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Multimodal Genetic Approach for Molecular Imaging of Vasculature in a Mouse Model of Melanoma

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Abstract

Purpose—In this study, we evaluated a genetic approach for *in vivo* multimodal molecular imaging of vasculature in a mouse model of melanoma.

Procedures—We used a novel transgenic mouse, *Ts-Biotag* that genetically biotinylates vascular endothelial cells. After inoculating these mice with B16 melanoma cells, we selectively targeted endothelial cells with (strept)avidinated contrast agents to achieve multimodal contrast enhancement of Tie2-expressing blood vessels during tumor progression.

Results—This genetic targeting system provided selective labeling of tumor vasculature and showed *in vivo* binding of avidinated probes with high specificity and sensitivity using microscopy, near infrared, ultrasound and magnetic resonance imaging. We further demonstrated the feasibility of conducting longitudinal three-dimensional (3D) targeted imaging studies to dynamically assess changes in vascular Tie2 from early to advanced tumor stages.

Conclusions—Our results validated the *Ts-Biotag* mouse as a multimodal targeted imaging system with the potential to provide spatio-temporal information about dynamic changes in vasculature during tumor progression.

INTRODUCTION

The formation of blood vessels is required for normal development and for pathogenesis in many human diseases. In cancer, angiogenesis plays a crucial role for sustained tumor growth and metastasis [1–2]. Furthermore, the degree of vascularization of some tumors has been correlated with their grade, malignancy and prognosis and novel treatments have been

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

developed to inhibit or normalize tumor vessels in order to halt tumor growth. For these reasons, there has been a tremendous effort in pre-clinical and clinical cancer studies to characterize the vascular profile of tumors and to investigate the molecular mechanisms underlying blood vessel growth.

Traditionally, studies of tumor vasculature have relied on histological methods in which tissue sections are stained with endothelial cell markers and the number of vessels per unit area is quantified to derive estimates of microvessel density (MVD). Even though this is a standard and widely used method, its reliability is frequently compromised by factors such as variable section thickness, the ambiguous appearance of vessels in histological sections, and inter and intra-observer variability [3]. Moreover, the information obtained from histology is two-dimensional (2D) and static, reflecting only the density of vessels, not their functional properties. In this regard, *in vivo* micro-imaging methods are powerful tools for the noninvasive, longitudinal, three-dimensional (3D) assessment of tumor neovasculature at all stages of tumorigenesis.

Advances in the field of molecular imaging have allowed the *in vivo* detection of key cellular and molecular markers specifically expressed at the surface of actively growing tumor vessels, including vascular endothelial growth factor receptor 2 (VEGFR2) [4–10] and vascular integrin $\alpha_v\beta_3$ [11–17]. The usefulness of this approach hinges on its ability to distinguish actively angiogenic endothelium from normal vasculature, as well as to elucidate the key molecular pathways being altered in different tumor phenotypes. As a result, molecular imaging has great potential for the study of tumor progression at an early point when anatomic or functional changes may not yet be present, for a more accurate monitoring of tumor vascular changes during disease progression and for the assessment of response to specific treatments.

Molecular imaging modalities include fluorescence, bioluminescence, and near infrared imaging (NIR), magnetic resonance imaging (MRI) and spectroscopy (MRS), ultrasound, computerized tomography (CT), single photon emission computed tomography (SPECT) and positron emission tomography (PET). Technological improvements have made all these modalities available for pre-clinical small animal imaging, enabling biological studies that would not be feasible in humans [18–21]. Despite the utility of these imaging methods, no single modality offers broad applicability to a wide range of biological processes, due to differences among them in terms of sensitivity, specificity, spatial and temporal resolution, cost and accessibility. For this reason, many advancements in the field of molecular imaging rely on the development of hybrid or multimodal approaches that can provide complementary information and offer advantages over imaging using a single imaging modality.

Most *in vivo* molecular imaging of tumor neovasculature involves functionalizing intravascular contrast agents with ligands such as antibodies, proteins, peptides, oligonucleotides or small molecules; and then targeting them to specific membrane proteins selectively expressed on tumor endothelial cells [22]. This targeted imaging strategy has been successful for several endothelial cell markers in tumors and other vascular diseases, but challenges remain related to target specificity, binding affinity, stability and the

pharmacokinetics of the imaging probes. In addition, this approach is inherently limited to extracellular surface proteins accessible by contrast agents delivered intravascularly while many genes and proteins crucial to tumor growth and neovascularization are located intracellularly and are therefore not accessible to targeted agents. Previously, genetic approaches have been used to overcome this limitation by expressing reporter proteins such as green fluorescent protein (GFP), using vascular specific regulatory elements to drive expression in genetically-engineered reporter mice. Imaging of such fluorescent or bioluminescent reporter genes has been valuable in studies of cellular markers during normal and pathological angiogenesis, but analysis has been mostly restricted to *ex vivo* histological studies or *in vivo* whole body imaging at low resolution [23–26].

There is a clear need for new approaches that combine the ability to both detect and dynamically study cellular and biological abnormalities underlying tumor neovascularization using multimodal imaging platforms that help address the challenges of high-resolution, high-specificity, *in vivo*, targeted molecular imaging in tumors. To this end, we previously developed a novel *Biotag* transgene and demonstrated its utility by generating lines of *Ts-Biotag* transgenic mice that biotinylate the surface of Tie2-expressing vascular endothelial cells [27]. In brief, the *Biotag* transgene co-expresses two interacting proteins: BirA, a biotin ligase; and a surface display protein containing 12 biotinylation sequences that are recognized by BirA. The expression of this bicistronic transgene is driven by a minimal (short) version of the Tie2 promoter (*Ts*). When endothelial cells express Tie2, the BirA protein ligates a cluster of biotins onto each display protein, which is then directed to the cell surface. Multiple biotins will then be present in the endothelial cell membranes where they can be targeted using several commercially available (strept)avidinated-imaging probes to obtain specific tissue contrast in the vasculature.

Our prior studies showed that *Ts-Biotag* mice efficiently label developing vasculature in both embryos and a matrigel model of adult angiogenesis [27]. The overall goal of the current study was to further validate the potential of this system for *in vivo* targeted imaging of vasculature in B16 mouse melanomas, a well established and highly vascularized tumor model. We aimed to establish the feasibility of conducting *in vivo* multimodal imaging using NIR, MRI and high frequency ultrasound (HFU) imaging, including 3D longitudinal analysis of contrast enhancement of labeled vessels in order to assess vascular changes in Tie2-expression at different stages of tumor progression.

