Supplemental data

Methods

Cell culture media formulations

A-431 and U-87 MG cells were maintained in Dulbecco's Modified Eagle's Medium. FaDu cells were cultured in Eagle's Minimum Essential Medium, containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Huvec were cultured in EGM-2 medium (Lonza, Verviers, Belgium).

Flow cytometry and western blot

For flow cytometry analysis, cells were harvested using trypsin and washed with PBS. $1x10^6$ cells were incubated with mouse antibodies against human αvβ3 integrin conjugated to FITC (clone LM609, Millipore) in flow cytometry staining buffer (eBiosciences) for 30 minutes on ice. After 2 washes, cells were resuspended in PBS and analyzed using FACSCanto II flow cytometer (BD Biosciences). Cells stained with mouse IgG conjugated to FITC (BD Biosciences) served as a negative control and U-87 MG cells, expressing high amounts of αvβ3 integrin, were used as a positive control.

For western blot, cells were washed twice with PBS and lysed in RIPA lysis buffer, containing EDTA and protease/phosphatase inhibitor cocktail (Thermo Scientific). Lysates were centrifuged 12 minutes at 12000g and protein concentration was determined using DC protein assay (BIO-RAD). 40 ug of proteins were separated using 8% SDS-PAGE (Anamed) and transferred to Immubilon

FL membrane (Millipore). After blocking in 5% nonfat dry milk dissolved in PBS containing 0.1% Tween 20, membranes were incubated with rabbit anti-β3 integrin subunit antibodies (Cell Signaling) and mouse anti-β actin antibodies (Sigma), followed by the incubation with the secondary antibodies: donkey anti-rabbit-IRDye 680 and donkey anti-mouse IRDye 800 (Li-COR Biosciences). To visualize the positive bands membranes were scanned using the Odyssey infrared imaging system (Li-COR Biosciences).

Quality control for ^{68}Ga-DOTA labeling

Briefly 2 µl of the product were spotted onto TLC (Whatman) and ^{68}Ga-DOTA was separated from unincorporated ^{68}Ga in the mixture of 10% Ammonium acetate and methanol (1:1). The TLC plate was exposed to MS phosphor screen (Perkin Elmer) and scanned with the Cyclone Plus Phosphoimager (Perkin Elmer). The TLC was cut one centimeter above the start and radioactivity on the bottom (^{68}Ga) and top (^{68}Ga-DOTA) parts was measured using a γ-counter (Cobra 5003; Packard Instruments).

Quantification of radiotracer uptake

PET sinograms were reconstructed after Fourier rebinning using a 2D ordered-subset expectation maximization (OSEM) algorithm provided by the manufacturer. Image counts per pixel per second were calibrated to activity concentrations (Bq/mL) by measuring a 3.5 cm cylinder phantom filled with a known concentration of radioactivity (*1*). No measured attenuation correction was performed. The PET and CT images of the animal studies were fused using the Rover software (ABX, Radeberg, Germany) and CoRegistration 1.0.1 software (CT Imaging GmbH,

Erlangen, Germany). For quantification of tumor radioactivity uptake in the static PET scans small ellipsoid volumes of interest (VOIs, 7 mm^3) were drawn on the fused PET/CT studies using AMIDE Medical Image Data Examiner software (*2*). These VOIs were placed on the region of the tumor with the highest radioactivity concentration on the PET images. The mean standardized uptake value (SUV) within the VOIs was then determined. For this calculation the decay corrected mean activity concentration in the VOI was divided by the injected activity and multiplied with the body weight of the mouse. For the dynamic PET studies with ^{68}Ga-DOTA the following frames were reconstructed from the list mode data set: 30 x 10 s, 10 x 30 s, and 10 x 60 s. In order to assess the radiotracer input function a VOI was placed in mediastinal blood pool using the CT images to avoid the inclusion of lung tissue. For the tumors a VOI was placed in the region with the highest uptake on summed images from 0-20 min p.i. The VOIs were then applied to all the frames of the dynamic study and the mean SUV within the blood pool and the tumor VOI determined for each frame as described above for the static PET scans. The area under a time activity curves was calculated by trapezoidal numerical integration.

1 Chow PL, Rannou FR, Chatziioannou AF. Attenuation correction for small animal PET tomographs. *Phys Med Biol.* 2005;50:1837-1850.

2 Loening AM, Gambhir SS. AMIDE: a free software tool for multimodality medical image analysis. *Mol Imaging.* 2003;2:131-137.

