Supplemental Data

Tissue Culture and Cell Lines

Luciferase-expressing GPC3-positive HepG2 HCC cells were purchased from PerkinElmer (Bioware HT1080-luc2) and maintained in Eagle minimum essential medium. GPC3-negative HLF HCC cells were acquired from the Japanese Cancer Research Resources Bank and maintained in Dulbecco modified Eagle medium (DMEM). GPC3-negative RH7777 HCC cells were purchased from American Type Culture Collection (ATCC no. CRL-1601). RH7777 cells were transfected with Luciferase-pcDNA3 (Addgene Plasmid 18964) using Lipofectamine 2000 (Invitrogen) per manufacturer's protocol and selected with DMEM containing 0.5 mg/mL G418. All cell lines were maintained in a monolayer at 37°C in their respective media (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) in a humidified atmosphere of 95%/5% air/CO_2. Relative GPC3 mRNA expression was determined via real-time PCR using 2 sequence-exclusive primer pairs.

Real-Time PCR

1×10^7 HepG2, HLF, and RH7777 cells were detached from a monolayer using TrypLE (Invitrogen), pelleted, and mRNA was isolated using an RNeasy Plus mini kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad), and template amplification was performed using iQ SYBR Green Supermix (BioRad) as per the manufacturer's instructions. Thermocycling for all targets was carried out in a solution of 20 μL containing 0.25 μM primers (Integrated DNA Technologies) under the following conditions: 15 s at 94°C, 30 s at 55°C, and 30 s at 72°C for 40 cycles. The reaction was monitored in real time using a CFX96 thermocycler (BioRad). Two sequence-exclusive sets of GPC3 primers with gene product sizes of 250 and 430 base-pairs were used to confirm the presence of this gene. Primer nucleotide sequences were as follows: GPC3-250 forward 5′-GATACAGCCAAAAGGCAG-3′, reverse 5′-ATCATTCCATCACCAGAG-3′, GPC3-430 forward 5′-GCAAGTATGTCTCCCTAAGG-3′, reverse 5′-AGGTCACGTCTTGCTCCTC-3′. GPC3 expression was normalized to expression of the housekeeping gene GAPDH: forward 5′-TCGACAGTCAGCCGCATCTTCTTT-3′, reverse 5′-ACCAAATCCGTTGATCCGACCTT-3′.

αGPC3 IgG1 Generation

RBF/DnJ mice were immunized with recombinant carrier-free human GPC3 protein in Freund adjuvant solution. After several boost injections, antiserum ELISAs confirmed the presence of the αGPC3 IgG. Additional boost injections were delivered to ensure IgM/IgG switch, which was verified on ELISA with IgG titrated to 1:10,000. After final prefusion boost injections, the mice were euthanized, their spleens were harvested, 10^8 splenocytes were fused 1:1 with FOX-NY myeloma cells, and the resultant hybridomas were resuspended in adenine/aminopterin/thymidine FBS solution. Clones producing high titers of GPC3 IgG1 were selected using capture ELISA with goat antimouse IgG1 for isotyping.

Antibody Internalization

The internalization of αGPC3 was evaluated using confocal microscopy. Briefly, HepG2 cells were plated on glass coverslips in 6-well plates, and after attachment each well was treated for 1 h with 1 μg αGPC3 conjugated to Alexa Fluor 647. The cells were then washed with PBS and fixed with 4% formaldehyde in PBS after 1 and 24 h. Cells fixed 24 hours after treatment with αGPC3 showed antibody internalization confirmed by z stack imaging (Supplemental Fig. 3).

Production of ^{89}Zr-Labeled αGPC3

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. PD-10 and Superdex-200 (10/300 GL) chromatography columns were purchased from GE Healthcare. In-process radioactivity (>0.2 MBq) was measured using a Capintec ion chamber (CRC-25R, setting 465) and radio-HPLC was performed with a Carroll Ramsey and Associates unit (105-S) equipped with a Si-PIN diode radiation detector. ITLC was performed with paper strips from Biodex (catalog no. 150-001) eluted with buffered 0.02 M sodium citrate.

