Published in final edited form as:

J Pharm Sci. 2015 August ; 104(8): 2600–2610. doi:10.1002/jps.24518.

Vascular targeting of a gold nanoparticle to breast cancer metastasis

Pubudu M. Peiris^{1,2,3}, Partha Deb^{1,2,3}, Elizabeth Doolittle^{1,2,3}, Gilad Doron^{1,3}, Amy Goldberg^{1,3}, Priya Govender^{1,3}, Shruti Shah^{1,3}, Swetha Rao^{1,3}, Sarah Carbone^{1,3}, Thomas Cotey^{1,3}, Meilyn Sylvestre^{1,3}, Sohaj Singh^{1,3}, William P. Schiemann⁴, Zhenghong Lee^{2,3,4}, and Efstathios Karathanasis^{1,2,3,4,*}

¹Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio

²Department of Radiology, Case Western Reserve University, Cleveland, Ohio

³Case Center for Imaging Research, Case Western Reserve University, Cleveland, Ohio

⁴Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio

Abstract

The vast majority of breast cancer deaths are due to metastatic disease. While deep tissue targeting of nanoparticles is suitable for some primary tumors, vascular targeting may be a more attractive strategy for micrometastasis. This study combined a vascular targeting strategy with the enhanced targeting capabilities of a nanoparticle to evaluate the ability of a gold nanoparticle to specifically target the early spread of metastatic disease. As a ligand for the vascular targeting strategy, we utilized a peptide targeting $\alpha(v)\beta(3)$ integrin, which is functionally linked to the development of micrometastases at a distal site. By employing a straightforward radiolabeling method to incorporate Technetium-99m into the gold nanoparticles, we used the high sensitivity of radionuclide imaging to monitor the longitudinal accumulation of the nanoparticles in metastatic sites. Animal and histological studies showed that vascular targeting of the nanoparticle facilitated highly accurate targeting of micrometastasis in the 4T1 mouse model of breast cancer metastasis using radionuclide imaging and a low dose of the nanoparticle. Due to the efficient targeting scheme, 14% of the injected AuNP deposited at metastatic sites in the lungs within 60 min after injection, indicating that the vascular bed of metastasis is a viable target site for nanoparticles.

Keywords

gold nanoparticle; vascular targeting; breast cancer micrometastasis; nuclear imaging

Introduction

Nanoparticles provide many potential benefits and new opportunities to address the complexity of micrometastasis. To date, though, applications of nanotechnology have mainly focused on primary tumors. While conventional agents with small molecular weight

* Author to whom correspondence should be addressed: Efstathios Karathanasis, 2071 Martin Luther King Jr. Drive, Wickenden Building, Cleveland, Ohio 44106, USA, Phone: +1-216-844-5281, Fax: +1-216-844-4987, stathis@case.edu.

are distributed within cancer and healthy tissues in a non-specific manner, nanoparticles have been developed to exploit the leaky vasculature of primary tumors to enhance the intratumoral delivery due to the so-called 'Enhanced Permeability and Retention' (EPR) effect¹. However, the early spread of cancer cells at metastatic sites lacks leaky vasculature for targeting by passive mechanisms. While the EPR strategy may be effective in well-vascularized tumors of several millimeters in diameter, it is ineffective against micrometastasis, which presents small clusters of malignant cells within variable tissue types². Micrometastasis is defined as cohesive deposits of tumor cells of 2 mm or less but larger than 0.2 mm. Thus, to design a nanoparticle with high sensitivity and specificity, targeting schemes must be devised according to the unique microenvironment of the target site³.

Targeting occult micrometastases hidden within a large population of normal cells presents a unique challenge. Micrometastases are nearly inaccessible to molecular or nanoscale agents due to several biobarriers, including tiny size and high dispersion to organs compared to primary or metastatic tumors, making them nearly inaccessible. However, micrometastatic disease upregulates specific cell-surface molecules that differ from the rest of the host organ. It is well known that extravascular metastases are preceded by metastatic cancer cells residing inside the lumen of blood vessels^{4,5}. Circulating tumor cells use the adhesion molecules of the leukocyte adhesion cascade to attach to the endothelium at the site of future metastasis. After the initial colonization, intravascular micrometastases continue to proliferate by producing their own microenvironment⁴⁻⁷. Specifically, integrins are functionally linked to the early development of metastatic disease at a distal site^{4,8}. Following the initial adhesion onto endothelium⁹⁻¹³, the micrometastatic site transitions from P-selectin-dependent cell rolling on the endothelium to firm attachment that is mediated by $\alpha_v\beta_3$ integrin on both cancer and endothelial cells. Overall, tumor cell $\alpha_v\beta_3$ integrin promotes adhesion, migration and invasiveness in cooperation with platelets^{14,15}. While $\alpha_v\beta_3$ integrin mediates the adhesion of cells to a large number of extracellular matrix proteins, it is minimally expressed on normal resting blood vessels¹⁶⁻¹⁸. Thus, $\alpha_v\beta_3$ integrin-mediated vascular targeting can be highly specific towards blood vessels associated with micrometastases^{19,20}.

To circumvent the limitations of today's agents in targeting micrometastatic disease, we combined a vascular targeting strategy with the enhanced targeting capabilities of a gold nanoparticle (AuNP) as illustrated in Fig. 1. First, vascular targeting of micrometastasis may be more effective than deep tissue targeting, which requires the EPR effect as a prerequisite. Most notably, the size of nanoparticles makes them ideal for vascular targeting due to their significantly increased targeting avidity (*i.e.*, latching on vascular target) resulting in geometrically enhanced multivalent attachment on the vascular bed¹⁹. This work shows that AuNP can effectively gain access to and be deposited at micrometastatic sites. The target site of the selected vascular targeting strategy involved $\alpha_v\beta_3$ integrin on metastatic foci resident in blood vessels as well as $\alpha_v\beta_3$ integrin on the remodeled endothelium of micrometastasis (inset in Fig. 1). Second, we employed an efficient radiolabeling method to incorporate Technetium-99m into the AuNP nanoparticles for single-photon emission computed tomography (SPECT). Since accurate detection requires sufficient signal

difference between the lumen of the blood vessels and the blood vessel walls (*i.e.*, target site), imaging must be delayed for days after injection of a long-circulating nanoparticle to allow the agent to clear from the bloodstream^{19,21}. To decrease ‘interfering signals’, we identified a low dose of the agent that generated significantly detectable signal on the tumor-associated vascular bed and insignificant background “noise” due to the blood circulation of the AuNP radiotracer or its non-specific uptake in various organs (*e.g.*, liver, spleen). In this work, the combination of these complementary features facilitated accurate targeting of micrometastasis in the 4T1 mouse model of breast cancer metastasis using a low dose of the nanoparticles.

Experimental Section

Materials

The 4T1-GFP-luc cell line was received as a gift from Dr. Ruth Keri (Case Western Reserve University, Cleveland, OH). Female Balb/c mice were purchased from Charles River (Wilmington, MA). The primary antibody for the specific endothelial antigen CD31 was purchased from BD Biosciences Pharmingen (San Diego, CA). Secondary antibodies and cell culture media were obtained from Invitrogen (Carlsbad, CA). The cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) peptide was purchased from Peptides International Inc (Louisville, KY). The bifunctional chelator 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (*p*-SCN-Bn-DTPA) was obtained from Macrocyclics (Dallas, TX). The crosslinker sulfo-succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was obtained from Thermo Fisher Scientific (Cleveland, OH). Polyethylene glycol (PEG) conjugates were purchased from Laysan Bio (Arab, AL). General solvents and chemicals were obtained from Thermo Fisher Scientific (Cleveland, OH).

