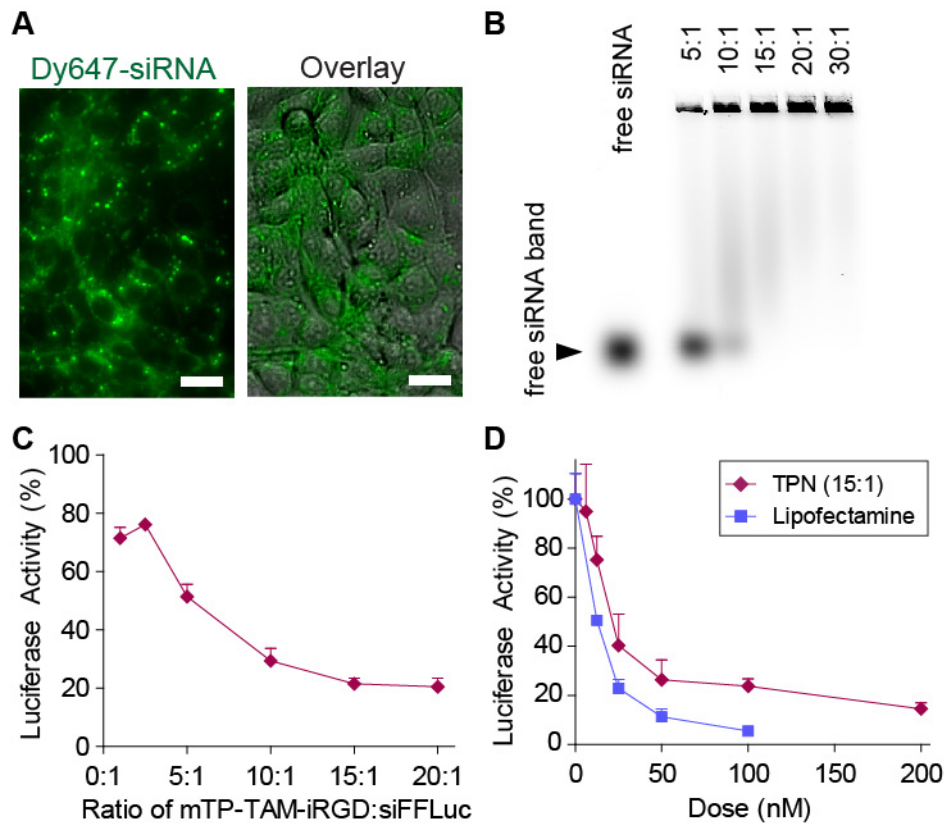
Supplemental Information

Figure S1. *In vitro* **optimization of iRGD TPNs in PDAC cell lines. (A)** DyLight647-tagged siRNA delivery by mTP-iRGD particles after 24 hours, showing cytosolic distribution. Scale bars: 20 μm. **(B)** Electrophoretic mobility shift assay showing encapsulation of siRNA at different ratios of peptide to siRNA. **(C)** 48-hour firefly luciferase knockdown in B22 cells as a function of peptide:siRNA ratio. **(D)** 48-hour luciferase knockdown dose curve for iRGD TPNs compared to lipofectamine RNAiMax positive control.
