**Tumor cell-selective *in vivo* imaging of syngeneic, spontaneous and xenograft tumors using a novel Hsp70 peptide-based probe**

Stefan Stangl, Julia Varga, Bianca Freysoldt, Marija Trajkovic-Arsic, Jens T. Siveke, Florian R. Greten, Vasilis Ntziachristos, and Gabriele Multhoff

**Supplementary Methods**

**Biodistribution measurements.** After the mice had been sacrificed, the contents of TPP[Cy5.5] in the tumors and organs (spleen, pancreas, liver, lung, duodenum, kidney, heart and blood) were assessed. For this, standardized 0.25 cm3 cubes of the tissues were cut using a 0.25 x 0.25cm x 0.25 cm form cutter with an open end and a scalpel to cut at this defined height. Biodistribution measurements were performed at time points of 12, 24 and 48 h of circulation in the body. Fluorescence intensities of the tissues were measured as described in the imaging procedures chapter. Quantification of the Cy5.5 contents in the defined tissue volumes was calculated using fluorescence signal intensities of standards with defined concentrations. To generate these standards, 5, 2.5, 1.25 and 0.625 µg/ml TPP[Cy5.5] were dissolved in 1% agarose gel cubes, supplemented with 10% milk powder to mimic the optical properties of body tissue, and cut in the manner described above. Fluorescence signal intensity was measured using the same instrument settings than for the tissue specimen (Supplementary Fig. S6, upper panel). A control-cube containing 10% milk-powder in 1% agarose did not emit any fluorescence in the Cy5.5 channel. Linear regression equation of the standards was calculated with Microsoft Excel 2007 (Supplementary Fig. S6, lower panel). The concentration of TPP[Cy5.5] in organs was calculated using the regression equation.

**Imaging procedures.** In order to acquire fluorescent images during selective illumination of the specimen with white light or diode lasers with wavelengths of 670 nm or 750 nm a back illuminated EM-CCD camera was used. For visual survey of the tumor borders and a superior ability to align the fluorescence signal with the tumor tissue, as well as to enhance the precision of fluorescence signal quantification, we performed intraoperative *in vivo* imaging experiments on exposed tumors. Light from the tissue was collected using a fixed focal length objective lens optimized for infrared transmission (Planar 1.4/50 ZF-IR, Carl Zeiss, Oberkochen, Germany). The light collected by the objective was filtered using a 780/10 nm band pass filter for Cy5.5 dye and a 800 nm long pass filter for DyLight750 or IntegriSense 750. 670 nm and 750 nm CW diode lasers (B&W Tek, Newark, DE, USA) at 300 mW were selectively used for the excitation of Cy5.5 and DyLight750 or IntegriSense 750, respectively. The laser light beam was guided through a multimode fiber (200 µm core/ 0.22 NA) to a collimator and a diffuser (F260SMA-b, ED1-S20, Thorlabs, Newton, NJ, USA) for beam expansion and uniform illumination. For color images, light was filtered by red, green and blue dichroic color filters. A 250 W halogen lamp (KL-2500 LCD, Edmund Optics) was used for white light illumination. To measure fluorescence of the Cy5.5 and DyLight750 / IntegriSense-750 signals, exposure times of 0.2, 0.5, 1 and 2 s were applied using the 780/10 nm band pass filter / 670 nm laser combination or the 800 nm LP filter/750 nm laser combination, respectively. In all experiments, the specimen was excited with laser sources adjusted to a current of 0.7 mA.

The selection of the regions for tumor and background was done by two independent researchers in a blinded way. Within the field of view (1.2 x 1.2 cm2) the complete tumor and normal tissue areas were gated macroscopically as separate regions of interest (ROI) in the true color image mode. ROI were then transferred one-to-one to the fluorescence images. Mean fluorescence intensities of the respective ROIs using unprocessed images were determined by using image J 1.48v software (NIH, USA) to calculate the tumor-to background ratios. A verification of the different tissue types (tumor and surrounding normal tissue) was done by a histological inspection of representative sections of both ROIs by a pathologist.

A single injection of the control peptide CP or TPP coupled to either DL750 (CP[DL750] or Cy5.5 (TPP[Cy5.5] did not result in different tumor-to-background ratios when compared to mice that were co-injected with both peptides CP[DL750] and TPP[Cy5.5] simultaneously. A comparison of the unspecific excitation of both coupled peptides (CP[DL750] and TPP[Cy5.5] showed no significant differences in primary lesions and lung metastases of mice as indicated in Figure S2A-B.

**Supplementary Figure legends**

**Figure S1**

**Specificity of TPP to Hsp70.** The binding specificity of TPP peptide to Hsp70 was tested in a peptide ELISA. Hsp70 protein was coupled to 96-well ELISA plates and incubated with carboxyfluorescein labeled TPP and CP peptides at indicated concentrations (25, 50, 100 ng/ml). After an incubation period at 27°C for 30 min and two washing steps the fluorescence of carboxyfluorescein-bound peptides was measured in a multiplate ELISA reader. The data show one representative experiment out of three with similar results.