Synthesis and characterization of gold nanoparticles

Gold nanoparticles (AuNP) were synthesized using the Brust-Schiffrin method with some modification²². Briefly, HAuCl₄ solution (367 μ L) and tetraoctylammonium bromide (TOAB, 0.1367g) were dissolved in toluene at room temperature. Dodecylamine (0.112 g) was added and stirred at room temperature for 10min. A reducing agent, NaBH₄ solution in ice-cold water was added slowly in to the reaction mixture. Following the addition of NaBH₄, the solution turned from orange to wine red solution. The resulting AuNP were precipitated with ethanol, centrifuged at 6,000 rpm for 15 min, and dried under nitrogen for 30 min. Upon dissolving the nanoparticles in chloroform, SH-PEG-NH₂ was added in large excess and allowed to react for 2 days at room temperature. Chloroform was removed by air-drying. The AuNP nanoparticles were suspended in water and the excess PEG was removed by a two-day dialysis against water.

The AuNP nanoparticles were characterized in terms of their size and structure using dynamic light scattering (DLS, Brookhaven Instruments) and transmission electron microscopy (TEM, Tecnai F30, FEI). The concentration of gold was determined *via* inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7000 DV;

Perkin-Elmer) and UV spectroscopy based on AuNP's absorption at 520 nm (Synergy HT; BioTek Instruments).

Functionalization of nanoparticles

The amines on the distal end of the PEG on the nanoparticle's surface were used to functionalize AuNP with the c(RGDfC) peptide and the DTPA chelating agent. As shown in Fig. 2, step 1 consisted of the conjugation of about 50 DTPA molecules per AuNP particle. Briefly, the bifunctional chelator, *p*-SCN-Bn-DTPA agent (25 µg), was conjugated to a fraction of the surface amines on AuNP (80 pmol) in saline solution for 6 h. Excess DTPA was removed by a one-day dialysis against saline solution. In step 2, the thiol of the cysteine residue on the c(RGDfC) peptide (15 µg) was used to conjugate the peptide to the remaining amines on the surface of nanoparticles *via* the sulfo-SMCC cross-linker for 2 h.

To determine the number of available amine groups on the surface of the AuNP nanoparticles, we used a fluorescence-based method by reacting the amines on the nanoparticle's surface with a fluorophore reactive towards amines (Alexa Fluor 488 NHS ester; Invitrogen)²³. The assay was employed before and after steps 1 and 2. The amount of fluorophore on the surface of AuNP was analyzed by a fluorescence plate reader (Synergy HT; BioTek Instruments).

Radiolabeling of nanoparticles with ^{99m}Tc

We developed a shake-and-bake radiolabeling method to stably incorporate ^{99m}Tc into pre-formed targeted AuNP. For radiolabeling, the AuNP nanoparticles contained a 10-fold excess of the chelating agent DTPA over the added ^{99m}Tc radionuclide. Briefly, the reducing agent, SnCl₂ (0.3 mg in 0.2 M HCl, purged with nitrogen), was added to 0.5 ml of the AuNP suspension (also purged with nitrogen), while the pH of the solution was adjusted close to 7 using a 1M NaOH solution. Sodium pertechnetate (3 mCi in 1 mL of saline) was added to the mixture in a shielded vial. The reaction vial was purged with nitrogen, shaken, and stirred for 30 min at 80 °C. Radiochemical purity was examined by thin-layer chromatography (radio-TLC). TLC was performed on silica gel coated fiber sheets with 85% aqueous ethanol as the mobile phase. The sheets were dried and cut into 1-cm segments and radioactivity was measured.

Tumor animal model

We used the 4T1-luc-GFP cell line, which stably expressed firefly luciferase (luc) and green fluorescent protein (GFP) to allow noninvasive tracking and quantification of the cells *in vivo* and in histology^{19,20}. The 4T1 mammary carcinoma is a transplantable tumor cell line that can spontaneously metastasize from the primary tumor in the mammary gland to distant sites including blood, liver, lung, brain²⁴. Briefly, we inoculated 0.5×10^6 4T1 cells orthotopically in a no. 9 mammary fat pad of female BALB/c mice. The primary tumor was surgically removed, so that metastatic disease could be studied in an animal setting comparable to the clinical scenarios where the primary tumor was removed, and metastatic foci remained intact. We have previously established that disseminated metastases are developed within two weeks of 4T1 cell engraftment^{19,20}. Therefore, primary tumors were

resected 14 days after tumor inoculation. The animals were used in the *in vivo* studies 5 days after surgery to allow the animals to recover.

Bioluminescence *in vivo* imaging

Starting 7 days after tumor inoculation, 200 μ L of D-luciferin (10 mg/ml) were intraperitoneally administered and imaged after 10 min using Bioluminescence Imaging (BLI) with a preclinical In Vivo Imaging System (IVIS, Perkin Elmer). The animals were imaged every 3-5 days until the terminal point of the study. At the endpoint, organs were extracted and processed for histological analysis.

Angiography using contrast-enhanced micro-CT imaging

Contrast-enhanced high-resolution angiography was performed using a Siemens Inveon micro-CT system (isotropic 99 μ m resolution, 80 kVp, 500 μ A) and a long-circulating liposomal imaging agent encapsulating a high cargo of an iodinated contrast agent^{21,25}. Following IV injection of the agent at a dose of 2.6 g iodine per kg of body weight (b.w.), the animals were imaged with the micro-CT system. Fiducial markers placed around the animal were visible in both the micro-CT and gamma scintigraphy images, which facilitated seamless co-registration of the two sets of images.

Radionuclide imaging

After tail-vein injection of 50 μ Ci of ^{99m}Tc-AuNP in 200 μ L, whole-body planar gamma scintigraphy images were acquired using a Gamma Medica FLEX small animal-imaging instrument (X-SPECT system) at multiple time points after injection (30 sec acquisition). To verify the findings of the *in vivo* imaging and confirm the presence of metastases in organs, we imaged the lungs *ex vivo* using the Gamma Medica system and a CRi Maestro fluorescence imaging system to detect 4T1 cells expressing GFP (green channel). Furthermore, to quantitatively validate nuclear imaging, the gold concentration in tissues was measured *post mortem*. After digestion of the tissues, the gold concentration in the lungs was measured using ICP-OES. Healthy animals were used as controls to correct for background levels.

Histological evaluation

Immunohistochemistry was performed on a subset of the animals to further validate the homing of AuNP nanoparticles and location of metastatic cancer cells with respect to blood vessels and associated expression of $\alpha_v\beta_3$ integrin. After the last imaging acquisition, tissues were collected from the mice for histological studies. The animals were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS. Organs were explanted and post-fixed overnight in 4% paraformaldehyde in PBS. The tissues were soaked in 30% sucrose (w/v) in PBS at 4 °C for cryosectioning. Serial tissue sections of 12 μ m in thickness were stained for the specific endothelial antigen CD31 and with the nuclear stain DAPI. Direct fluorescence of GFP (green) imaging was performed for imaging the location of metastatic lesions. Silver staining was used to image AuNP in bright field. The tissue sections were imaged at 5, 10 or 40 $\times$ on the Zeiss Axio Observer Z1 motorized FL inverted microscope. To obtain an image

of the entire large tissue section (*i.e.*, lung lobe), a montage of each section was made using the automated tiling function of the microscope.