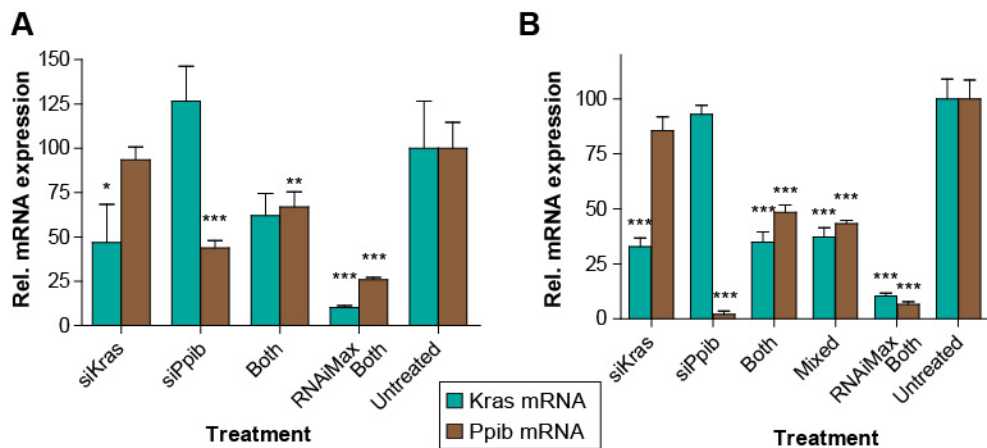
Figure S2. Dual knockdown using TPNs. (A) Knockdown of Kras and Ppib by iRGD TPNs containing siKras, siPpib, or both siRNAs encapsulated simultaneously, dosed through forward transfection. **(B)** Knockdown of Kras and Ppib by iRGD TPNs containing siKras, siPpib, or both siRNAs simultaneously, dosed through reverse transfection (addition of suspended cells over particle solution). "Both" indicates particles encapsulating both types of siRNAs pooled together, while "Mixed" indicates a mixed solution of particles encapsulating one siRNA or the other. In all panels, statistical significance was computed by two-way ANOVA comparing to the untreated mRNA level, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$.
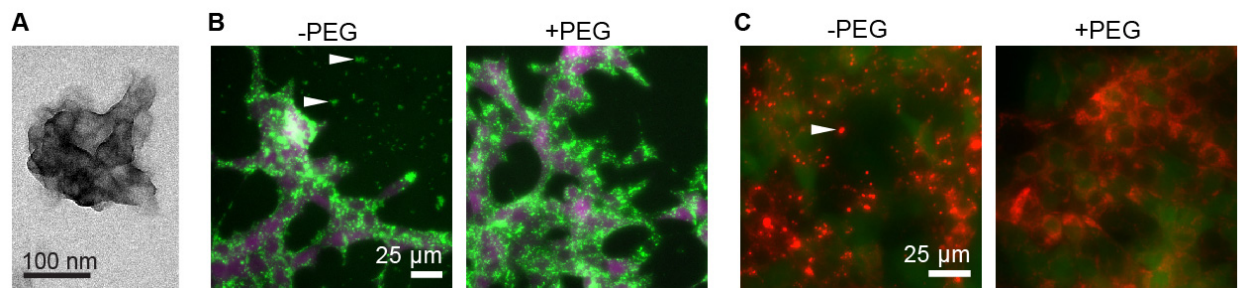
Figure S3. *In vitro* and *in vivo* properties and function of PEGylated iRGD TPNs **(A)** TEM image of iRGD TPNs formed at a 15:7.5:1 peptide:TP-PEG-iRGD:siRNA ratio in 1x PBS. Scale bar: 100 nm. **(B)** Localization of fluorescently-tagged mTP-iRGD 5 hours after transfection with 10:0:1 (left) or 10:10:1 (right) iRGD TPNs. White arrowheads indicate aggregates that are not localized to any cells. Green: TAMRA-labeled peptide; Magenta: GFP (cells) **(C)** Localization of fluorescently-tagged siRNA 24 hours after transfection with 10:0:1 (left) or 10:10:1 (right) iRGD TPNs. Red: DyLight-647-labeled siRNA; Green: GFP (cells).