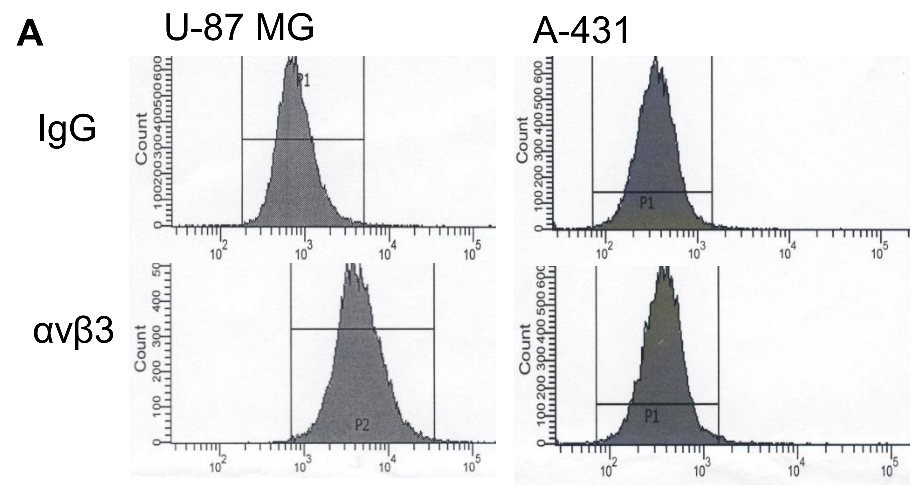
Cell binding studies

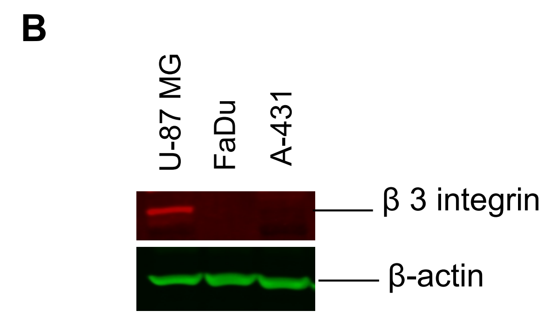
A-431, FaDu and U-87 MG cells were seeded at densities of 700,000 per well in 6-well plates coated with poly-D-Lysine (Sigma), Huvec's were plated at a density of 500,000 per well on gelatin-coated 6-well plates. Next day the growth media was replaced with the media containing 1% FBS for A-431 and U-87 MG cells and 2% FBS for the Huvecs and 100 µl of ^{68}Ga-NODAGA-c(RGDfK) in PBS was added to three wells to yield peptide concentration of 100 nM (100 nM concentration of ^{68}Ga-NODAGA-c(RGDfK) was used, because it is similar to the maximum concentration of the tracer in the blood after injection). To define the nonspecific binding additional three wells received 100 nM of labelled peptide and 20 µM of the cold c(RGDfK) (Bachem). Cells were incubated for 45 minutes at 37°C/5% CO_2 and placed on ice. The media was removed and cells were washed twice with ice-cold PBS (2x1 ml); total bound peptide was collected with 1 N NaOH (3x1 ml). The cell-associated radioactivity along with the standards in triplicate, containing the total activity added to the cells, were measured using a γ-counter (Cobra 5003; Packard Instruments) and the amount of total specifically bound peptide was expressed in counts per minute (cpm) per one million of cells. The experiment was repeated twice in triplicates.

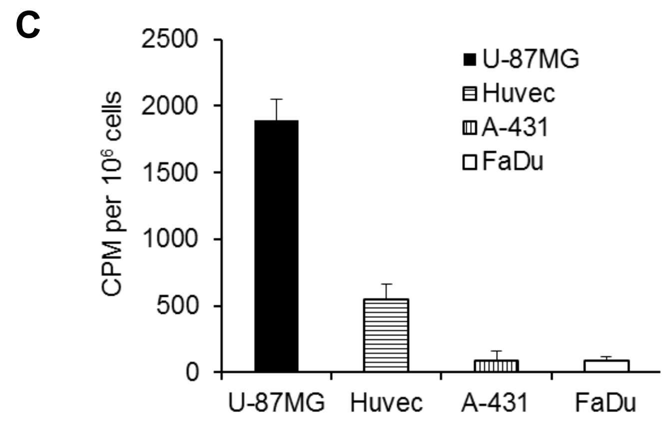
SUPPLEMENTAL TABLE 1. Biodistribution of ^{68}Ga-NODAGA-c(RGDfK) in mice bearing A-431 xenografts at day 7 after start of the treatment[#].