Statistical analysis

Means were determined for each variable in this study and the resulting values from each experiment were subjected to one-way analysis of variance with post hoc Bonferroni test (SPSS 15, Chicago, IL). Statistical significance was set at the 5% level ($p < 0.05$). Normality of each data set was confirmed using the Anderson-Darling test.

Results

Synthesis of the AuNP radiotracer

Following synthesis of the PEGylated AuNP nanoparticles, the amines on the distal end of the PEG were used to functionalize the nanoparticles with the c(RGDfC) peptide and the DTPA chelating agent. Fig. 2 shows that step 1 utilized the bifunctional chelator, *p*-SCN-Bn-DTPA to functionalize a fraction of the surface amines on AuNP. This isothiocyanate compound reacted efficiently with the primary amine-containing AuNP with no leaving group involved at pH 7 in saline. In step 2, the thiol of the cysteine of the c(RGDfC) peptide was conjugated to the remaining amines on AuNP *via* the sulfo-SMCC cross-linker. Sulfo-SMCC contains an amine-reactive *N*-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group to form stable amide and thioether bonds. The number of amines on the AuNP nanoparticle's surface was evaluated quantitatively using fluorescence-based assay^{19,23}. The parent AuNP was found to have ~400 amine groups (per particle), whereas step 1 consumed 50 of them. We confirmed that step 2 utilized the remaining 350 amines for the conjugation of the c(RGDfC) peptide.

Once the AuNP was functionalized, we developed an efficient and straightforward radiolabeling method to stably incorporate ^{99m}Tc into pre-formed AuNP nanoparticles (*i.e.*, shake-and-bake method). One major issue with ^{99m}Tc-radiolabeling is the formation of radiocolloids, which is an undesirable result of the reduction of pertechnetate ^{99m}TcO₄⁻ (generator eluate) with stannous chloride (SnCl₂). We performed detailed studies to optimize the pH, SnCl₂ concentration, Sn/DTPA ratio, incubation time and temperature to achieve maximal labeling with minimal colloid formation. For example, colloid formation is increased and radiolabeling efficiency is decreased, when the levels of Sn is insufficient to completely reduce the added pertechnetate, or the DTPA or Sn levels are too low or too high, respectively. Considering the excess of DTPA (50 DTPAs per AuNP), we were able to label the nanoparticle with ~5 ^{99m}Tc radionuclides per AuNP (depending on the age of the generator). Using radio-TLC as a quality control method, the radiolabeling yield of the AuNP formulation was 99% with negligible colloid formation. Using an ethanol-water mobile phase, ^{99m}Tc-AuNP remained at the origin of the sheets (retention factor $R_f = 0$), whereas free ^{99m}TcO₄⁻ migrated to the front of the TLC sheet at an R_f value of 0.9-1.0. The amount of free ^{99m}TcO₄⁻ 6 h after radiolabeling was less than 1%. Using pyridine-acetic acid-water (3:5:2) as mobile phase, the absence of a spot at $R_f = 0$ indicated that no radiocolloids were formed. Since the labeling efficiency was more than 99%, no further purification was required. Importantly, the *in vivo* studies with healthy mice showed the

stable radioisotope incorporation into the AuNP nanoparticles for at least 12 h as indicated by undetectable ^{99m}Tc accumulation in the thyroid and kidneys, where free ^{99m}Tc is found, or lungs, where radiocolloids are typically found. DLS measurements showed that the hydrodynamic size of the AuNP nanoparticle before and after radiolabeling was unchanged and equal to about 22 nm, while TEM images showed that the size the Au core was about 5 nm (Fig. 3).

Characterization of the mouse tumor model

For this study, we selected the murine 4T1 breast cancer cells, which represent a model of triple-negative breast cancer (TNBC). The 4T1 model is a highly aggressive and metastatic TNBC tumor. It is a mouse mammary adenocarcinoma model displaying genetic compatibility of the tumor microenvironment with the host tissues in immune-competent animals. Since intact immune system is critical for metastatic development, this is one of the few breast cancer models that metastasis occurs due to infiltration of leukocytes into the primary tumor²⁶. Thus, the 4T1 cells facilitated studies of TNBC development and metastatic disease in immunocompetent mice, which ultimately develop metastases at organ sites similar to those observed in human patients^{24,26}.

To use the animal model at a stage that is more informative and relevant to the human disease, we monitored the progression of metastatic disease using non-invasive, longitudinal imaging for rodents (BLI imaging) and histological analysis. Fig. 4A provides the timeline and schedule of the different *in vivo* and terminal analyses. In order to more carefully recapitulate clinical breast cancer settings, we surgically resected primary 4T1 tumors, developed in the mammary fat pad of mice, whose micrometastatic foci remained intact. BLI imaging of 4T1-*luc*-GFP cells showed that the primary tumor of untreated animals grew rapidly within 12 days after 4T1 cell engraftment (Fig. 4B). While surgical resection of the primary tumor at day 14 resulted in elimination of BLI signal, early metastatic spread quickly became detectable in the thoracic region.

Radionuclide imaging

To test the detection accuracy of the AuNP radiotracer in identifying metastatic disease, we performed gamma scintigraphy imaging in a group of mice harboring 4T1 metastasis (n=6) and a group of healthy mice (n=6). Fig. 5A shows a representative coronal gamma scintigraphy image of a mouse bearing 4T1 metastasis 30 min after administration of 50 μCi of ^{99m}Tc -AuNP radiotracer, which is a ~ 6 -fold lower dose than typically used for integrin imaging in preclinical studies²⁷⁻³⁰. The amount of gold injected in each animal was 6 μg , which corresponded to a dose of 272 μg gold per kg of body weight.

Since our objective was to detect signals due to efficient targeting of AuNP on metastasis-associated vascular bed, there should be low levels of ‘interfering signal’ from circulating AuNP in the bloodstream. To achieve this, we sought to identify the lowest dose capable of generating significant and detectable signal at the target sites, while at the same time there was insignificant background “noise” due to blood circulating nanoparticles or non-specific uptake of the agent in various organs (*e.g.*, liver, spleen). We have successfully developed similar methods for other imaging modalities^{19,21}. As shown in Fig. 5A, vascular targeting

of the AuNP radiotracer resulted in 2-3 “hot spots” in the lungs of mice with 4T1 metastasis (yellow arrows in Fig. 5B). To provide an anatomical reference, the scintigraphy image was co-registered with a high-resolution angiogram obtained using an iodinated nanoparticle contrast agent and a micro-CT (Fig. 5B)²⁵. Importantly, the findings of *in vivo* imaging were verified by *ex vivo* organ imaging (Fig. 5C) and histological analysis (next section). While injection of the AuNP radiotracer resulted in significant signal from micrometastatic sites, negligible signal was generated in the lungs of healthy mice at such low dose of injection (representative image is shown in Fig. 5D). Not surprisingly, the majority of the AuNP radiotracer was found in the liver due to the known clearance of AuNP nanoparticle by the reticuloendothelial system (RES).