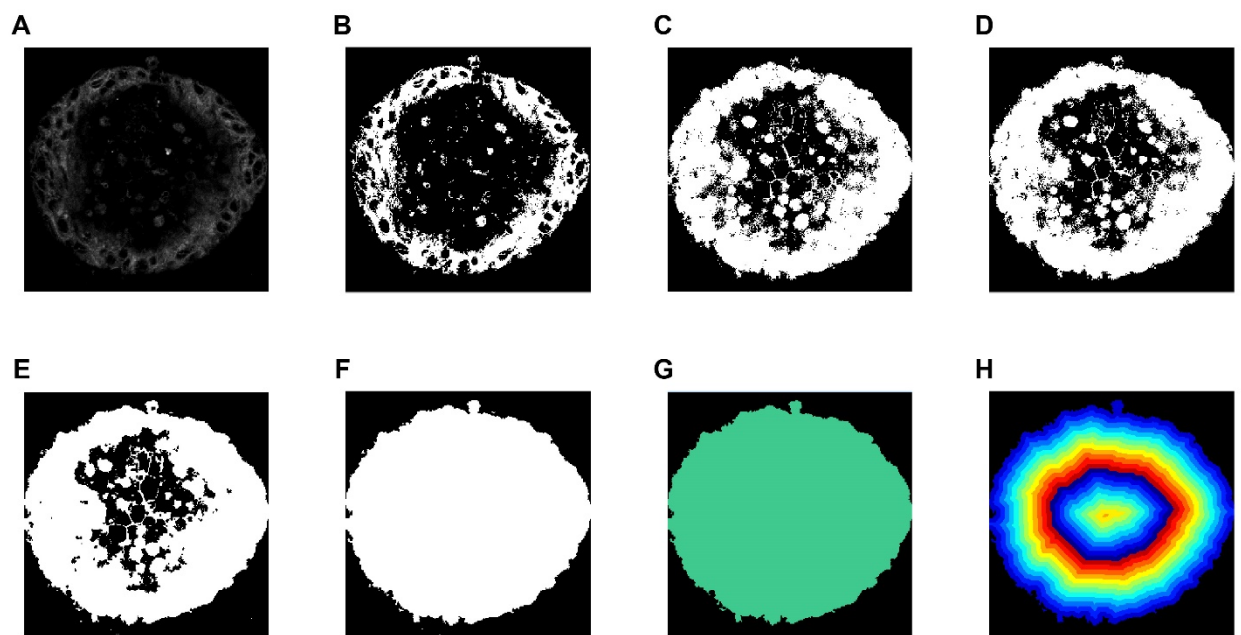
Figure S4. Overview of organoid image analysis. (A) Raw image of cytoplasmic stain **(B)** Binary (black and white) conversion of cytoplasmic stain **(C)** Binary conversion of nuclear stain **(D)** Overlaid cytoplasmic and nuclear stains **(E)** Eroded and dilated overlay to fill small gaps **(F)** Filled holes **(G)** Identification of individual organoids (if there were more than one in the image, each would be color-coded separately) **(H)** Computation of distance from the edge of the organoid, with each colored band representing a "bin" for which an average siRNA intensity is computed.
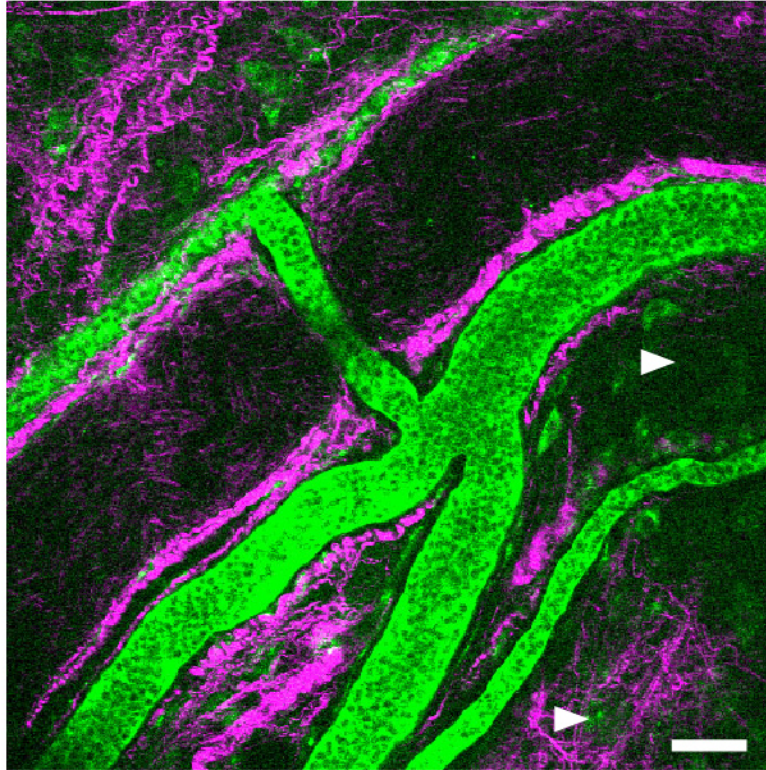
Figure S5. iRGD TPN-mediated delivery of siRNA in models of pancreatic cancer. Still image from intravital imaging of a subcutaneous pancreatic cancer xenograft showing PEGylated iRGD TPNs (green) in the bloodstream and accumulation of the particles in tumor cell compartments (white arrowheads) amidst stromal collagen fibrils (magenta). This is a still image taken 8 minutes following injection, at a depth of 80 µm from the surface of the tumor, part of a timelapse acquisition (see also Supplemental Video 1, which displays a timelapse of the particles in the tumor at a different depth). Scale bar = 50 µm.