METHODS

Mouse melanoma model

All mice used in this study were maintained under protocols approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. *Ts-Biotag* transgenic mice [27] were bred to C57BL/6 mice (Taconic) for at least five generations prior to conducting experiments. The genotype of each mouse was confirmed by PCR of tail biopsy using previously reported primers [27]. Melanoma tumors were established by subcutaneous injection of a suspension of 10^6 B16-BL6 mouse melanoma cells in 100- μ l sterile phosphate-buffered saline (PBS) into a shaved area in the right lower flank of anesthetized 5–9 week-old wild type (WT) and *Ts-Biotag* mice. Tumors were allowed to

grow between 9 and 20 days for volumetric and targeted imaging studies. For all imaging sessions, mice were anesthetized with Isoflurane (5% in air for initial induction followed by 1.0–1.5% in air delivered *via* nosecone during imaging) while maintaining physiological body temperature and monitoring respiration and heart rates using integrated equipment on each imaging system. For targeted imaging, previously optimized doses of avidinated contrast agents specific to each modality were administered intravenously (IV) via tail vein injection or tail vein catheterization using a 27g needle [27]. To confirm specific labeling of endothelial cells, *Ts-Biotag* mice were injected IV with 100 μ g (1mg/ml) of Avidin-FITC (Av-FITC; A02050, Sigma). One hour later the mice were perfused transcardially and the tumors were extracted for histological analysis.

Near Infrared (NIR) Imaging

For *in vivo* targeted NIR imaging, WT and *Ts-Biotag* mice were anesthetized and administered a commercially available IRDye® 680 Streptavidin, denoted here as SAV-680 (926-32231, LI-COR Biosciences) at a 100- μ g dose (1mg/ml). Immediately after injection, mice recovered from anesthesia for 1h until being scanned, which allowed for circulation of the agent under physiological conditions. Mice were then anesthetized and placed on the NIR imaging scanner (Odyssey; LI-COR) and whole body scans were acquired using the 700 nm wavelength channel at 180 μ m resolution in approximately 15 min. After imaging, selected mice were perfused and melanoma tumors excised to be immediately re-scanned *ex vivo*. Subsequently, these tumors were fixed and prepared for cryosectioning, and histological slides with 7 μ m thick serial tumor sections were later re-scanned at a higher 21 μ m resolution. For quantification, NIR images were exported and mean signal intensity was analyzed in regions-of-interest (ROIs) covering the entire tumor area using ImageJ software (v.1.49, NIH freeware).

Magnetic Resonance Imaging (MRI)

Mice were injected with chelated gadolinium (DTPA-Gd) conjugated to avidin (Av-DTPA-Gd) as previously described [27], at a dose of 10-mg (40-mg/ml). As in the NIR experiments, mice were allowed to recover from anesthesia and had normal physiology for 1h prior to the acquisition of post-injection scans. MRI was performed on a 7 Tesla, 200 mm diameter horizontal bore magnet (MagneX Scientific) interfaced to a Bruker Biospec Avance II console (Bruker BioSpin MRI) with actively shielded gradients (750-mT/m; BGA9S, Bruker) and volume-coil-transmit / surface-coil-receive systems (Bruker). To reduce artifacts from the breathing motion, the surface coil was secured to the mouse flank with tape. *In vivo* pre- and post-contrast MR images were obtained using a 3D gradient echo sequence (3DGE), with parameters optimized empirically for T1-weighted contrast (echo and repetition time, TE/TR = 3.27 ms/20 ms; flip angle, FA = 30°; isotropic resolution = 200 μ m; number of averages = 6; total imaging time = 27 min), and scans were exported and analyzed using ImageJ software. Signal intensities from ROIs covering the entire tumor area were divided by the standard deviation of the background noise in order to calculate signal-to-noise ratio (SNR). Contrast was then computed as $(\text{SNR}_{\text{Post}} - \text{SNR}_{\text{Pre}}) / \text{SNR}_{\text{Pre}}$. Targeted 3D vascular maps were constructed to observe the distribution of the vascular enhancement using AMIRA Imaging software (V5.2; Visage Imaging). To generate renderings of the overall 3D tumor surface, selected MRI datasets of individual animals were analyzed using

segmentation tools according to previously reported protocols [28–30]. A histogram analysis then allowed for the segmentation of enhanced voxels based on their intensities, which were displayed as a color map overlaid on the segmented tumor volume.

High Frequency Ultrasound (HFU) Imaging

HFU imaging was performed using two small animal ultrasound scanners (Fujifilm VisualSonics): the Vevo-770 system for 3D tumor volumetric studies at 40 MHz (RMV704 single element transducer); and the Vevo 2100 for 2D and 3D targeted contrast-enhanced ultrasound (CEU) studies at 18 MHz (MS-250 linear array transducer). After tumor-bearing mice were anesthetized, a tail vein catheter was inserted (for targeted CEU) and mice were positioned on a heated platform to maintain physiological body temperature during the experiments. The skin over and around the tumor was carefully shaved to remove the hair, and an acoustic gel was applied to couple the transducer to the skin. On both the Vevo 770 and Vevo 2100 scanners, the transducer was secured over the mouse/tumor in the 3D imaging mount on the Vevo rail system. 2D images were acquired from the central section of each tumor, while 3D images were acquired as a series of sequential sections (step size = 0.1 mm) covering the entire tumor.

Tumor Volume and Growth Rate Analysis

To assess tumor volume and growth rate, longitudinal 3D-HFU images were acquired at 9, 13, 16 and 20 days post tumor cell inoculation, and analyzed using semi-automated segmentation tools in AMIRA. Linear regression analysis was applied to the time-dependent volumetric data, and the doubling time T_d (in days) was estimated from the fitted slope b (in mm^3/day) as:

$$T_d = V_0/b, \quad (1)$$

where V_0 was the initial volume (in mm^3) measured at day 9. The correlation coefficient R^2 was also computed for each fit. For comparison of tumor volume between groups, mean $\pm$ standard deviation (SD) were plotted.

Microbubbles for Targeted CEU

Targeted microbubbles (MBs) (Target-Ready MicroMarker; Visualsonics, Toronto, Canada) were reconstituted according to manufacturer's specifications using 700 μL of sterile saline (0.9% sodium chloride) to yield a stock concentration of 2×10^9 MB/ml. These MBs are coated with streptavidin and were administered directly to *Ts-Biotag* mice to achieve targeted labeling by binding the biotinylated endothelial cell membranes [27], and are referred to as Av-MBs in this paper. Av-MBs were also conjugated conventionally, using the vendor's protocol, to a biotinylated Tie2-antibody (Abcam, ab9572) prior to *in vivo* injection in an attempt to target the endogenous Tie2 receptors in WT B16 melanomas. Controls for these experiments included Av-MBs alone and Av-MBs conjugated to a biotinylated (non-specific) isotype-matched polyclonal immunoglobulin G antibody (Abcam, ab18445), both injected into WT mice. For each type of MB, 1×10^8 MBs were injected as an IV bolus via a tail vein catheter.