To quantitatively evaluate the ability of the AuNP radiotracer to target metastasis, the time-course of the signal intensity in the lungs was measured using manually drawn regions of interest enclosing the thoracic region. Comparing mice bearing 4T1 metastasis and healthy mice after injection of the radiotracer (n=6 per group), quantification of the lung signal intensity indicated significant accumulation of the AuNP radiotracer in metastatic sites (Fig. 6A). Due to the highly efficient vascular targeting of AuNP to metastasis, the signal reached its maximum plateau within 30 min after injection. At 60 min after injection, 14% of the injected AuNP radiotracer accumulated in the lungs of mice with 4T1 metastasis. In conjunction to the undetectable levels of circulating AuNP in the blood at 60 min after injection, the negligible signal in the thoracic region of healthy animals indicated the absence of non-specific accumulation of the AuNP radiotracer in the lungs. Thus, at 60 min after injection, we were able to precisely identify all 6 mice with metastatic disease (true positives) with a high degree of assurance, while no false positives were recorded. At all time points, we measured less than 0.5% of the injected dose in the kidney, bladder or thyroid regions, indicating the absence of free ^{99m}Tc. Using the signal from the heart of healthy mice as an indication of blood circulation, at the selected low dose of 272 µg Au/ kg b.w., the blood t_{1/2} is ~47 min. Previous studies have reported that non-targeted AuNP of similar composition as our formulation exhibit significantly longer blood circulation (blood t_{1/2} ~20 h)³¹. Thus, while the RGD ligands on the AuNP provided selective targeting of metastasis, there was a significant penalty in the blood circulation of the agent. However, a previous study showed that the blood t_{1/2} of a cRGD-targeted AuNP (with a similar number of ligands per NP; ~400) was only decreased by 25% compared to its non-targeted variant³². In addition to the cRGD ligands, the observed blood circulation in our studies suggests that the DTPA chelating molecule also contributed to the recognition and clearance of the nanoparticle by the RES organs.

Furthermore, we compared the nanoparticle radiotracer to an analogue with small molecular weight. Following radiolabeling of a c(RGDfC)-DTPA with ^{99m}Tc, the small molecule radiotracer was injected in a group of mice with 4T1 metastasis (n=5) at a two-fold higher dose than the ^{99m}Tc-AuNP (*i.e.*, 100 µCi). In both healthy mice and mice with 4T1 metastasis, the ^{99m}Tc-labeled RGD peptide exhibited similar pharmacokinetics with rapid elimination from blood circulation. Using the signal from the heart in scintigraphic images as an indication of blood circulation, the blood t_{1/2} was <10 min with the majority of the agent being found in the bladder and kidneys after 10 min post-injection³³. Sufficiently high

tumor-to-blood ratio was observed after 45 min. Fig. 6B shows that the AuNP radiotracer achieved a 5.2-fold higher signal in lung metastases than the small molecule tracer. Furthermore, the *in vivo* nuclear imaging-based measurements of AuNP concentration were validated by direct measurement of gold concentration in lung tissues of animals using ICP-OES. While small molecule tracers are capable in highlighting relatively large metastatic lesions, the multivalent targeting avidity and large radioisotope cargo of AuNP resulted in highlighting the early spread of metastatic disease.

To confirm the selective targeting of the c(RGDfC)-targeted AuNP to lung metastasis, we compared the targeted AuNP to its non-targeted variant in healthy mice and mice with 4T1 metastasis (n=5 mice per condition). At 72 h post-injection of AuNP nanoparticles at a dose of 12.7 μ g in each animal, the entire lung from each animal was excised, digested, and gold concentration was measured using ICP-OES. Since gold is not a natural element found in animals, a major advantage in using gold is that the accumulated gold in tissues can be quantitatively determined by elemental mass spectrometry. The deposition of non-targeted AuNP was similar in healthy lungs and lungs with metastasis (Fig. 7). Most notably, the levels of AuNP were found to be negligible ($\sim$ 500 ng per g of tissue), which corresponded to $\sim$ 0.16 % of the injected dose. Furthermore, the targeted AuNP exhibited similar deposition in the lungs of healthy animals.

Histological evaluation

We performed detailed histological analysis to evaluate the localization of the AuNP radiotracer with respect to metastatic cancer cells, blood vessels and associated expression of $\alpha_v\beta_3$ integrin. First, we confirmed the presence of metastasis in the lungs of all the animals bearing 4T1 tumors. Images of entire histological sections of the lungs were obtained at a low magnification (5 $\times$) using the automated tiling function of the microscope. A representative image of a lung lobe is shown in Fig. 8A displaying the presence of clusters of metastatic cells dispersed in lung tissues. Imaging at high magnification showed that metastatic cancer cells were localized primarily on the endothelium (Fig. 8B), which were exactly the locations that overexpressed $\alpha_v\beta_3$ integrins (Fig. 8C). As expected, negligible integrin expression was observed on the endothelium of normal blood vessels. Most importantly, the AuNP nanoparticles were almost exclusively distributed around those same blood vessels colonized by 4T1 cells (Fig. 8D).

Discussion

As a case study for this study, triple-negative breast cancer (TNBC) metastasis was selected^{6,7,34}. Among the subtypes of breast cancer³⁵, TNBC is an extremely metastatic and difficult-to-treat subtype with frequent relapse and shorter metastasis-free-survival. Even though TNBC represents 15-25% of invasive breast cancers, their aggressive phenotype results in disproportional mortality among breast cancer patients³⁴. Here, we demonstrated the design of a nanoparticle capable of recognizing the microenvironment of micrometastasis in the 4T1 mouse model, which is a model of TNBC that metastasizes similarly to human disease.

First, the biological mechanisms of metastasis had to be carefully considered to design a metastasis-targeted nanoparticle. During cancer progression, tumor cells show changes in their plasticity, including the epithelial to mesenchymal transition (EMT), which is strongly associated with cell invasion and migration. Numerous factors have been identified to induce EMT including substrate-binding integrins, resulting in a mesenchymal-like cell that is able to move freely by forming focal adhesions. Furthermore, circulating tumor cells use the adhesion molecules of the leukocyte adhesion cascade to attach to the endothelium at the site of future metastasis. In the first steps of micrometastatic development, the initial transient interactions of circulating tumor cells with the endothelium occur in a similar fashion to the recruitment of leukocytes in inflammation. Following the rolling and tethering of circulating tumor cells to the microvascular bed, the micrometastatic site transitions from cell rolling on the endothelium to firm attachment that is mediated by $\alpha_v\beta_3$ integrin⁴. Besides primary and metastatic tumors, the $\alpha_v\beta_3$ integrin is expressed in the extracellular matrix, since this integrin mediates the adhesion of normal cells to a large number of extracellular matrix proteins, including fibronectin, fibrinogen, collagen, and laminin. However, due to its size, the AuNP particle cannot extravasate through intact endothelium into normal tissues. Considering that our strategy depended on vascular targeting of the upregulated $\alpha_v\beta_3$ integrin on the metastasis-associated endothelium, $\alpha_v\beta_3$ integrin is minimally expressed on normal resting blood vessels¹⁶⁻¹⁸. Thus, besides $\alpha_v\beta_3$ integrin in blood vessels during wounds healing, vascular targeting of the AuNP particle provides selective binding to metastatic sites. In this context, the selected c(RGDfC) peptide has been found to provide high specificity and high affinity towards $\alpha_v\beta_3$ integrin³⁶.