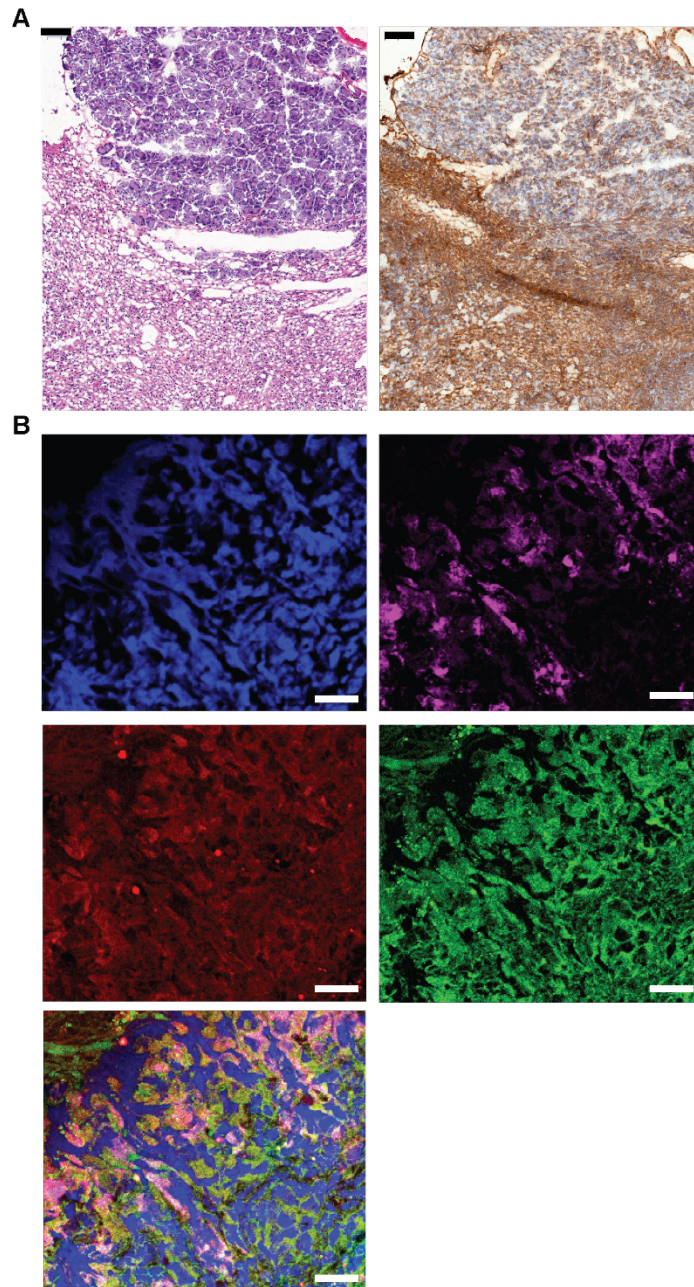
Figure S6. iRGD-mediated TPN uptake and NRP-1 expression in the tumor vasculature.
(A) Orthotopic pancreatic tumor xenografts in NCR/nude mice. Histology and IHC staining of NRP-1 indicate the high receptor level in orthotropic tumor tissue (bottom left) as compared to normal pancreatic tissue (upper right). Scale bar = 100 μm. **(B)** Multi-color confocal imaging of a KPC-derived orthotopic tumor section, showing nuclear staining (blue), fluorescein-conjugated *Lycopersicon Esculentum* (tomato) lectin indicating microvessels (magenta), TAMRA-labeled TPNs (red), and immunofluorescent staining of NRP-1 (green). An overlay of all four channels is shown at the bottom. Scale bar = 20 μm.