Targeted CEU

2D and 3D targeted CEU studies were performed using the Vevo-2100 scanner. Images were acquired using both B-mode for tumor morphology, and Nonlinear Contrast (NLC) mode to detect the presence of MBs. Tumor imaging settings best suited for NLC mode imaging were followed and maintained constant throughout all imaging sessions: 18 MHz center frequency, 4% transmit power, 35 dB dynamic range and 28 dB contrast gain.

After acquiring a non-enhanced 3D baseline scan, the central transverse section of each tumor was identified and a baseline 2D scan was acquired at this location. Each mouse then received an IV bolus of a single type of MB slowly over 2 seconds followed by a 20 μ l flush with sterile saline. Real-time 2D imaging was performed during the IV injection to observe delivery of the contrast agent into the tumor. A second 3D scan was acquired immediately post injection, the transducer repositioned at the previously identified central 2D section and the acquisition paused for 4 min, the vendor-recommended time to allow optimal binding of targeted MBs. During this time, body temperature and heart rate were monitored and maintained at normal physiological values to ensure proper circulation of the contrast agent in the vasculature. After 4 min, a set of 2D image frames (cineloop) was acquired to obtain signal from bound and freely circulating MBs, followed by a high power destructive pulse (100% transmit power) applied for 1 second to destroy the MBs in the field of view. Two seconds after the destruction, another set of 2D frames was acquired to account for signal from freely circulating MBs replenishing the tumor. Since the applied high power pulse only destroyed MBs in the 0.1mm thickness of a particular 2D slice, a similar protocol was conducted to obtain 3D pre- and post-destruction datasets, but in this case, after destruction of all bound bubbles within the whole tumor volume using a 3D high power destructive pulse.

In a subset of *Ts*-Biotag mice, 2–4 sequential 2D and 3D CEU scans were obtained between 9–20 days post tumor inoculation to evaluate changes in targeting at different tumor stages in individual animals. Due to tumor growth rate and size variability, tumors were segregated by volume for analysis in the following groups: I - (0–50mm³), II- (50–150mm³) and III- (>150mm³).

Analysis of Targeted CEU Data

Datasets from individual tumors were exported as RAW B-mode and NLC-mode cineloops and analyzed offline using ImageJ and Amira image analysis software. Motion artifacts present in 2D and 3D datasets were corrected using an ImageJ multistack rigid registration macro applied to the B-mode images to generate a transformation matrix, which was then applied to both the B-mode and NLC-mode images. Since there was additional displacement in the acquisition of 3D datasets as the motor moved along the tumor axis, a subsequent 3D registration step was applied using the affine registration tool in Amira. These steps allowed all pre- and post-destruction datasets, for both 2D and 3D scans, to be aligned and registered in the same space prior to analysis of targeting. The signal from bound MBs was obtained by calculating the difference between signals from NLC pre- and post-destruction datasets. For 2D images, the signals between one hundred twenty NLC frames from both pre-destruction and post-destruction cineloops were averaged and digitally subtracted in order to derive a

differential image. For 3D datasets, the registered whole tumor pre-destruction and post-destruction images were subtracted directly. Targeting was quantified within ROIs covering the entire tumor area (2D) or volume (3D) from the resulting differential image and was expressed as number of pixels or voxels for 2D and 3D imaging, respectively. For visual representation, the difference in signal from the subtraction of pre- and post- destruction NLC-mode images was shown as a green overlay on the greyscale B-mode images and on the 3D surface rendering of the tumors for 2D and 3D images, respectively.

Histological Analysis

For immunohistochemistry (IHC), mice were perfused transcardially with 4°C phosphate buffered saline mixed with heparin (5000 units/l), followed by 4°C 4% paraformaldehyde (PFA). After perfusion, tumors were dissected from the flank and post-fixed with 4°C, 4% PFA overnight. Tissue was then cryoprotected in a 15% to 30% sucrose gradient and frozen in blocks of embedding compound (Tissue-Tek OCT, Sakura Finetek USA). Serial sections were cut at 7- μ m thickness on a cryostat (CM3050S, Leica Microsystems). The primary antibodies used were rat anti-CD31 (1:50/ 550274; BD Biosciences) as a marker of endothelial cells and mouse anti-Tie2 (1:250 / 24859; Abcam). Secondary antibody staining was performed with polyclonal goat anti-rat Alexa-555 (1:1000) and anti-mouse Alexa-488 (1:1000). Slides were counter stained with 4,6-diamidino-2-phenylindole (DAPI, 1:10,000 / Sigma) for nuclear visualization. After staining, slides were cover-slipped and imaged using a fluorescence microscope (Leica DM RXE) or a digital slide scanner (Leica SCN400F). Captured images were further analyzed using ImageJ and SlidePath Digital Image Hub software (Leica Biosystems).

Quantitative analysis of vascular density in melanoma tumors was performed using one representative CD31 stained histological section from the center of each melanoma tumor, which were digitally scanned to cover the entire tumor region with the Slidepath software. Using a grid as a reference, we acquired representative images within the whole tumor region (21 ± 8 ROIs per tumor depending on size), each ROI corresponding to a 0.01 mm² field. Captured ROI's were imported and analyzed using ImageJ. For this, vascular signal was evaluated via histogram analysis to identify areas of positive staining as vessels. The —analyze particles tool in ImageJ was used to generate outlines of all vessels contained within each ROI and to automatically count the number of vessels with an area ≥ 100 pixels. The total number of CD31 expressing vessels per tumor was then obtained as the sum of the counted vessels in all ROIs per whole tumor section. Tie2 expression per tumor was analyzed using dual CD31/Tie2 staining, measuring signal from Tie2 staining within the previously defined CD31-expressing vasculature. Then a ratio of Tie2:CD31 was obtained and used to calculate the number of Tie2 expressing vessels per tumor. Mean value and SD were subsequently calculated for further comparison among tumor groups.

Statistical Analyses

All data were represented as mean value $\pm$ SD. The two-tailed Student's t-test was used to determine statistical significance ($P < 0.05$).