Various receptors have been exploited to target nanoparticles to cancer cells (*e.g.*, folate, EGF, HER2, integrin receptors). Even though attention of the field has recently shifted towards metastasis, there are still very few examples of metastases². In particular, even though the importance of integrins to the development of metastasis is well documented^{4,15}, this target has been predominantly used to direct nanoparticles to primary tumors but not metastases. Specifically, AuNP nanoparticles have been extensively studied as possible drug delivery carriers for various anticancer therapies and a wide range of cancers³⁷. For example, AuNP have been used for hyperthermia and tumor ablation³⁸ and the delivery of chemotherapy³⁹, a variety of hydrophobic drugs⁴⁰ and photosensitizers for photodynamic therapies⁴¹. While AuNP were initially investigated to enhance intratumoral delivery due to the so-called 'Enhanced Permeability and Retention' (EPR) effect¹, various targeting schemes and ligands have been exploited to direct nanoparticles to cancerous sites that upregulate specific cell-surface molecules that differ from the rest of normal tissues. However, since deep tissue targeting of cancer cells depends on the EPR effect, inefficient delivery and poor uptake of therapeutic drugs to primary tumors have been observed³². However, this is even more profound in the case of highly aggressive metastatic tumors at the early and transient stages of their development that do not display the leaky vasculature found in many solid tumors at their primary site. Here, we investigated vascular targeting as a more effective strategy to target metastasis than deep tissue targeting. To investigate targeting of AuNP to metastases at their very early stages of development *in vivo*, we considered the various types of nanoparticle labeling for different imaging modalities and their adjustable ability to generate detectable signals. Since metastases present target sites

with small sizes, we selected gamma scintigraphy using a radionuclide due to its quantitative nature and extraordinary contrast sensitivity (down to the picomolar level)⁴². Previous studies have explored radiolabeled AuNP for targeting integrins in animal models of glioblastoma or melanoma^{43,44} or breast cancer in cell culture experiments⁴⁵, but there has been no applications of integrin-targeting AuNP to targeting metastasis.

To differentiate from traditional deep tissue targeting, we designated our strategy as vascular targeting with the target sites including both endothelial cells and cancer cells that colonize the endothelium. Contrary to targeting nanoparticles to primary tumors, the vascular bed of micrometastasis presents a highly suitable and targetable docking site for a nanoparticle. Not surprisingly, our *in vivo* imaging studies and histological analysis showed that vascular targeting of a nanoparticle to micrometastasis provided enhanced selectivity. While there are better performing low-MW integrin-binding probes, such as ¹⁸F-Galacto-RGD⁴⁶, our objective was to evaluate the multivalent avidity of a nanoparticle over a single ligand for vascular targeting. Thus, we directly compared the performance of the RGD-targeted AuNP to the RGD peptide itself.

The vast majority of breast cancer deaths are due to metastatic disease⁴⁷. Considering that systemic therapies are usually used to eradicate distant micrometastases in breast cancer patients, site-specific targeting is essential for both effective drug delivery to metastasis (reduced off-target side effects) and accurate detection of disease (high signal-to-noise ratio). Regarding therapeutic applications, vascular targeting of AuNP to metastases can be used in many therapeutic scenarios including hyperthermia and tumor ablation, chemotherapy, and photodynamic therapies³⁸⁻⁴¹. Here, we combined the AuNP nanoparticle's targeting capabilities with nuclear imaging to achieve accurate diagnosis of hard-to-detect micrometastases. In comparison to nanoparticle therapeutics, consideration of safety is even more important with nanoparticle imaging agents, since these agents could be administered to healthy individuals. While current clinical modalities (*e.g.*, CT, MRI, FDG-PET) can detect large metastatic tumors (>1 cm) with accuracy⁴⁸⁻⁵⁰, today's imaging techniques rarely detect disseminated disease at an early stage (*i.e.*, micrometastasis)⁴⁸⁻⁵¹, which prohibits early and effective interventions. However, by the time metastatic disease becomes clinically evident, long-term patient outcome is not favorable. For example, the 5-year survival rate of breast cancer patients sharply decreases from 98% in cases with localized primary lesions to 23% in cases of distant metastases⁴⁷. The concept of occult disease or "micrometastasis" emphasizes that when cancer patients have distant micrometastatic disease at the time of diagnosis, their risk for later development of overt metastatic disease is very high⁵². In fact, numerous retrospective analyses with long-term follow-up have shown an increased risk of breast cancer-related death in women with micrometastasis⁵³⁻⁵⁷. Thus, it is essential to evaluate the risk of an individual patient of harboring clinically silent micrometastatic disease. It is equally important to be able to select the optimal adjuvant therapy for an individual patient based on accurate diagnostic testing. While it is standard practice to administer systemic therapy to all patients with node-positive disease⁵⁸, it is clear that detection of micrometastatic disease in distant organs can significantly influence the selection of patients, regimen of chemotherapy and, in many cases, may warrant more aggressive therapeutic approaches. Furthermore, radiation therapy

and new therapeutic strategies, such as thermal and radiofrequency ablation, require knowledge of the precise location of the disease. Thus, accurate detection of micrometastasis can become one of the most consistent prognostic factors in therapy decision-making, leading to an overall increase in quality of life and patient outcome.

Considering the various types of nanoparticle for different imaging modalities and their adjustable ability to target a specific site and generate detectable signals, we selected nuclear imaging. The major advantage of radionuclide imaging over other modalities is that they exhibit extraordinary contrast sensitivity (down to the picomolar level)⁴². Contrary to imaging of anatomical abnormalities (*i.e.*, detection of primary tumors), we should emphasize that contrast sensitivity is a critical factor in molecular imaging of cancer biomarkers (*i.e.*, detection of micrometastasis).

For our studies, we selected the short-lived metastable isotope of technetium, ^{99m}Tc, which is one of the most commonly used radionuclide in SPECT and diagnostic nuclear medicine. This can be attributed to its ideal physical properties, such as its manageable half-life ($t_{1/2}$ of 6 h) and low γ -photon emission of a single energy at 140 keV, which are beneficial for both effective imaging and patient safety perspectives. Furthermore, to make a range of targeted radiotracers, a commercially available chelator such as DTPA enables easy and rapid ^{99m}Tc labeling using the so-called “shake and bake” method.

However, the use of a nanoparticle radiotracer presents challenges. Contrary to small molecule radiotracers that are cleared relatively quickly from blood circulation, the prolonged blood residence time of PEGylated nanoparticles generates sufficient ‘interfering’ signal in the lumen of blood vessels to prohibit discrimination of signal from metastasis-associated vascular bed with a high degree of confidence. In pilot imaging studies, where health mice were injected with different doses of the AuNP radiotracer, we identified that a dose of 50 μ Ci of ^{99m}Tc-AuNP radiotracer resulted in elimination of the signal from the blood vessels within 60 min. This dose of ^{99m}Tc-AuNP (50 μ Ci; 1.8 MBq) is a $\sim$ 6-fold lower dose than typically used in preclinical evaluation of integrin-targeting radiotracers²⁷⁻³⁰. Thus, this dose was the threshold that allowed visualization of the radiotracer at the target sites as early as 30 min post-injection, with no interference from the vascular signal. We have successfully developed similar imaging protocols for other imaging modalities (*e.g.*, X-ray imaging)^{19,21,25}.