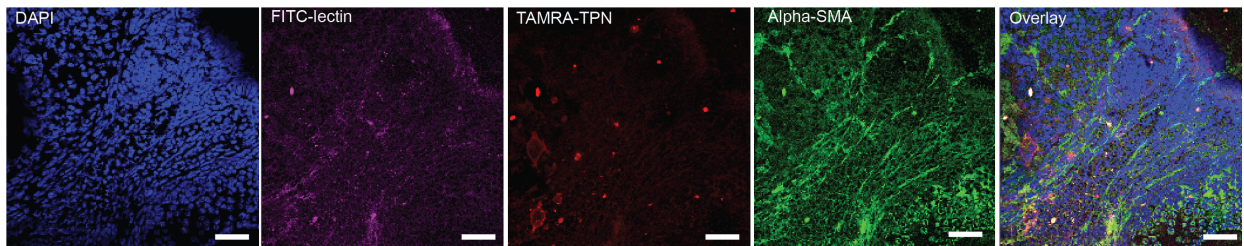
Figure S7. Stromal elements in a KPC-derived orthotopic tumor section following iRGD TPN administration. From left to right, nuclear staining (blue), FITC-conjugated tomato lectin staining to indicate microvessels (magenta), TAMRA-labeled iRGD TPNs (red), and alpha-smooth muscle actin to indicate stroma (green). Multichannel overlay is displayed rightmost, showing the relationship between particle accumulation and the stromal and vascular networks. Scale bar = 20 μm.

Supplemental Video 1. Intravital imaging of iRGD TPNs in a xenograft model of pancreatic cancer

Intravital imaging of a subcutaneous pancreatic cancer xenograft (MIA PaCa-2) showing distribution of PEGylated iRGD TPNs (green) in tumor cell compartments surrounding abnormal tumor blood vessels. Collagen visualized through second-harmonic generation is displayed in magenta. Images in this video were captured at a depth of 60 μm from the edge of the exposed tumor. Each frame of 0.25 sec represents 5 min of elapsed time, starting 3 minutes after injection of the particles. Please refer to Figure S5 for reference scale bar.

Supplemental MATLAB code for organoid analysis:

```matlab
threshold_level = [0.1, 0.2, 0.2]; % Threshold for R G B channels,
respectively, scale 0-1, for use in conversion to binary
bin_width = 10; % width of each ring
warning('off','Images:initSize:adjustingMag') % turning off the resizing
warning that arises due to large images

%%% PART 1 %%%
figure(1)
img_green = imread('ms_z14_Cells.tif'); % cytoplasmic pixels
img_blue = imread('ms_z14_Nuclei.tif'); % nuclear pixels (Hoechst)
img_red = imread('ms_z14_siRNA.tif'); % siRNA pixels

bg_green = mean(mean(img_green(500:512,500:512))) % background computation
for cytoplasmic channel from area of image without organoids
bg_red = mean(mean(img_red(500:512,500:512))) % background computation for
siRNA channel from area of image without organoids

BW_green = im2bw(img_green,threshold_level(2)); % B&W conversion of cytoplasm
BW_blue = im2bw(img_blue,threshold_level(3)); % B&W conversion of nuclei
BW_combined = 1-((1-BW_green).*(1-BW_blue)); % overlaying the cytoplasmic and
nuclear pixels to get complete cells
se = strel('disk',3);
organoid = imdilate(BW_combined,se); % dilation to fill small gaps
organoid2 = imerode(organoid,se); % erosion to returning to original
dimensions
organoid3 = imfill(organoid2,'holes'); % filling of interior holes to include
space within organoids without cells

subplot(2,4,1); imshow(img_green); title('Original');
subplot(2,4,2); imshow(BW_green); title('BW Cytoplasm');
subplot(2,4,3); imshow(BW_blue); title('BW Nuclei');
subplot(2,4,4); imshow(BW_combined); title('BW combined');
subplot(2,4,5); imshow(organoid2); title('BW dilated and eroded');
subplot(2,4,6); imshow(organoid3); title('BW filled holes');
organoidCandidates = bwlabel(organoid3); % catalogue of all potential
organoids in the image
statsAll = regionprops(organoidCandidates,'Area','Centroid'); % measurement
of area and centroid of each organoid candidate
sizeLowerLimit = 400; % minimum area (in pixels) to be considered an organoid
organoids = find([statsAll.Area] > sizeLowerLimit)
color_filtered = zeros(size(img_green,1),size(img_green,2),3);