	Control, 1H (n=4)	Bevacizumab, 1h (n=5)	Bevacizumab, blocking, 1h (n=3)
Blood	0.21±0.05	0.16±0.04	0.11±0.01
Heart	0.45±0.06	0.39±0.08	0.05±0.01
Lung	1.08±0.10	0.99±0.09	0.22±0.04
Liver	2.14±0.52	1.91±0.48	0.20±0.03
Spleen	1.67±0.34	1.41±0.82	0.16±0.08
Kidney	3.10±0.67	2.70±0.47	1.46±0.11
Muscle	0.58±0.64	0.44±0.42	0.03±0.00
Bone	0.66±0.39	0.43±0.30	0.09±0.08
Tumor	**1.64±0.24**	**2.60±0.11***	**0.17±0.02**

[#] The results are presented as %IA/g±SD; *p<0.005 for treated versus control tumors.







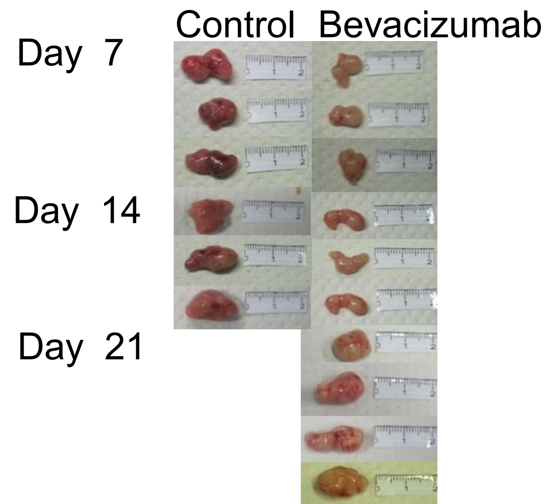
SUPPLEMENTAL FIGURE 1. A-431 and FaDu cells do not express αvβ3 integrin.

A. Analysis of αvβ3 protein expression in A-431 cells using flow cytometry. The top panel shows samples stained with IgG-FITC (negative control), lower panel shows samples stained with LM609-FITC antibodies.The ration of the mean fluorescent signal in samples stained with LM609 antibodies to that of samples stained with IgG was 1.04 for A-431 cells and 6.07 for U-87 MG cells.

B. Western blot analysis of β3 integrin protein expression in A-431 and FaDu tumor cells (upper panel). β-actin served as loading control (lower panel). β3 integrin positive band was only detected in U-87 MG cells, used as positive control. A-431 and FaDu cells are both negative for β3 integrin protein expression.