RESULTS

***Biotag* transgene expression does not affect melanoma progression**

Previously, we established *Ts-Biotag* transgenic mice for *in vivo* multimodal imaging of *Tie2* expressing vasculature during normal vascular development [27]. The goal of the current study was to utilize the *Ts-Biotag* mice for multimodal and longitudinal vascular imaging of melanoma tumors. Since *Tie2* expression and proper vascularization is crucial for tumor growth, it was first important to establish that melanoma progression was similar in WT and *Ts-Biotag* mice, and that tumor vasculature was not affected by expression of the *Biotag* transgene.

Longitudinal 3D HFU (40 MHz) imaging was used to analyze tumor volume and growth rate in WT (N = 7) and *Ts-Biotag* mice (N = 8). Each tumor was imaged at 9, 13, 16 and 20 days after inoculation of B16 melanoma cells (Fig. 1a). At the beginning of the imaging studies (9 days) tumor volume was the same ($P = 0.9$) in WT ($29.6 \pm 11.6 \text{ mm}^3$) and *Ts-Biotag* mice ($30.5 \pm 15.9 \text{ mm}^3$). Quantitative analysis showed variability in individual tumor volumes at the subsequent imaging time points, but no significant difference was detected between WT and *Ts-Biotag* at any stage (Fig. 1b). These volumetric data were also used to calculate doubling time (Td) for each melanoma tumor. While individual tumors exhibited different growth rates, each tumor had a good fit to the linear model employed ($R^2 > 0.9$ in all cases; mean $\pm$ SD = 0.94 ± 0.05). There was no significant difference in Td ($P = 0.4$) comparing WT (1.9 ± 1.6 days; min = 0.3, max = 4.9) and *Ts-Biotag* mice (1.4 ± 0.9 days; min = 0.4, max = 2.6). We concluded that the growth characteristics of the B16 melanoma model, although variable between individuals was not affected by *Biotag* transgene expression.

B16 melanomas are highly vascularized (Fig. 1c, Suppl. Video 1) and thus constitute an excellent system for testing our targeted vascular imaging approaches. To rule out any differences in vascularization due to *Biotag* transgene expression, we analyzed CD31 stained sections of similar stage tumors from WT (volume = $394.3 \pm 260.9 \text{ mm}^3$) and *Ts-Biotag* (volume = $434.3 \pm 209.4 \text{ mm}^3$) mice (N = 7 for each genotype), harvested at 16 and 20 days post B16 cell inoculation. There was no significant difference in the qualitative vessel integrity or measured MVD, comparing tumors in WT and *Ts-Biotag* mice (Fig. 1d; $P = 0.6$). These results were consistent with the growth rate analyses, and confirmed prior results showing that vascular development is not compromised in *Ts-Biotag* transgenic mice [27]

Vascular endothelial cells were biotinylated in *Ts-Biotag* B16 melanomas

Previous studies established that the surface of vascular endothelial cells in *Ts-Biotag* mice were labeled with biotin during angiogenesis in embryos and in (adult) VEGF-supplemented matrigel plugs [27]. We investigated whether tumor vessels in *Ts-Biotag* melanomas were also biotinylated. At 16–20 days post B16 cell inoculation, Av-FITC was injected IV (via tail vein) in WT and *Ts-Biotag* mice (N = 3 for each genotype). One hour after injection of Av-FITC, each mouse was perfused transcardially and the tumors were excised, histologically processed and stained for the endothelial cell marker CD31. Qualitative analysis of the histological sections showed the presence of the Av-FITC signal, which

clearly co-localized to CD31 expression in *Ts-Biotag* melanomas, and was minimal or absent in WT melanomas (Fig. 2). These results confirmed the biotinylation of vascular endothelial cells in *Ts-Biotag* melanomas.

Multimodal targeted imaging of tumor vasculature in *Ts-Biotag* mice

To explore the utility of the *Ts-Biotag* mouse as a novel genetic system for noninvasive multimodal targeted imaging of tumor vasculature, melanoma tumors were imaged after IV injection of avidinated probes with three different modalities: NIR, MRI and CEU. At 2–3 weeks post B16 cell inoculation, both WT (N = 18) and *Ts-Biotag* (N = 20) tumors were macroscopically obvious and judged to be well-vascularized based on our initial CEU studies (Fig. 1c), and were therefore considered to be an appropriate stage for imaging. The average tumor volumes, measured by 3D imaging, were similar ($P = 0.8$) comparing WT ($330.8 \pm 135.3 \text{ mm}^3$) and *Ts-Biotag* ($290.5 \pm 151.4 \text{ mm}^3$) mice for these studies.

In vivo whole-body NIR scans were acquired prior to NIR agent injection and showed no autofluorescence being emitted from the tumor or mouse flank. A second set of images were acquired 1 h after the injection of the SAv-680 probe and showed greater labeling in the tumor and surrounding skin areas in *Ts-Biotag* mice compared to WT mice (Fig. 3a & b). Quantitative analysis showed that the mean NIR signal (in arbitrary scanner units) in *Ts-Biotag* melanomas was approximately 6-fold higher (Fig. 3c; $P < 0.001$) in *Ts-Biotag* melanomas (3567 ± 625 ; N = 4) than in WT melanomas (621 ± 470 ; N = 6). After *in vivo* imaging, a subset of mice (N = 3 per genotype) were perfused transcardially and the melanoma tumors were excised from them for *ex vivo* imaging. NIR images of the whole-specimen and subsequent histological tumor sections showed similar results to the *in vivo* imaging and confirmed that the higher signal seen in tumors was most likely a result of the targeted labeling and retention of SAv-680 in tumor vasculature (Inserts in Fig. 3a & b).

3D *in vivo* MRI images were obtained before and after IV injection of a synthesized avidinated chelated gadolinium agent, Av-DTPA-Gd. As previously shown, this agent is a macromolecular MR probe that is retained intravascularly, thus allowing for a more efficient targeting of the biotinylated endothelial cells and less nonspecific signal from contrast agent leakage into the tumor interstitium [27]. Post-contrast T1-weighted MRI of melanoma tumors were obtained 1 h after injection (Fig. 3d & e), demonstrating increased contrast (post to pre) in *Ts-Biotag* (0.79 ± 0.36 ; N = 8) compared to WT (0.07 ± 0.04 ; N = 4) melanomas (Fig. 3f, $P < 0.003$). In *Ts-Biotag* melanomas, labeling was heterogeneous, with the major enhancement observed along the surface of the tumors and around hypointense areas inside the tumor core. 3D segmentation of selected *Ts-Biotag* post-injection images were created to better visualize the distribution of the contrast enhancement. Qualitative analysis of these 3D vascular maps confirmed the clustered distribution of high-intensity voxels in the tumor periphery, sparse contrast in the rest of the tumor interior and some vascular labeling surrounding circular necrotic areas (Suppl. Video 2).