The amount of injected ^{99m}Tc-AuNP corresponded to a low dose of gold (6 μ g gold per animal; 272 μ g/kg b.w.). Previous studies have evaluated the toxicologic effects of gold nanoparticles *in vivo* at a range of different doses (137.5–2,200 μ g/kg)^{59,60}. Similar to other PEGylated nanoparticles of similar size, PEGylated AuNP are cleared by the RES organs. For example, regarding the hepato-biliary clearance, PEGylated AuNP showed an increase from 1 h to 24 h in gastrointestinal tract and feces (0.42–6.5%)⁶⁰. Most importantly, it was shown that very high doses of gold nanoparticles were required to start observing negative consequences (such as reduced body weight and red blood cells), while the low doses (comparable to the dose of ^{99m}Tc-AuNP) had no effect⁵⁹. Furthermore, gold nanoparticles completed or currently undergo clinical trials in various therapeutic contexts (ClinicalTrials.gov identifiers: NCT00436410, NCT00356980, NCT01270139). However,

since the field of nanomedicine presents its own challenges that differ substantially from conventional pharmaceutical ingredients, concerns surrounding the *in vivo* safety and short and long-term toxicity of AuNP nanoparticles need to be considered and is subject of future studies.

In this study, we performed a preclinical study to demonstrate the potential of an AuNP nanoparticle that combines the advantages of vascular targeting and nanotechnology to effectively target metastatic disease.

Acknowledgments

This work was partially supported by grants from the National Cancer Institute (R01CA177716), the Ohio Cancer Research Associates, and the Prayers from Maria Children's Glioma Foundation (E.K.). E.D. was supported by a fellowship from the NIH Interdisciplinary Biomedical Imaging Training Program (T32EB007509) administered by the Department of Biomedical Engineering, Case Western Reserve University.

References

1. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release*. 2000; 65(1-2):271–284. [PubMed: 10699287]
2. Schroeder A, Heller DA, Winslow MM, Dahlman JE, Pratt GW, Langer R, Jacks T, Anderson DG. Treating metastatic cancer with nanotechnology. *Nat Rev Cancer*. 2012; 12(1):39–50. [PubMed: 22193407]
3. Toy R, Bauer L, Hoimes C, Ghaghada KB, Karathanasis E. Targeted nanotechnology for cancer imaging. *Adv Drug Deliv Rev*. 2014; 76:79–97. [PubMed: 25116445]
4. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat Rev Cancer*. 2011; 11(2):123–134. [PubMed: 21258396]
5. Zhang Q, Yang M, Shen J, Gerhold LM, Hoffman RM, Xing HR. The role of the intravascular microenvironment in spontaneous metastasis development. *Int J Cancer*. 2010; 126(11):2534–2541. [PubMed: 19847811]
6. van Zijl F, Krupitza G, Mikulits W. Initial steps of metastasis: cell invasion and endothelial transmigration. *Mutat Res*. 2011; 728(1-2):23–34. [PubMed: 21605699]
7. Coghlin C, Murray GI. Current and emerging concepts in tumour metastasis. *J Pathol*. 2010; 222(1): 1–15. [PubMed: 20681009]
8. Felding-Habermann B, Habermann R, Saldivar E, Ruggeri ZM. Role of beta3 integrins in melanoma cell adhesion to activated platelets under flow. *J Biol Chem*. 1996; 271(10):5892–5900. [PubMed: 8621462]
9. Kim YJ, Borsig L, Han HL, Varki NM, Varki A. Distinct selectin ligands on colon carcinoma mucins can mediate pathological interactions among platelets, leukocytes, and endothelium. *Am J Pathol*. 1999; 155(2):461–472. [PubMed: 10433939]
10. Ludwig RJ, Boehme B, Podda M, Henschler R, Jager E, Tandi C, Boehncke WH, Zollner TM, Kaufmann R, Gille J. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. *Cancer Res*. 2004; 64(8):2743–2750. [PubMed: 15087389]
11. Nierodzik ML, Karpatkin S. Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell*. 2006; 10(5):355–362. [PubMed: 17097558]
12. Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc Natl Acad Sci U S A*. 2001; 98(6):3352–3357. [PubMed: 11248082]
13. Mousa SA, Petersen LJ. Anti-cancer properties of low-molecular-weight heparin: preclinical evidence. *Thromb Haemost*. 2009; 102(2):258–267. [PubMed: 19652876]

14. Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A, Mueller BM. Integrin activation controls metastasis in human breast cancer. *Proc Natl Acad Sci U S A*. 2001; 98(4):1853–1858. [PubMed: 11172040]
15. Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer*. 2010; 10(1):9–22. [PubMed: 20029421]
16. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science*. 1994; 264(5158):569–571. [PubMed: 7512751]
17. Brooks PC, Stromblad S, Klemke R, Visscher D, Sarkar FH, Cheresh DA. Antiintegrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. *J Clin Invest*. 1995; 96(4):1815–1822. [PubMed: 7560073]
18. Peiris PM, Bauer L, Toy R, Tran E, Pansky J, Doolittle E, Schmidt E, Hayden E, Mayer A, Keri RA, Griswold MA, Karathanasis E. Enhanced Delivery of Chemotherapy to Tumors Using a Multicomponent Nanochain with Radio-Frequency-Tunable Drug Release. *ACS Nano*. 2012; 6(5):4157–4168. [PubMed: 22486623]
19. Peiris PM, Toy R, Doolittle E, Pansky J, Abramowski A, Tam M, Vicente P, Tran E, Hayden E, Camann A, Mayer A, Erokku BO, Berman Z, Wilson D, Baskaran H, Flask CA, Keri RA, Karathanasis E. Imaging metastasis using an integrin-targeting chain-shaped nanoparticle. *ACS Nano*. 2012; 6(10):8783–8795. [PubMed: 23005348]
20. Peiris PM, Toy R, Abramowski A, Vicente P, Tucci S, Bauer L, Mayer A, Tam M, Doolittle E, Pansky J, Tran E, Lin D, Schiemann WP, Ghaghada KB, Griswold MA, Karathanasis E. Treatment of cancer micrometastasis using a multicomponent chain-like nanoparticle. *J Control Release*. 2014; 173:51–58. [PubMed: 24188960]
21. Karathanasis E, Suryanarayanan S, Balusu SR, McNeeley K, Sechopoulos I, Karellas A, Annapragada AV, Bellamkonda RV. Imaging nanoprobe for prediction of outcome of nanoparticle chemotherapy by using mammography. *Radiology*. 2009; 250(2):398–406. [PubMed: 19188313]
22. Brust M, Walker M, Bethell D, Schiffrin DJ, Whyman R. Synthesis of Thiol-derivatised Gold Nanoparticles in a Two-phase Liquid-Liquid System. *J Chem Soc Chem Commun*. 1994:801–802.
23. Peiris PM, Schmidt E, Calabrese M, Karathanasis E. Assembly of linear nano-chains from iron oxide nanospheres with asymmetric surface chemistry. *PLoS One*. 2011; 6(1):e15927. [PubMed: 21253600]
24. Pulaski BA, Ostrand-Rosenberg S. Mouse 4T1 breast tumor model. *Curr Protoc Immunol*. 2001; Chapter 20:20–22. Unit.
25. Toy R, Hayden E, Camann A, Berman Z, Vicente P, Tran E, Meyers J, Pansky J, Peiris PM, Wu H, Exner A, Wilson D, Ghaghada KB, Karathanasis E. Multimodal in vivo imaging exposes the voyage of nanoparticles in tumor microcirculation. *ACS Nano*. 2013; 7(4):3118–3129. [PubMed: 23464827]
26. Tao K, Fang M, Alroy J, Sahagian GG. Imagable 4T1 model for the study of late stage breast cancer. *BMC Cancer*. 2008; 8:228. [PubMed: 18691423]
27. Zhou Y, Kim YS, Chakraborty S, Shi J, Gao H, Liu S. 99mTc-labeled cyclic RGD peptides for noninvasive monitoring of tumor integrin alphaVbeta3 expression. *Molecular imaging*. 2011; 10(5):386–397. [PubMed: 21521559]
28. Shi J, Zhou Y, Chakraborty S, Kim YS, Jia B, Wang F, Liu S. Evaluation of In-Labeled Cyclic RGD Peptides: Effects of Peptide and Linker Multiplicity on Their Tumor Uptake, Excretion Kinetics and Metabolic Stability. *Theranostics*. 2011; 1:322–340. [PubMed: 21850213]
29. Zhou Y, Chakraborty S, Liu S. Radiolabeled Cyclic RGD Peptides as Radiotracers for Imaging Tumors and Thrombosis by SPECT. *Theranostics*. 2011; 1:58–82. [PubMed: 21547153]
30. Kim YH, Jeon J, Hong SH, Rhim WK, Lee YS, Youn H, Chung JK, Lee MC, Lee DS, Kang KW, Nam JM. Tumor targeting and imaging using cyclic RGD-PEGylated gold nanoparticle probes with directly conjugated iodine-125. *Small*. 2011; 7(14):2052–2060. [PubMed: 21688390]
31. Perrault SD, Walkey C, Jennings T, Fischer HC, Chan WC. Mediating tumor targeting efficiency of nanoparticles through design. *Nano Lett*. 2009; 9(5):1909–1915. [PubMed: 19344179]
32. Huang X, Peng X, Wang Y, Shin DM, El-Sayed MA, Nie S. A reexamination of active and passive tumor targeting by using rod-shaped gold nanocrystals and covalently conjugated peptide ligands. *ACS Nano*. 2010; 4(10):5887–5896. [PubMed: 20863096]

33. Wedeking P, Eaton S, Covell DG, Nair S, Tweedle MF, Eckelman WC. Pharmacokinetic analysis of blood distribution of intravenously administered ^{153}Gd -labeled $\text{Gd}(\text{DTPA})_2$ - and $^{99\text{m}}\text{Tc}(\text{DTPA})$ in rats. *Magn Reson Imaging*. 1990; 8(5):567–575. [PubMed: 2082126]
34. Bertucci F, Finetti P, Birnbaum D. Basal breast cancer: a complex and deadly molecular subtype. *Curr Mol Med*. 2012; 12(1):96–110. [PubMed: 22082486]
35. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. *Nature*. 2000; 406(6797):747–752. [PubMed: 10963602]
36. Haubner R, Maschauer S, Prante O. PET Radiopharmaceuticals for Imaging Integrin Expression: Tracers in Clinical Studies and Recent Developments. *BioMed research international*. 2014; 2014:871609. [PubMed: 25013808]
37. Thakor AS, Jokerst J, Zavaleta C, Massoud TF, Gambhir SS. Gold nanoparticles: a revival in precious metal administration to patients. *Nano Lett*. 2011; 11(10):4029–4036. [PubMed: 21846107]
38. O'Neal DP, Hirsch LR, Halas NJ, Payne JD, West JL. Photo-thermal tumor ablation in mice using near infrared-absorbing nanoparticles. *Cancer Lett*. 2004; 209(2):171–176. [PubMed: 15159019]
39. Vigderman L, Zubarev ER. Therapeutic platforms based on gold nanoparticles and their covalent conjugates with drug molecules. *Adv Drug Deliv Rev*. 2013; 65(5):663–676. [PubMed: 22613038]
40. Cheng Y, Meyers JD, Agnes RS, Doane TL, Kenney ME, Broome AM, Burda C, Basilion JP. Addressing Brain Tumors with Targeted Gold Nanoparticles: A New Gold Standard for Hydrophobic Drug Delivery? *Small*. 2011; 7(16):2301–2306. [PubMed: 21630446]
41. Cheng Y, Meyers JD, Broome AM, Kenney ME, Basilion JP, Burda C. Deep penetration of a PDT drug into tumors by noncovalent drug-gold nanoparticle conjugates. *J Am Chem Soc*. 2011; 133(8):2583–2591. [PubMed: 21294543]
42. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev*. 2003; 17(5):545–580. [PubMed: 12629038]
43. Ng QK, Olariu CI, Yaffee M, Taelman VF, Marincek N, Krause T, Meier L, Walter MA. Indium-111 labeled gold nanoparticles for in-vivo molecular targeting. *Biomaterials*. 2014; 35(25):7050–7057. [PubMed: 24840614]
44. Morales-Avila E, Ferro-Flores G, Ocampo-Garcia BE, De Leon-Rodriguez LM, Santos-Cuevas CL, Garcia-Becerra R, Medina LA, Gomez-Oliván L. Multimeric system of $^{99\text{m}}\text{Tc}$ -labeled gold nanoparticles conjugated to c[RGDFK(C)] for molecular imaging of tumor $\alpha(\text{v})\beta(3)$ expression. *Bioconjug Chem*. 2011; 22(5):913–922. [PubMed: 21513349]
45. Arosio D, Manzoni L, Araldi EM, Scolastico C. Cyclic RGD functionalized gold nanoparticles for tumor targeting. *Bioconjug Chem*. 2011; 22(4):664–672. [PubMed: 21434651]
46. Beer AJ, Haubner R, Wolf I, Goebel M, Luderschmidt S, Niemeyer M, Grosu AL, Martinez MJ, Wester HJ, Weber WA, Schwaiger M. PET-based human dosimetry of ^{18}F -galacto-RGD, a new radiotracer for imaging $\alpha \text{v} \beta 3$ expression. *J Nucl Med*. 2006; 47(5):763–769. [PubMed: 16644745]
47. American Cancer Society. Cancer Facts and Figures. 2012
48. Kinkel K, Lu Y, Both M, Warren RS, Thoeni RF. Detection of hepatic metastases from cancers of the gastrointestinal tract by using noninvasive imaging methods (US, CT, MR imaging, PET): a meta-analysis. *Radiology*. 2002; 224(3):748–756. [PubMed: 12202709]
49. Bipat S, van Leeuwen MS, Comans EF, Pijl ME, Bossuyt PM, Zwinderman AH, Stoker J. Colorectal liver metastases: CT, MR imaging, and PET for diagnosis--meta-analysis. *Radiology*. 2005; 237(1):123–131. [PubMed: 16100087]
50. Niekel MC, Bipat S, Stoker J. Diagnostic imaging of colorectal liver metastases with CT, MR imaging, FDG PET, and/or FDG PET/CT: a meta-analysis of prospective studies including patients who have not previously undergone treatment. *Radiology*. 2011; 257(3):674–684. [PubMed: 20829538]
51. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol*. 2009; 6(6):339–351. [PubMed: 19399023]