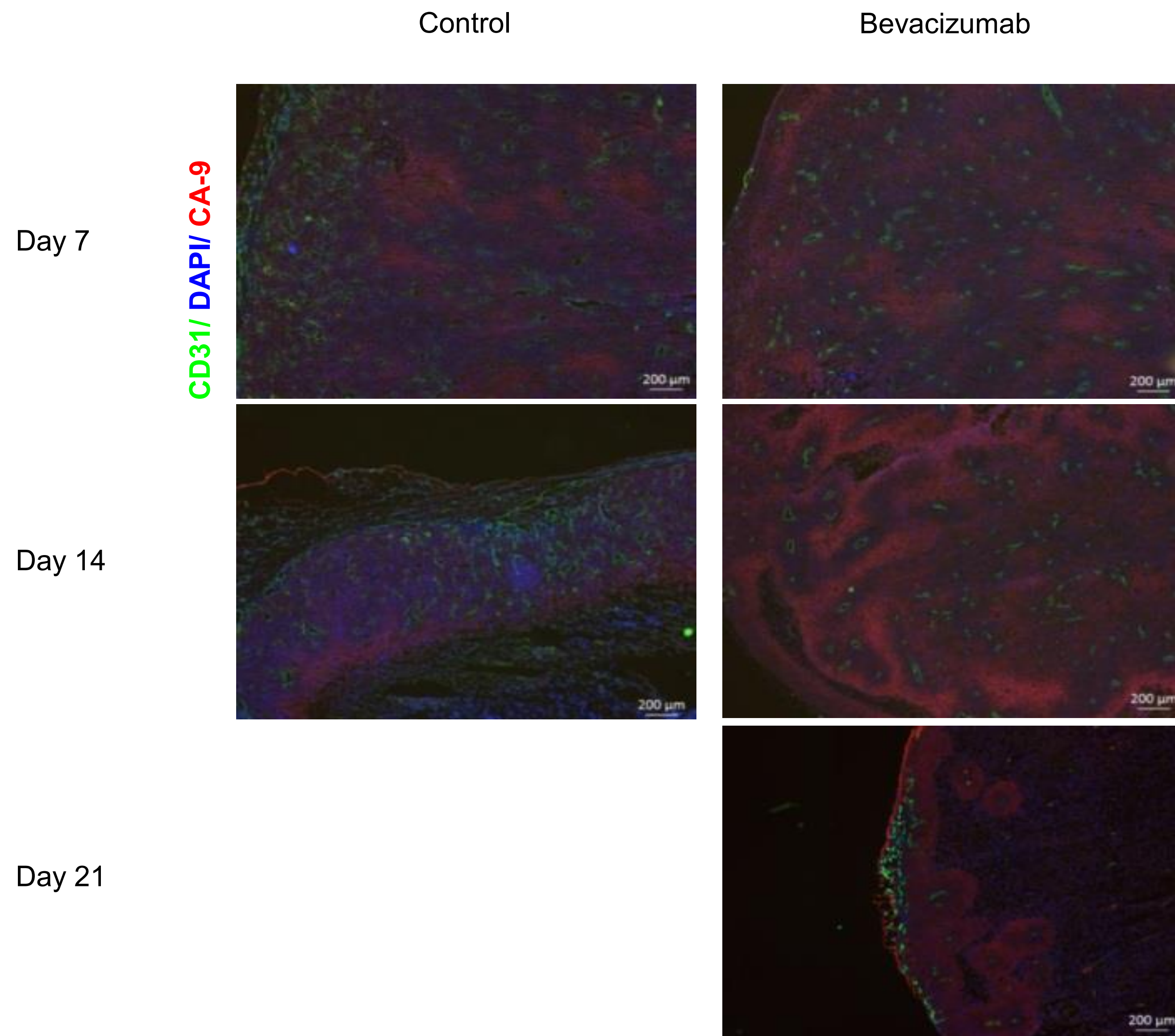
C. ^{68}Ga-NODAGA-c(RGDfK) binds to Huvec cells *in vitro.*

Binding of 100 nM ^{68}Ga-NODAGA-c(RGDfK) to A-431, FaDu and Huvec cells *was* assayed as described in the supplemental methods and expressed in CPM per 1 million of cells after substraction of unspecific binding (i.e. binding in the presence of 20 uM unlabeled c(RGDfK)). Values are shown as Mean±SD. U-87 MG cells were used as positive control.



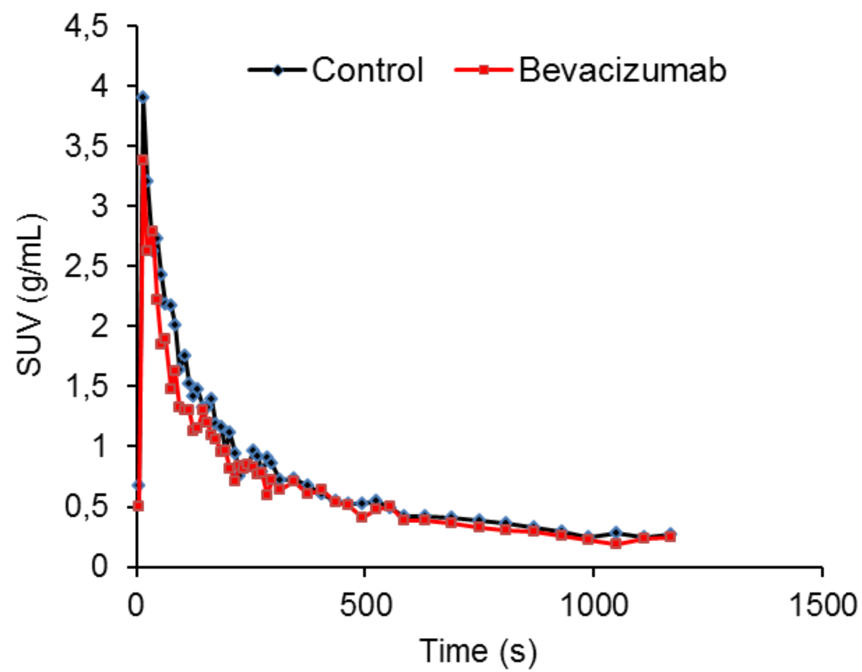
SUPPLEMENTAL FIGURE 2. Bevacizumab inhibits A-431 tumor growth.