Finally, we evaluated whether targeted CEU allowed assessment of vascular labeling in *Ts-Biotag* mice. Using 2D NLC HFU (18-MHz) imaging, images were obtained using a destruction-reperfusion protocol, 4 min after the IV injection of Av-MBs. Signals from pre- and post-destruction images were averaged and subtracted in each pixel to obtain a

differential image, displaying the differential signals as a green overlay on the grey-scale (B-mode) images (Fig. 3g & h). Targeting was analyzed in ROIs covering the entire tumor and the number of targeted pixels was quantified. The results showed an obvious and significant increase in the number of labeled pixels in *Ts-Biotag* vs WT melanomas (Fig. 3g & h; N = 8 per group), confirmed by quantitative analysis (Fig. 3i, $P < 0.001$). Taken together with the results from NIR and MRI, these results show that *Ts-Biotag* mice can be used for *in vivo* targeted molecular imaging and analyses of tumor vasculature with multiple imaging modalities.

***In vivo* assessment of Tie2 expression was only possible in *Ts-Biotag* mice**

Targeted CEU has been used previously for *in vivo* detection of several important molecules expressed on the surface of tumor vascular endothelial cells, including VEGFR2, $\alpha_v\beta_3$ integrin, and endoglin. Notably, Tie2 has been absent from the list of surface proteins detected by targeted molecular imaging, using CEU or any other modality, most likely because of the ineffective binding of available Tie2 antibodies to the receptor. The *Biotag* system enables molecular imaging of potentially difficult targets like Tie2 by generating a highly effective biotin label on the surface of Tie2 expressing cells. To demonstrate this advantage, we compared CEU of *Ts-Biotag* melanomas using Av-MBs, with conventional targeted CEU using MBs conjugated to Tie2-antibodies (MBt). For this, mice bearing similar-sized tumors (N = 20, $P > 0.2$) were injected with three types of MBs and imaged with CEU to assess targeting (Fig. 4a–d). In WT mice, Av-MBs (Fig. 4c; N = 5) and isotype IgG-conjugated MBs (MBi, Fig. 4a; N = 5) served as controls, showing similar low-level baseline retention of these nonspecific MBs within tumor vessels. Conventional Tie2-targeting with MBt in WT mice did not demonstrate MB signals above control levels (Fig. 4b; N = 5), indicating that targeting was not effective. Only Av-MBs in *Ts-Biotag* mice showed effective targeting and retention of MBs in the vasculature (Fig. 4d; N = 5). Quantitative analysis of these images (Fig. 4e) showed significantly higher numbers of labeled pixels in the *Ts-Biotag* melanomas compared to conventional targeting with MBt ($P = 0.002$) or the controls ($P < 0.01$). Notably, targeting among the three MBs in WT mice did not differ significantly ($P > 0.1$).

Time-intensity curves were measured for each type of MB, revealing distinct binding dynamics in *Ts-Biotag* vs WT melanomas. The average signal intensity was higher in *Ts-Biotag* than in WT tumors, both before and after the destruction pulse (Fig. 5a). Quantification of the reperfusion curves demonstrated a significantly increased slope of the post-destruction signal from melanomas in *Ts-Biotag* mice injected with Av-MBs compared to the post-destruction signals in WT mice (Fig. 5b), again indicating that the targeted MB-binding in *Ts-Biotag* vasculature was much more effective than in WT.

Longitudinal imaging of Tie2-expression during melanoma progression

Longitudinal targeted CEU studies were conducted in *Ts-Biotag* mice (N = 5) to investigate temporal changes in vasculature during B16 melanoma progression. In most previous reports, targeted CEU studies have been performed using 2D images acquired in a central section of each tumor. To further investigate the utility of 3D analysis over the entire tumor volume, we acquired both 2D (central section) and 3D data (whole tumor) in each mouse

and in each imaging session, performed 9 to 20 days after B16 cell inoculation. Data from longitudinally imaged melanomas were segregated into stages according to tumor volume: I (0–50 mm³), II (51–150 mm³) and III (>150 mm³). 2D (Fig. 6a) and 3D (Fig. 6b; Suppl. Video 3) images showed dynamic changes in tumor size and targeted signal over time in each individual tumor. To quantify changes in targeted vascular density, we normalized the targeted signal enhancement by the tumor area (for 2D analysis) or volume (for 3D analysis) in each mouse. The results showed fairly constant 2D targeted signal as a function of tumor size (Fig. 6c), while the 3D targeted signal was greatest in the early stage I tumors and somewhat reduced in stages II and III (Fig. 6d). These results suggest that 3D analysis is required to fully characterize vascular changes during tumor progression, which may not be obvious in the conventional approach of a 2D analysis of the central tumor regions.

In order to validate the longitudinal targeted CEU results, IHC analyses of CD31 and Tie2 expression were performed in central sections of similar size tumors (N=3 for each group, I, II and III) in *Ts-Biotag* mice. Linear regression analysis showed that changes in Tie2 and CD31 expression during tumor progression were highly correlated ($R^2 = 0.992$), with approximately 70% of CD31+ vessels expressing Tie2 (data not shown). There was also good correlation between the number of Tie2-expressing vessels in the central sections and the number of pixels detected with targeted CEU in the *Ts-Biotag* mice ($R^2 = 0.88$). Taken together, these results confirmed that endothelial Tie2 expression was present throughout tumorigenesis, and that targeted CEU in the *Ts-Biotag* mice provided an accurate assessment of these changes during tumor progression.

DISCUSSION

Decades of research have provided a large body of evidence that establish the process of neovascularization as one of the central hallmarks of cancer progression. The use of genetically-engineered mice and the development of small animal imaging techniques have been crucial for the analysis of vascular formation in tumors, and further advances in the field of molecular imaging have allowed noninvasive and dynamic studies on the molecular factors underlying tumor vessel development [22, 31–32]. Despite these advances, there are still limitations in current molecular imaging approaches, which arise from the available biological markers and imaging techniques, and from the targeted contrast agents used in these studies. We have previously demonstrated the *Biotag* reporter system as a novel approach for the biotinylation of vascular endothelial cell membranes and demonstrated its suitability for conducting targeted imaging studies with many commercially available avidinated-imaging probes. These studies also confirmed the utility of the *Ts-Biotag* transgenic mouse as an effective tool for *in vivo* imaging of Tie2 expression during vascular development [27].