A polypropylene conical Falcon tube (50 mL, Blue-Max; Becton-Dickinson) was successively charged with ^{89}Zr in 1 M oxalate (400 μL, 270 MBq [7.28 mCi]); 2 M sodium carbonate (180 μL, slowly); 1 M HEPES buffer (300 μL); water (210 μL); Df-(NCS)-antibody (P5F8) conjugate (200 μL, 1 mg protein in 0.25 M sodium acetate trihydrate buffer with 5 mg/mL gentisic acid); 1 M HEPES buffer (355 μL); and water (355 μL). The final mixture (2.0 mL) was incubated at room temperature for 1 h, with intermittent

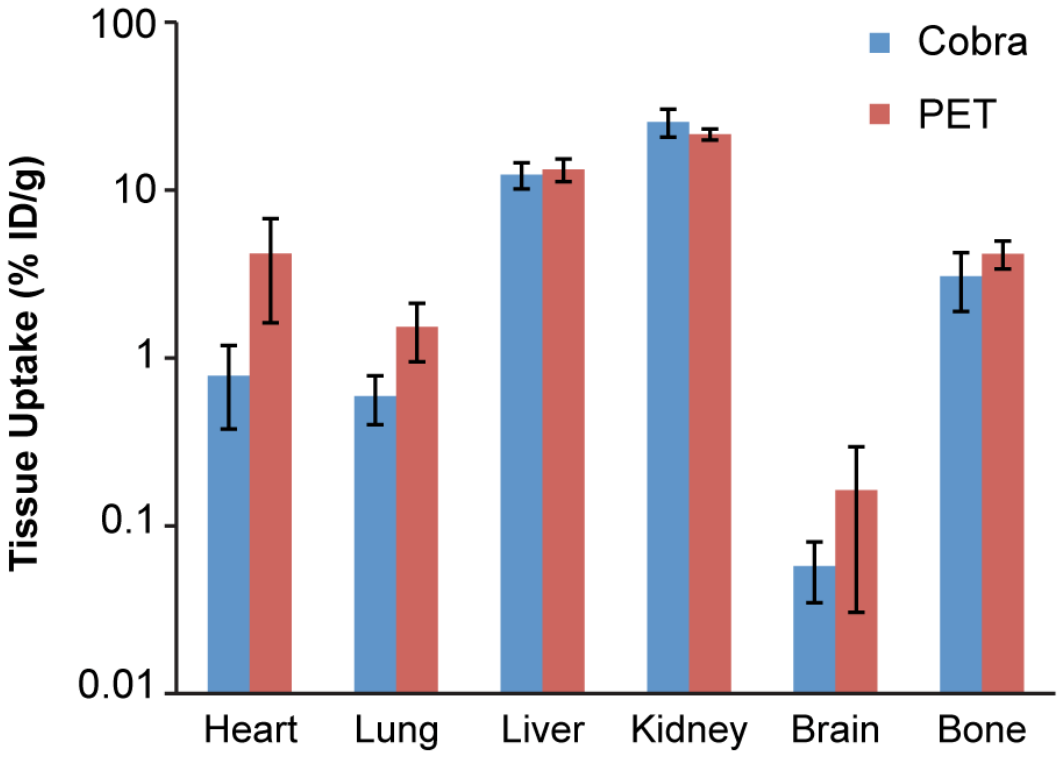
swirling, then loaded onto a prewashed (0.25 M sodium acetate trihydrate with 5 mg/mL gentisic acid, pH 5.4) PD-10 column, in addition to 2 × 0.5 mL reaction tube rinses (0.25 M NaOAc-gentisic acid buffer). The column was next eluted with the acetate-gentisic buffer, and fractions (0.5 mL) were serially collected.