**Figure S2**

**Discrimination of TPP[Cy.5.5] and CP[DL750] fluorescence using the NIRF imaging system.** Spectral unmixing of CP[DL750] and TPP[Cy5.5] was tested with two 1.5 ml centrifugation tubes, filled with 0.1 µg/ml of CP[DL750] or TPP[Cy5.5], respectively (Fig. S2A, upper panel). Left, color image of both tubes, middle, fluorescence signal of TPP[Cy5.5] and [CP[DL750] (right) after spectral unmixing. Quantification of unspecifically excited dye fluorescence intensities resulted in values of 9.51±1.30% for CP[DL750] and 10.17±2.76% for TPP[Cy5.5] (n=2). Figure S2B, *in vivo* confirmation of dye discrimination. 100 µg of CP[DL750] and CP[Cy5.5] were simultaneously injected i.v. into 4T1 tumor-bearing mice. Comparable tumor-to- background ratios of DyLight750 and Cy5.5 fluorescence after 12, 24 and h of circulation in primary lesions (left panel) as well as in lung metastases (right panel) confirm the comparability of the different dyes using the imaging setup and thus provides reliable signal-to-background ratios when compared *in vivo.*

**Figure S3**

**Immunohistochemical staining of tumors using cmHsp70.1 mAb.** Upon immunohistochemical staining,cryosections of the PDAC modelshow cytosolic staining of Hsp70 in tumor cells, whereas fibroblasts of the tumor-microenvironment were negative for Hsp70. Analogous staining have been performed on tumor sections of other models with similar results. These data indicate that expression of Hsp70 only occurs in tumor cells.

**Figure S4**

**Primary tumor growth kinetics and size distribution of 4T1 lung metastases after s.c. CT26 and o.t. 4T1 mammary carcinoma cell implantation.** Primary Tumor volumes of s.c. CT26 tumors, located in the neck area (A) and o.t. 4T1 tumors (B), as determined by sonographic measurement. (C)Identification and size distribution of lung metastases after 25 d of primary tumor growth. Left, H&E stain of mouse lung containing small (S), medium-sized (M) and large (L) metastases (scale bar, 2 mm). Right, numbers of small (S, 0.001-0.05 mm3), medium-sized (M, 0.05-0.5 mm3) and large (L, 0.5-4.3 mm3) metastases of n=3 tumor-bearing mice.

**Figure S5**

**Time kinetics of TBR after intraoperative *in vivo* imaging of tumor-bearing mice after tail vein injection of TPP[Cy5.5] and CP[DL750].** NIRF imaging of CT26 tumors, subcutaneously implanted into the neck area (A), orthotopically implanted 4T1 tumors (B) and lung metastases derived thereof (C). Upper panels, true color image of tumor (t) and surrounding normal tissue including muscle (m), gut (g) and liver (li) (left) with corresponding pseudocolor fluorescence images 24 h after i.v. injection of TPP[Cy5.5] (right). Scale bars, 5 mm. Lower panels, kinetics of TBR after i.v. injection of TPP[Cy5.5] (black circles) and CP[DL750] (gray triangles), simultaneously measured in the corresponding mice. Mean signal intensities of the tumor and normal tumor-surrounding tissue were used to calculate the average tumor-to-background ratio. The TBRs indicated a progressive and tumor-specific accumulation of TPP[Cy5.5] within the first 24 h after injection of the probes in primary tumors of both models (TBR = 5.7±1.5 (CT26) and 5.8±1.5 (4T1)). The TBRs of CP[DL750] control peptide remained significant lower 24 and 48 h after probe injection. Kinetic studies of 4T1 metastases versus normal tissue ratios, calculated from epifluorescence images of dissected lungs, revealed an increase of the specific signal in tumor tissue during the first 48 h after i.v. application with a peaking ratio of 2.6±0.7 at this time point. TBRs of CP[DL750] remained significantly lower at the time-points 12, 24 and 48 h, compared to the specific probe. These data indicate that the optimal time-point for the detection of primary tumors using TPP is 24 h after i.v. application, whereas the detection of lung metastases is optimal after 48 h. The data are shown as mean ± SD (n (12h) = 2 (CT26) and 4 (4T1), n (24h) = 10 (CT26) and 6 (4T1), n (48h) = 3 (CT26) and 4 (4T1), n (72h, 4T1) = 1), \* indicates p<0.05, \*\* indicates p<0.01 and \*\*\* indicates p<0.001.

**Figure S6**

**Regression analysis of tissue-mimicking concentration-standard gel cubes.** To quantify the contents of TPP[Cy5.5] in tumor and organs, fluorescence intensities of standardized 0.25 cm3 cubes, supplemented with 10% milk powder and containing 5, 2.5, 1.25 or 0.625 µg/cm3 TPP[Cy5.5] were measured as described in the “imaging procedures” chapter (upper panel). Regression equation of linear regression analysis (lower panel). The concentration of TPP[Cy5.5] in organs was calculated using this regression equation. Scale bar, 5 mm.

**Figure S7**

**True color images and TPP[Cy5.5]-derived fluorescence signals of tumor-bearing mice.** True color images (left panels) of tumor-bearing mice after subcutaneous or orthotopical implantation of tumors, the fluorescence signals derived thereof, 24 h after i.v. injection of TPP[Cy5.5] (middle panel), as well as the overlay of color and fluorescence images (right panel). Figure S7A, dorsal view of a subcutaneous CT26 tumor-bearing mouse with a tumor located in the neck area. Figure S7B, ventral view of an orthotopic 4T1 mamma carcinoma-bearing mouse. Upper and middle panels: Exemplary whole-body overviews of the mice before and during intraoperative measurement (scale bar, 25 mm). Lower panels show tumor areas, captured in higher magnification in order to allow measurement of fluorescence signal intensities for calculation of TBR (scale bar, 5 mm).