Given the need for *in vivo* targeted imaging of biological markers in cancer, we assessed the potential of the *Ts-Biotag* mouse for multimodal molecular imaging studies of tumor vasculature. The results of the current study demonstrated that the *Biotag* system selectively labeled vasculature in a mouse melanoma model by targeting vascular endothelial cells expressing Tie2. The *Ts-Biotag* mouse also proved to be a sensitive multimodal targeting

platform with the potential to provide spatio-temporal information about vascular changes in tumors and their relationship to specific disease stages.

Several studies have successfully reported molecular imaging of tumor angiogenesis, using imaging probes targeted to surface receptors in vascular endothelial cells. However, in most receptor-ligand studies, the potential to label vasculature and the level of image contrast obtained is determined by the characteristics of the imaging probe, the target receptor and the affinity between them. In contrast, the *Ts-Biotag* mice were engineered to maximize labeling and contrast by adding multiple biotins to the surface of the Tie2-expressing endothelial cell membranes. Additionally, in endogenous receptor-ligand binding, blood flow and shear stress on endothelial cells can cause washout of the contrast agent, while, in the *Biotag* system, the increased binding between biotin and (strept)avidin minimizes this effect [33].

In the past, it has often been difficult to analyze and monitor *in vivo* molecular patterns in vasculature in a noninvasive, multimodal and 3D manner in order to gain a complete representation of the process of vascular development in tumors. Recent reports have shown promise for 3D CEU imaging of tumor vasculature [34–37]. Our results demonstrated a highly sensitive and versatile approach to study tumor vasculature that allows the use of several *in vivo* 2D and 3D imaging methods. Similar to other reports, the ability to conduct 3D targeted imaging studies provided additional important information when analyzing vessel labeling in tumors compared to traditional 2D imaging. This is especially relevant when measuring labeling in longitudinal tumor studies, where it can be difficult to consistently obtain the same imaging plane as tumors change in volume and morphology.

To our knowledge, this is the first report that demonstrates the feasibility of *in vivo* labeling and 3D longitudinal assessment of Tie2-expressing blood vessels in tumors. Our results, showing the ineffective binding of microbubbles targeted conventionally to Tie2 (Fig. 4), support the need for the genetic targeting approach provided by the *Biotag* transgene. In this study, the 3D renderings generated from longitudinal targeted ultrasound imaging datasets enabled the spatio-temporal visualization of labeled tumor vasculature in whole tumors as they progressed from early to advanced stages in individual *Ts-Biotag* mice. These changes were analyzed quantitatively, using NLC data and post-processing approaches that are now available on many clinical and pre-clinical scanners. There was variability in the targeted signal as tumors were growing, which could potentially correlate to changes in the Tie2 expression patterns among tumors or additional underlying molecular differences among them. This highlights the need for *in vivo* imaging methods to be part of pre-clinical studies of therapeutic agents for a more accurate stratification of treatment cohorts based on tumor growth behavior and molecular characteristics and not only the traditional metrics of days post inoculation or tumor size.

A full discussion of the biology of Tie2, including the complexity of the Tie2/angiopoietin signaling system and its context-dependent regulation in tumor vascularization and growth is beyond the scope of the current proof-of-concept study. However, similar imaging protocols to those used in this study can be implemented in future studies to provide insights into the role of Tie2 expression in lymphangiogenesis, wound-healing and inflammation, as well as

the potential role of Tie2 expression in the other cell types of the microenvironment [38–41]. Further studies could be conducted to analyze the expression of additional molecular markers in tandem with Tie2, or several imaging methods could be used simultaneously in one imaging session and co-registered to obtain complementary information on complex molecular and cellular processes.

Finally, in addition to studying the Tie2 pathway, the *Biotag* targeting system can be developed in future to target other important molecular markers of tumorigenesis such as VEGFR2, the receptor for VEGF-A [23–24]. Many important molecular markers, similar to Tie2, are currently difficult to study *in vivo* because they are located intracellularly or because there are no antibodies that target them adequately *in vivo*. By expanding the *Biotag* system to target other molecular markers, we expect to enhance our understanding of the mechanisms of diseases and to offer readouts of the levels of expression of molecular targets using multimodal imaging methods during tumor progression and therapeutic response through pre-clinical drug testing.

CONCLUSION

In summary, this study demonstrated the labeling and *in vivo* molecular imaging of Tie2-expressing tumor vasculature in a mouse melanoma model. We have validated the *Ts-Biotag* mouse as a genetic platform for the multimodal targeted imaging of vasculature in tumors using NIR, HFU and MRI. We also showed the usefulness of 2D and 3D longitudinal targeted imaging for detecting, quantifying, and monitoring the dynamic molecular changes in vessels at different stages of tumor progression. These results suggest the potential of the *Ts-Biotag* mouse for multi-parametric molecular studies and for pre-clinical applications, such as monitoring the vascular response to anti-angiogenic or other targeted drug therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005; 438:932–936. [PubMed: 16355210]
2. Folkman J. Tumor angiogenesis: therapeutic implications. *New Engl J Med*. 1971; 285:1182–1186. [PubMed: 4938153]
3. Hlatky L, Hahnfeldt P, Folkman J. Clinical Application of Antiangiogenic Therapy: Microvessel Density, What It Does and Doesn't Tell Us. *J Natl Canc Inst*. 2002; 94:883–893.
4. Angst E, Chen M, Mojadidi M, et al. Bioluminescence imaging of angiogenesis in a murine orthotopic pancreatic cancer model. *Mol Imaging Biol*. 2010; 12:570–575. [PubMed: 20376570]