Micro-PET

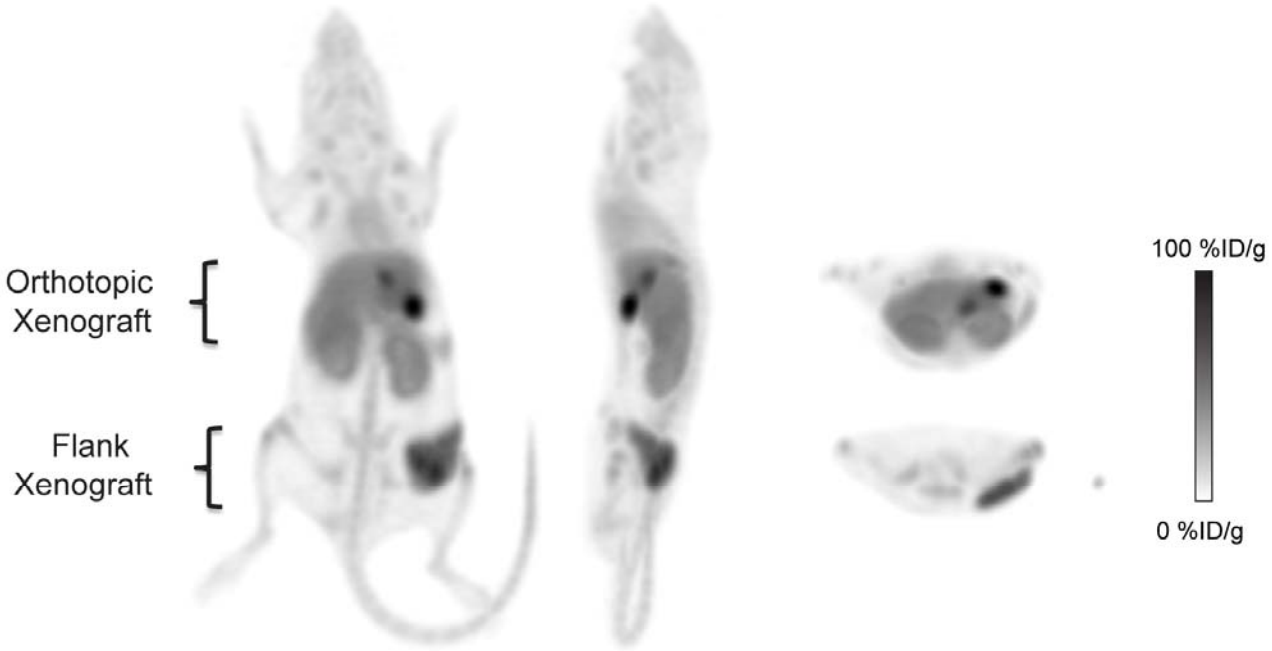
A transmission scan was collected for each mouse as part of each imaging session. The vendor's three-dimensional ordered-subset, maximum a posteriori (3D OSEM-MAP) image reconstruction with attenuation and scatter correction was used to create the mouse images. The image resolution was specified at 1.5 mm full width at half maximum (FHWM) for the 3D OSEM-MAP reconstruction. Data were exported via DICOM, and region of interest (ROI) analysis was conducted off-line using Osirix (open-source software, http://www.osirix-viewer.com/).