Images of representative A-431 tumors from control and bevacizumab treated group at days 7, 14 and 21.

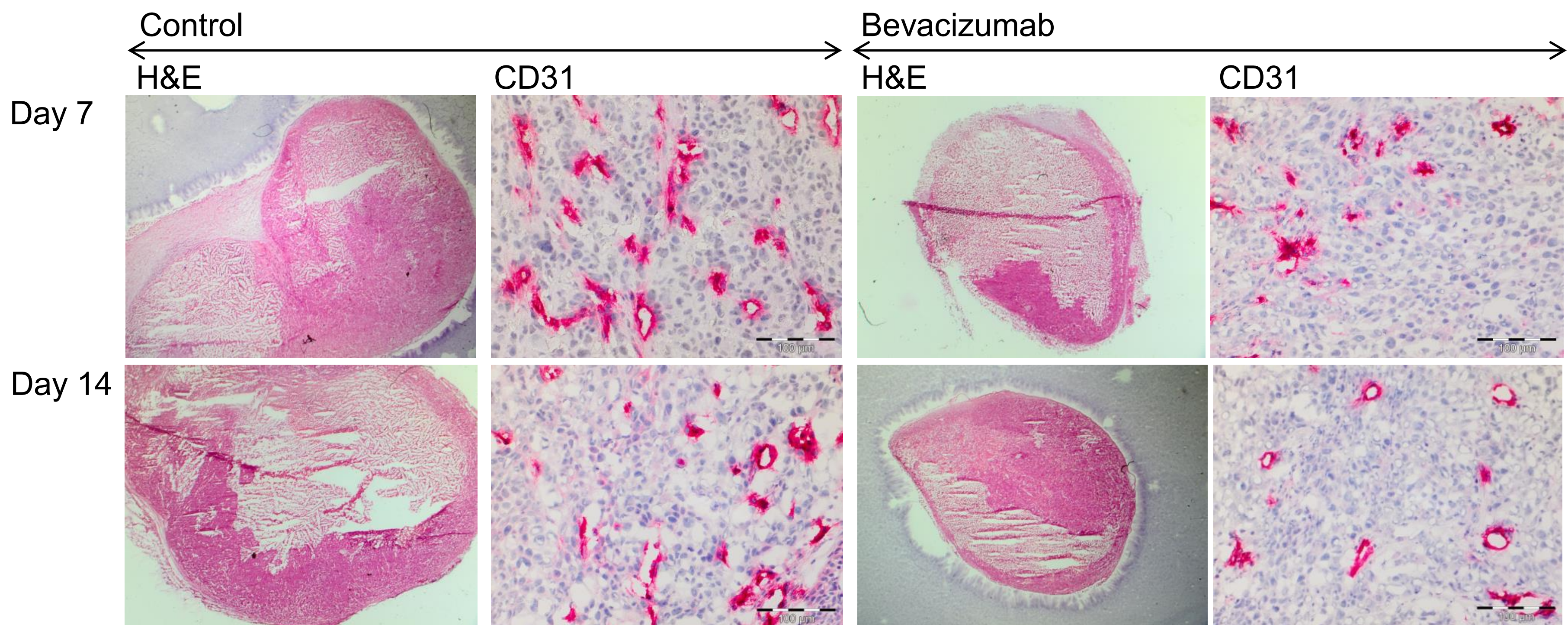


SUPPLEMENTAL FIGURE 3. Assessment of hypoxia in A-431 tumors during the course of bevacizumab treatment.

Representative fluorescence overlay images [blood vessels (green), CA-9 (red) , and nuclei (blue)] of sections from control and bevacizumab treated A-431 tumors at days 7, 14, and 21 (50x magnification).