5. De Leon-Rodriguez LM, Lubag A, Udugamasooriya DG, et al. MRI detection of VEGFR2 in vivo using a low molecular weight peptoid-(Gd)8-dendron for targeting. *J Am Chem Soc.* 2010; 132:12829–12831. [PubMed: 20795620]
6. Deshpande N, Pysz MA, Willmann JK. Molecular ultrasound assessment of tumor angiogenesis. *Angiogenesis.* 2010; 13:175–188. [PubMed: 20549555]
7. Deshpande N, Ren Y, Foygel K, et al. Tumor angiogenic marker expression levels during tumor growth: longitudinal assessment with molecularly targeted microbubbles and US imaging. *Radiology.* 2011; 258:804–811. [PubMed: 21339349]
8. Lee DJ, Lyshchik A, Huamani J, et al. Relationship between retention of a vascular endothelial growth factor receptor 2 (VEGFR2)-targeted ultrasonographic contrast agent and the level of VEGFR2 expression in an in vivo breast cancer model. *J Ultras Med.* 2008; 27:855–866.
9. Willmann JK, Lutz AM, Paulmurugan R, et al. Dual-targeted contrast agent for US assessment of tumor angiogenesis in vivo. *Radiology.* 2008; 248:936–944. [PubMed: 18710985]
10. Willmann JK, Paulmurugan R, Chen K, et al. US imaging of tumor angiogenesis with microbubbles targeted to vascular endothelial growth factor receptor type 2 in mice. *Radiology.* 2008; 246:508–518. [PubMed: 18180339]
11. Ellegala DB, Leong-Poi H, Carpenter JE, et al. Imaging tumor angiogenesis with contrast ultrasound and microbubbles targeted to $\alpha(v)\beta_3$. *Circulation.* 2003; 108:336–341. [PubMed: 12835208]
12. Hsu AR, Hou LC, Veeravagu A, et al. In vivo near-infrared fluorescence imaging of integrin $\alpha v\beta_3$ in an orthotopic glioblastoma model. *Mol Imaging Biol.* 2006; 8:315–323. [PubMed: 17053862]
13. Jarzyna PA, Deddens LH, Kann BH, et al. Tumor angiogenesis phenotyping by nanoparticle-facilitated magnetic resonance and near-infrared fluorescence molecular imaging. *Neoplasia.* 2012; 14:964–973. [PubMed: 23097630]
14. Pan D, Pramanik M, Senpan A, et al. Molecular photoacoustic imaging of angiogenesis with integrin-targeted gold nanobeacons. *FASEB J.* 2011; 25:875–882. [PubMed: 21097518]
15. Schmieder AH, Winter PM, Caruthers SD, et al. Molecular MR imaging of melanoma angiogenesis with $\alpha v\beta_3$ -targeted paramagnetic nanoparticles. *Magn Reson Med.* 2005; 53:621–627. [PubMed: 15723405]
16. van Tilborg GA, Mulder WJ, van der Schaft DW, et al. Improved magnetic resonance molecular imaging of tumor angiogenesis by avidin-induced clearance of nonbound bimodal liposomes. *Neoplasia.* 2008; 10:1459–1469. [PubMed: 19048124]
17. Winter PM, Caruthers SD, Allen JS, et al. Molecular imaging of angiogenic therapy in peripheral vascular disease with $\alpha v\beta_3$ -integrin-targeted nanoparticles. *Magn Reson Med.* 2010; 64:369–376. [PubMed: 20665780]
18. Schnall M, Rosen M. Primer on imaging technologies for cancer. *J Clin Oncol.* 2006; 24:3225–3233. [PubMed: 16829646]
19. Condeelis J, Weissleder R. In vivo imaging in cancer. *Cold Spring Harb Perspect Biol.* 2010; 2:a003848. [PubMed: 20861158]
20. Weissleder R. Scaling down imaging: molecular mapping of cancer in mice. *Nat Rev Cancer.* 2002; 2:11–18. [PubMed: 11902581]
21. Weissleder R. Molecular imaging in cancer. *Science.* 2006; 312:1168–1171. [PubMed: 16728630]
22. Choyke P. Science to Practice: Angiogenic Marker Expression during Tumor Growth—Can Targeted US Microbubbles Help Monitor Molecular Changes in the Microvasculature? *Radiology.* 2011; 258:655–656. [PubMed: 21339343]
23. Fraser ST, Hadjantonakis AK, Sahr KE, et al. Using a histone yellow fluorescent protein fusion for tagging and tracking endothelial cells in ES cells and mice. *Genesis.* 2005; 42:162–171. [PubMed: 15986455]
24. Larina IV, Shen W, Kelly OG, et al. A membrane associated mCherry fluorescent reporter line for studying vascular remodeling and cardiac function during murine embryonic development. *Anat Rec (Hoboken).* 2009; 292:333–341. [PubMed: 19248165]
25. Motoike T, Loughna S, Perens E, et al. Universal GFP reporter for the study of vascular development. *Genesis.* 2000; 28:75–81. [PubMed: 11064424]

26. Schlaeger TM, Bartunkova S, Lawitts JA, et al. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci USA*. 1997; 94:3058–3063. [PubMed: 9096345]
27. Bartelle BB, Berrios-Otero CA, Rodriguez JJ, et al. Novel genetic approach for in vivo vascular imaging in mice. *Circ Res*. 2012; 110:938–947. [PubMed: 22374133]
28. Dhenain M, Ruffins SW, Jacobs RE. Three-Dimensional Digital Mouse Atlas Using High-Resolution MRI. *Dev Biol*. 2001; 232:458–470. [PubMed: 11401405]
29. Deans AE, Wadghiri YZ, Berrios-Otero CA, Turnbull DH. Mn enhancement and respiratory gating for in utero MRI of the embryonic mouse central nervous system. *Magn Reson Med*. 2008; 59:1320–1328. [PubMed: 18506798]
30. Szulc KU, Nieman BJ, Houston EJ, et al. MRI analysis of cerebellar and vestibular developmental phenotypes in Gbx2 conditional knockout mice. *Magn Reson Med*. 2013; 70:1707–1717. [PubMed: 23400959]
31. McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med*. 2003; 9:713–725. [PubMed: 12778170]
32. Turkbey B, Kobayashi H, Ogawa M, et al. Imaging of tumor angiogenesis: functional or targeted? *AJR Am J Roentgenol*. 2009; 193:304–313. [PubMed: 19620425]
33. Laitinen OH, Nordlund HR, Hytonen VP, Kulomaa MS. Brave new (strept)avidins in biotechnology. *Trends Biotechnol*. 2007; 25:269–277. [PubMed: 17433846]
34. Eisenbrey JR, Sridharan A, Machado P, et al. Three-dimensional subharmonic ultrasound imaging in vitro and in vivo. *Acad Radiol*. 2012; 19:732–739. [PubMed: 22464198]
35. Wang H, Hristov D, Qin J, et al. Three-dimensional Dynamic Contrast-enhanced US Imaging for Early Antiangiogenic Treatment Assessment in a Mouse Colon Cancer Model. *Radiology*. 2015; 277:424–434. [PubMed: 26020439]
36. Wang H, Kaneko OF, Tian L, et al. Three-dimensional ultrasound molecular imaging of angiogenesis in colon cancer using a clinical matrix array ultrasound transducer. *Invest Radiol*. 2015; 50:322–329. [PubMed: 25575176]
37. Zhou J, Wang H, Zhang H, et al. VEGFR2-targeted three-dimensional ultrasound imaging can predict responses to anti-angiogenic therapy in preclinical models of colon cancer. *Cancer Res*. 2016; doi: 10.1158/0008-5472.CAN-15-3271
38. De Palma M, Naldini L. Angiopoietin-2 TIEs up macrophages in tumor angiogenesis. *Clin Cancer Res*. 2011; 17:5226–5232. [PubMed: 21576085]
39. Fukuhara S, Sako K, Noda K, et al. Tie2 is tied at the cell-cell contacts and to extracellular matrix by angiopoietin-1. *Exp Mol Med*. 2009; 41:133–139. [PubMed: 19293632]
40. Fukuhara S, Sako K, Noda K, et al. Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis. *Histol Histopathol*. 2010; 25:387–396. [PubMed: 20054809]
41. Shimoda H. Immunohistochemical demonstration of Angiopoietin-2 in lymphatic vascular development. *Histochem Cell Biol*. 2009; 131:231–238. [PubMed: 18836739]