colors = colormap(parula);

% The following loop creates an image that color-codes and labels individual
organoids for the user to choose more easily the organoid of interest
for i = organoids
    BW_organoid = ismember(organoidCandidates,i);
    RGB_organoid = zeros(size(img_green,1),size(img_green,2),3);
    colorIndex = mod(i*19,64);
    RGB_organoid(:,:,1) = BW_organoid*colors(colorIndex,1);
    RGB_organoid(:,:,2) = BW_organoid*colors(colorIndex,2);
    RGB_organoid(:,:,3) = BW_organoid*colors(colorIndex,3);
    color_filtered = color_filtered+RGB_organoid;
    pos = statsAll(i).Centroid;
```

```matlab
    stringF = sprintf('%2.0f',i); % labeling of organoids by number
    text(pos(1)+3,pos(2)-5,stringF,'Color',[1 0
0],'FontSize',7,'FontWeight','bold','FontName','Arial');
end
figure(1)
subplot(2,4,7); imshow(color_filtered); title('Organoids');

%%% PART 2 %%%
colors = colormap(jet);
BW_main = ismember(organoidCandidates,17); % select organoid of interest. In
this example, #17 is the organoid of interest

figure(4)
imshow(BW_main)
se2 = strel('disk',bin_width); % shape that will set the width of each
binning ring
color_rings = zeros(size(img_green,1),size(img_green,2),3);
counter = 0;
output_data = []; % collects average siRNA and cytoplasmic intensity by
binning ring
output_ratio = []; % collects the ratios of the above intensities

% The following loop computes the average siRNA and cytoplasmic signal, and
their ratio, for each "tree ring," eating its way outside-in. It will stop
when there is no longer any area left the process.
while sum(sum(BW_main)) ~= 0
    counter = counter+1;
    BW_main2 = imerode(BW_main,se2); % define the inner boundary of current
ring
    ring = BW_main-BW_main2; % binary mask of each binning ring

    % RING PROCESSING
    signal_red = ring.*double(img_red(:,:,1));
    signal_green = ring.*double(img_green(:,:,1));
    ring_area = sum(sum(ring)); % total area in pixels of each ring
    avg_signal_red = sum(sum(signal_red))/ring_area-bg_red; % average siRNA
intensity in the binning ring
    avg_signal_green = sum(sum(signal_green))/ring_area-bg_green; % average
cytoplasmic intensity in the binning ring
    ring_data = [counter*bin_width, avg_signal_red, avg_signal_green];
    output_data = [output_data; ring_data];
    output_ratio = [output_ratio; counter*bin_width,
avg_signal_red/avg_signal_green]; % ratio between siRNA and cytoplasmic stain
intensity

    colorIndex = mod(counter*5,64);
    RGB_ring = zeros(size(img_green,1),size(img_green,2),3);
    RGB_ring(:,:,1) = ring*colors(colorIndex,1);
    RGB_ring(:,:,2) = ring*colors(colorIndex,2);
    RGB_ring(:,:,3) = ring*colors(colorIndex,3);
    color_rings = color_rings+RGB_ring;
    BW_main = BW_main2; % move on to the next ring
end
figure(1)
subplot(2,4,8); imshow(color_rings); title('Regions binned by distance from
outer edge of organoid')
```

```matlab
figure(2)
subplot(1,2,1);
bar(output_data(:,1),output_data(:,2:3)); xlabel('Distance from edge (px)');
ylabel('Signal intensity'); legend('siRNA intensity','cell intensity');
title('Individual channel data');
subplot(1,2,2);
bar(output_ratio(:,1),output_ratio(:,2)); xlabel('Distance from edge (px)');
ylabel('siRNA/cytoplasm ratio'); title('Intensity Ratio');
```