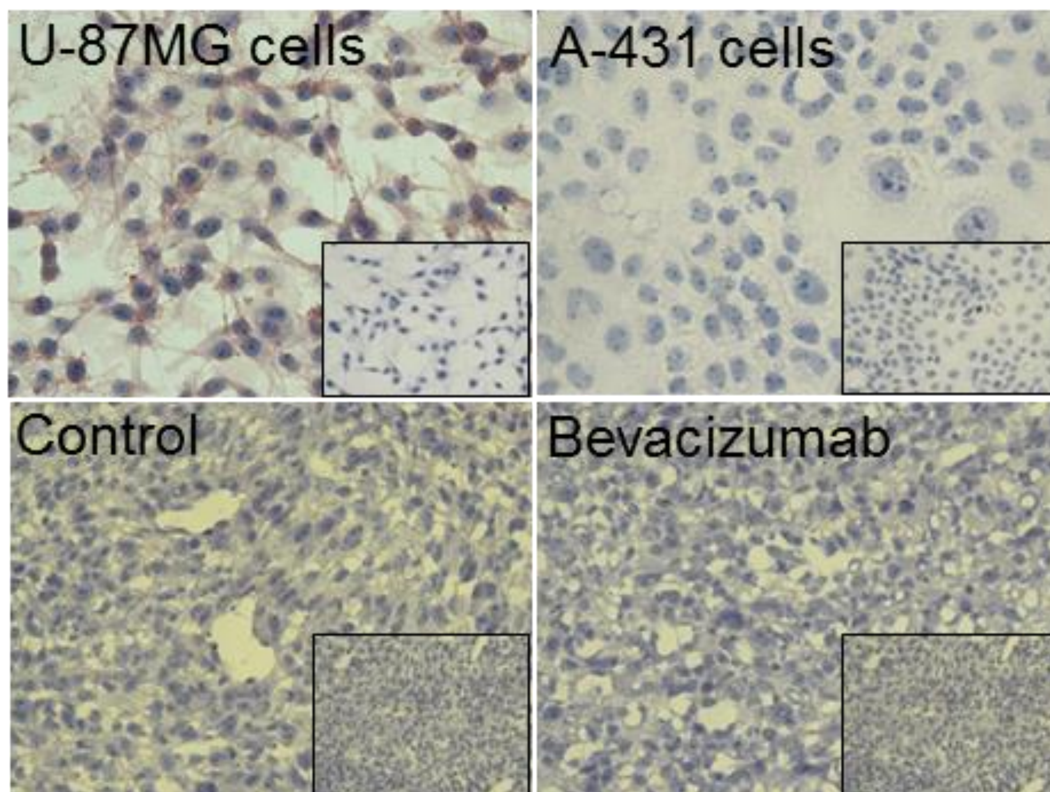


SUPPLEMENTAL FIGURE 4. Representative blood time-activity curves (0 to 20 minutes) for ^{68}Ga-DOTA in control and Bevacizumab-treated animals on day 7 after start of treatment.

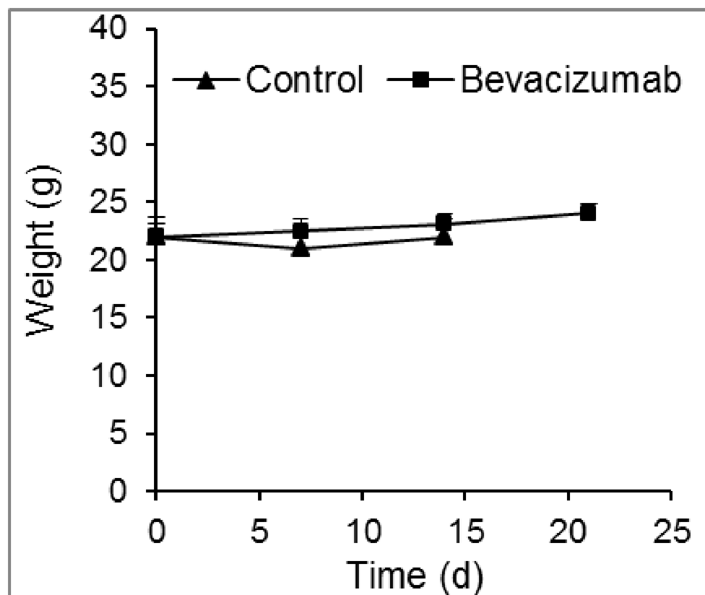


SUPPLEMENTAL FIGURE 5.

Representative images of FaDu tumor sections from control and bevacizumab treated animals at days 7 and 14 of treatment, stained with H&E and CD31 antibodies, specifically binding to blood vessels. The quantification of microvascular density, vessel diameter and necrosis index is presented in Table 2.



SUPPLEMENTAL FIGURE 6. Immunohistochemistry for human αvβ3 integrin in U-87 MG, A-431 cells (upper panel, 400x magnification) and tumor sections from control and bevacizumab treated A-431 tumor (lower panel, 200x magnification). Small inserts show negative controls. U-87 MG cells served as a positive control.



SUPPLEMENTAL FIGURE 7.

Body weights of the animals from control and bevacizumab treated groups. Values are shown as Mean±SD.