Figure 1. Tumor growth and vasculature in WT and *Ts-Biotag* melanomas

a Representative *in vivo* HFU images acquired longitudinally in each animal, 9–20 days post tumor cell inoculation, showed growth and changes in the morphology of each melanoma tumor. **b** Quantification of tumor volumes from longitudinal 3D HFU images showed no significant differences between WT (light gray; N = 7) and *Ts-Biotag* (dark gray; N = 8) tumors. **c** Representative 2D HFU (40MHz) images with overlaid MB signal (red) acquired during IV contrast injection (N = 4), showed detailed tumor and vascular morphology, and the high level of vascularization of the 2–3 week B16 melanomas (see Supplemental Video 1). **d** Quantification of CD31 staining in histological sections from size-matched WT (N = 7) and *Ts-Biotag* (N = 7) melanomas, harvested on days 16 and 20, confirmed the degree of tumor vascularization and showed no significant differences in MVD between tumors in the two groups. Scale bars = 2-mm.

Figure 2. Validation of endothelial cell labeling in *Ts-Biotag* melanomas

Immunohistochemistry analysis of sections from melanoma tumors extracted 1 h after *in vivo* injection of Av-FITC, and co-stained with **a** endothelial cell marker CD31, which **b** showed the presence of Av-FITC within the vasculature of *Ts-Biotag* but not WT tumors (N = 3 for each genotype). The WT and *Ts-Biotag* images in both the red (left) and green (middle) panels are displayed with the same window and level to facilitate qualitative comparison between the two genotypes. **c** Overlaid images demonstrated colocalization of Av-FITC and CD31, confirming labeling of vascular endothelial cells in *Ts-Biotag* melanomas (yellow, right panels). Magnification= 40 $\times$. Scale bar = 25- μ m.

Figure 3. *In vivo* multimodal vascular imaging in *Ts-Biotag* melanomas

In vivo targeted imaging studies, using: **a–c** NIR (N = 6 WT, N = 4 *Ts-Biotag*), **d–f** MRI (N = 4 WT, N = 8 *Ts-Biotag*) and **g–i** CEU (N = 8 WT, N = 8 *Ts-Biotag*), showed increased contrast in images of *Ts-Biotag* compared to WT tumors, after IV-injected avidinated contrast agents for each modality: SAV-680 (red) for NIR; Av-DTPA-Gd (white) for MRI; and Av-MB (green) overlaid on B-mode images for CEU. Quantitative analysis of images of WT (light gray) and *Ts-Biotag* (dark gray) melanomas for **c** NIR, **f** MRI and **i** CEU, demonstrated statistically significant differences in contrast enhancement for each modality

(* $P < 0.001$, ** $P < 0.002$). Additional *ex vivo* imaging of excised tumors was also performed for selected mice with NIR (Insets in **a** and **b**). Scale bars for panels e and h = 2 mm.

Figure 4. Comparison of conventional and *Biotag* Tie2-targeting with CEU

2D CEU images made in central sections of WT melanomas with control, isotype IgG-conjugated MBs, **a** MBi, (N = 5), conventional Tie2-targeted MBs, **b** MBt (N=5), and **c** Av-MBs (N = 5) all showed similar, sparsely distributed low-level contrast-enhancement, compared to the increased uptake and enhancement of Av-MBs in **d** *Ts-Biotag* melanomas (N = 5). Quantitative analysis, calculated as the number of labeled pixels within the tumor area, confirmed the obvious and significant increase in targeting using Av-MBs in *Ts-Biotag* tumors compared to any of the three MB types in WT tumors (e; * $P = 0.007$, ** $P < 0.001$). Scale bar for panels a–d = 2 mm.

Figure 5. Kinetics of Av-MB signal changes in WT and *Ts-Biotag* tumors

a Time-intensity curves were acquired 4 min after IV injection of Av-MBs in WT ($N = 5$) and *Ts-Biotag* ($N = 5$) with size-matched melanomas. NLC-signals (plotted as mean $\pm$ SD), measured in ROIs covering the entire tumor on each 2D HFU image, showed significantly higher contrast enhancement in the *Ts-Biotag* tumors, before and after the destruction pulse (vertical rectangle). Immediately after the high-power destruction pulse, the signal recovery using Av-MBs in the *Ts-Biotag* tumors was more rapid than in WT tumors, likely due to the increased binding efficiency of Av-MBs to *Ts-Biotag* blood vessels. This effect was

quantified by measuring the slope of the recovery curves within the first 2s (20 frames) after destruction. **b** Slopes were measured with Av-MBs in WT and *Ts-Biotag* mice, as well as with MBi and MBt in WT mice (N = 5 for each group; * P = 0.008; ** P = 0.007) and plotted as mean $\pm$ SD for each group. A linear regression analysis of the post-destruction slope of representative WT (R^2 = 0.781) and *Ts-Biotag* (R^2 = 0.938) mice is also shown (inset in b panel).

Figure 6. Longitudinal CEU of vascular changes in *Ts-Biotag* melanomas

a Longitudinal 2D and **b** 3D CEU images were acquired in each tumor from 9 to 20 days post B16 cell inoculation (N = 5; Av-MB signal in green, superimposed on 2D or 3D HFU images). Quantitative analysis of **c** 2D and **d** 3D targeting (number of labeled pixels/voxels) were performed after assigning tumors to three stages according to their measured volumes: I (< 50 mm³), II (51–150 mm³) and III (>150 mm³), after normalization to tumor volume in each mouse. The results showed **c** a fairly constant 2D targeted relative signal while **d** a

higher targeting at the early tumor stage (group I) was observed on the more comprehensive 3D targeted analysis. Scale bars for panels a and b = 1-mm

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