Histology

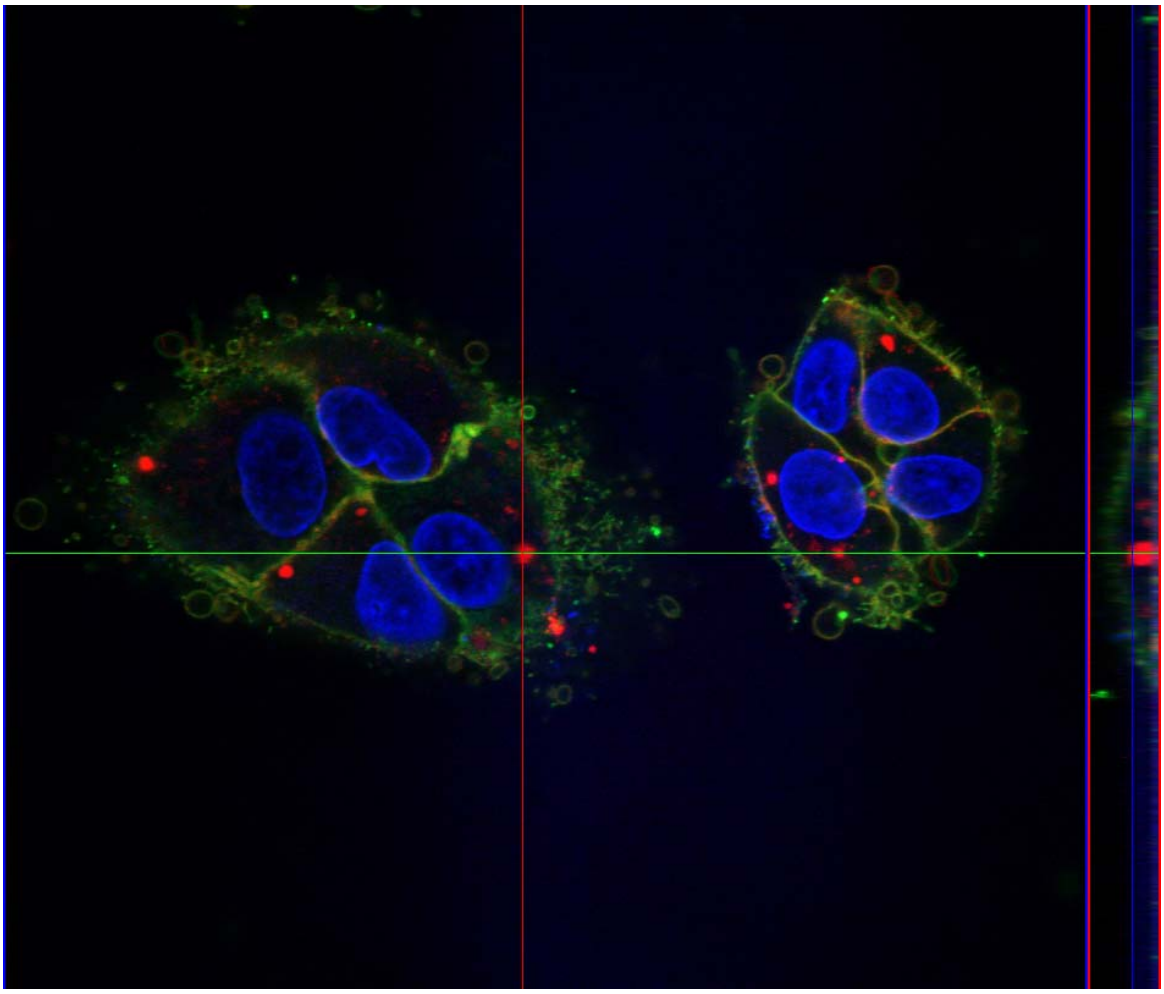
After whole-body perfusion and organ harvesting, selected mouse livers containing tumor were fixed in formalin and allowed to decay 10 half-lives (~33 d) as per University of Washington Environmental Health and Safety Policy. Serial sections were taken every 100 μm using 4-μm-thick sections, subjected to a standard H&E stain, and imaged to determine maximum tumor diameter. Additional slides were pretreated for 18 min in pH 9.0 EDTA buffer, microwaved according to heat-induced epitope retrieval (HIER) techniques, and then treated with a 1:500 dilution of Glypican-3 envision mouse antibody (DAKO) in PBS. These were then subjected to a secondary antibody conjugated to horseradish peroxidase and subsequently imaged. Furthermore, immunofluorescence (IF) immunohistochemistry (IHC) was performed to evaluate the presence of αGPC3 in tumor tissue. Specimens were treated in 10% FBS in PBS with goat antimouse IgG1 conjugated to Cy5.5 (Abcam ab6563) as a secondary antibody for 2 h. Nuclei were counterstained with DAPI for 1 min. Sections were then washed and imaged with a fluorescent microscope using appropriate filters.



Supplemental Figure 1: Comparison of day 7 tissue biodistribution showing statistical equivalence between activity levels obtained from Cobra γ-counter and micro-PET images. All *P* values >0.05.



Supplemental Figure 2: Comparison HepG2 orthotopic and flank xenografts in the same 8-wk-old NU/J mouse. Average activity in the orthotopic xenograft is >3-fold higher than average activity in the much larger flank xenograft.



Supplemental Figure 3: Confocal microscopy of HepG2 cells demonstrating internalization of αGPC3 (red) 24 h after treatment.