52. Fisher B, Gebhardt MC. The evolution of breast cancer surgery: past, present, and future. *Semin Oncol.* 1978; 5(4):385–394. [PubMed: 366753]
53. Susnik B, Frkovic-Grazio S, Bracko M. Occult micrometastases in axillary lymph nodes predict subsequent distant metastases in stage I breast cancer: a case-control study with 15-year follow-up. *Ann Surg Oncol.* 2004; 11(6):568–572. [PubMed: 15150071]
54. Cote RJ, Peterson HF, Chaiwun B, Gelber RD, Goldhirsch A, Castiglione-Gertsch M, Gusterson B, Neville AM. Role of immunohistochemical detection of lymph-node metastases in management of breast cancer. International Breast Cancer Study Group. *Lancet.* 1999; 354(9182):896–900. [PubMed: 10489948]
55. Braun S, Kantenich C, Janni W, Hepp F, de Waal J, Willgeroth F, Sommer H, Pantel K. Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. *J Clin Oncol.* 2000; 18(1):80–86. [PubMed: 10623696]
56. Mansi JL, Gogas H, Bliss JM, Gazet JC, Berger U, Coombes RC. Outcome of primary-breast-cancer patients with micrometastases: a long-term follow-up study. *Lancet.* 1999; 354(9174):197–202. [PubMed: 10421301]
57. Gerber B, Krause A, Muller H, Richter D, Reimer T, Makovitzky J, Herrnring C, Jeschke U, Kundt G, Friese K. Simultaneous immunohistochemical detection of tumor cells in lymph nodes and bone marrow aspirates in breast cancer and its correlation with other prognostic factors. *J Clin Oncol.* 2001; 19(4):960–971. [PubMed: 11181658]
58. Fehm T, Muller V, Alix-Panabieres C, Pantel K. Micrometastatic spread in breast cancer: detection, molecular characterization and clinical relevance. *Breast Cancer Res* 10 Suppl. 2008; 1:S1.
59. Zhang XD, Wu HY, Wu D, Wang YY, Chang JH, Zhai ZB, Meng AM, Liu PX, Zhang LA, Fan FY. Toxicologic effects of gold nanoparticles in vivo by different administration routes. *Int J Nanomedicine.* 2010; 5:771–781. [PubMed: 21042423]
60. Lipka J, Semmler-Behnke M, Sperling RA, Wenk A, Takenaka S, Schleh C, Kissel T, Parak WJ, Kreyling WG. Biodistribution of PEG-modified gold nanoparticles following intratracheal instillation and intravenous injection. *Biomaterials.* 2010; 31(25):6574–6581. [PubMed: 20542560]

Fig. 1. Illustration of the vascular targeting strategy of a AuNP radiotracer to metastatic sites. Inset: Interactions of circulating tumor cells and vascular bed.

Fig. 2.
Reaction scheme of the functionalization of AuNP with (A) the chelating agent DTPA and (B) the c(RGDfC) peptide.

Fig. 3. In vitro characterization of the AuNP nanoparticles. **(A)** TEM images of the AuNP nanoparticles. **(B)** Size distribution of AuNP particles obtained by DLS. **(C)** Summary of the size measurements before and after radiolabeling (data presented as mean $\pm$ s.d.).

Fig. 4. Synopsis of *in vivo* and terminal analyses. **(A)** The timeline and schedule of the *in vivo* imaging studies and *post mortem* analyses. **(B)** An example of longitudinal bioluminescence imaging of a mouse bearing a 4T1 tumor.

Fig. 5.

Detection of micrometastasis using an $\alpha_v\beta_3$ integrin-targeting ^{99m}Tc -AuNP radiotracer and nuclear imaging. **(A)** Representative coronal image using whole-body planar gamma scintigraphy shows the detection of micrometastatic sites in the lungs in a mouse bearing 4T1 breast cancer metastasis at 30 min after injection of the AuNP radiotracer (Gamma Medica FLEX small animal-imaging instrument, X-SPECT system). The animal was injected with a dose of 50 μCi of the ^{99m}Tc -AuNP radiotracer. **(B)** The gamma scintigraphy image was co-registered with a 99- μm -resolution micromorphological image obtained by using a Siemens Inveon micro-CT and a liposome-based iodinated contrast agent (yellow arrows indicate the location of metastatic sites). **(C)** At the end of the nuclear imaging session, the lungs of the animals were perfused, excised and imaged *ex vivo* using the Gamma Medica X-SPECT system and a CRi Maestro fluorescence imaging system, indicating the colocalization of the AuNP radiotracer and 4T1 metastatic cells expressing GFP. **(D)** Representative coronal image using planar gamma scintigraphy shows a healthy mouse 30 min after injection of the AuNP radiotracer. Regions of interest are drawn indicating the location of different organs.

Fig. 6.

Quantitative analysis of radionuclide imaging. **(A)** Quantification of the time-course of signal intensity in the thoracic region is shown after injection of the AuNP radiotracer in animals bearing 4T1 metastasis ($n=6$) and healthy animals ($n=6$). Each animal in the group with 4T1 metastasis presented 2-3 hot spots. **(B)** The signal of the AuNP radiotracer is compared to that of a small molecule analogue 60 min after injection in animals with 4T1 metastasis. At the end of imaging, the lungs of animals injected with AuNP were perfused, excised, and weighed. After digestion of the tissues, the gold concentration was measured using ICP-OES. Control animals were used to correct for background levels. Data points marked with asterisks are statistically significant relative to all groups ($n = 6$ animals per formulation; $*P < 0.05$).

Fig. 7.

Comparison of the deposition of non-targeted and targeted AuNP in lungs. The deposition of targeted AuNP and its non-targeted variant was compared in healthy mice and mice with 4T1 metastasis (n=5 mice per condition). Each animal was systemically injected with AuNP at a dose of 12.7 μ g.

Fig. 8.

Histological evaluation of the distribution of the $^{99\text{m}}\text{Tc}$ -AuNP radiotracer in the lungs of a mouse with 4T1 metastasis. **(A)** Fluorescence image of a histological section of a lung lobe (5 $\times$ magnification; blue: nuclear stain (DAPI); green: 4T1 cancer cells expressing GFP). Images of entire histological sections of the lungs were obtained using the automated tiling function of the microscope. The location of metastatic cancer cells is shown with respect to **(B)** endothelial cells and **(C)** expression of $\alpha_v\beta_3$ integrin in the same histological section (10 $\times$ magnification; green: 4T1 cancer cells; red: endothelial cells; purple: $\alpha_v\beta_3$ integrin). **(D)** In the same histological section, the accumulation of AuNP nanoparticles is shown to be exclusively in locations with 4T1 cells (10 $\times$ magnification; green: 4T1 cancer cells; burgundy: AuNP).