

# Molecular imaging of VEGF receptors in angiogenic vasculature with single-chain VEGF-based probes

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**We describe a new generation of protein-targeted contrast agents for multimodal imaging of the cell-surface receptors for vascular endothelial growth factor (VEGF). These receptors have a key role in angiogenesis and are important targets for drug development. Our probes are based on a single-chain recombinant VEGF expressed with a cysteine-containing tag that allows site-specific labeling with contrast agents for near-infrared fluorescence imaging, single-photon emission computed tomography or positron emission tomography. These probes retain VEGF activities *in vitro* and undergo selective and highly specific focal uptake into the vasculature of tumors and surrounding host tissue *in vivo*. The fluorescence contrast agent shows long-term persistence and co-localizes with endothelial cell markers, indicating that internalization is mediated by the receptors. We expect that multimodal imaging of VEGF receptors with these probes will be useful for clinical diagnosis and therapeutic monitoring, and will help to accelerate the development of new angiogenesis-directed drugs and treatments.**

Preclinical and clinical research indicates that anti-angiogenic drugs can improve the outcome in individuals with tumors. The development of such drugs might be facilitated by the introduction of imaging agents that bind selectively to angiogenic vasculature<sup>1–3</sup>. VEGF receptors, the main target for emerging anti-angiogenic therapies<sup>4,5</sup>, are particularly attractive targets for molecular imaging because they are overexpressed in angiogenic endothelium<sup>6–9</sup>, accessible directly from the bloodstream, and internalized when they bind VEGF<sup>10</sup>, allowing contrast agents to accumulate and be retained.

Most of the VEGF-based imaging agents that have been reported<sup>11–17</sup> are unsuitable for clinical development because of uncertain binding activity of the protein, owing to damage by random radiolabeling<sup>11–13</sup>; unacceptably high liver uptake<sup>14</sup>; or complex probe design<sup>15,16</sup>. As a new approach to the labeling of targeting proteins, we developed a 15-amino-acid humanized fusion tag, named Cys-tag, which contains a unique cysteine for site-specific modification by facile thiol-directed chemistries<sup>17,18</sup>. We found that Cys-tagged dimeric VEGF, site-specifically labeled with technetium-99m (<sup>99m</sup>Tc), was useful for

imaging tumor vasculature<sup>17,18</sup> but was still too complex to be used as a targeting protein. Here, we describe molecular imaging agents based on a new, robust, single-chain Cys-tagged VEGF (scVEGF)<sup>19</sup>, a fusion protein that combines two fragments (amino acids 3–112) of human VEGF<sub>121</sub> cloned head to tail. We show that scVEGF-based imaging agents can be readily prepared for near-infrared fluorescence imaging (NIRF), single-photon emission computed tomography (SPECT) or positron emission tomography (PET) imaging of VEGF receptors in angiogenic vasculature.

## RESULTS

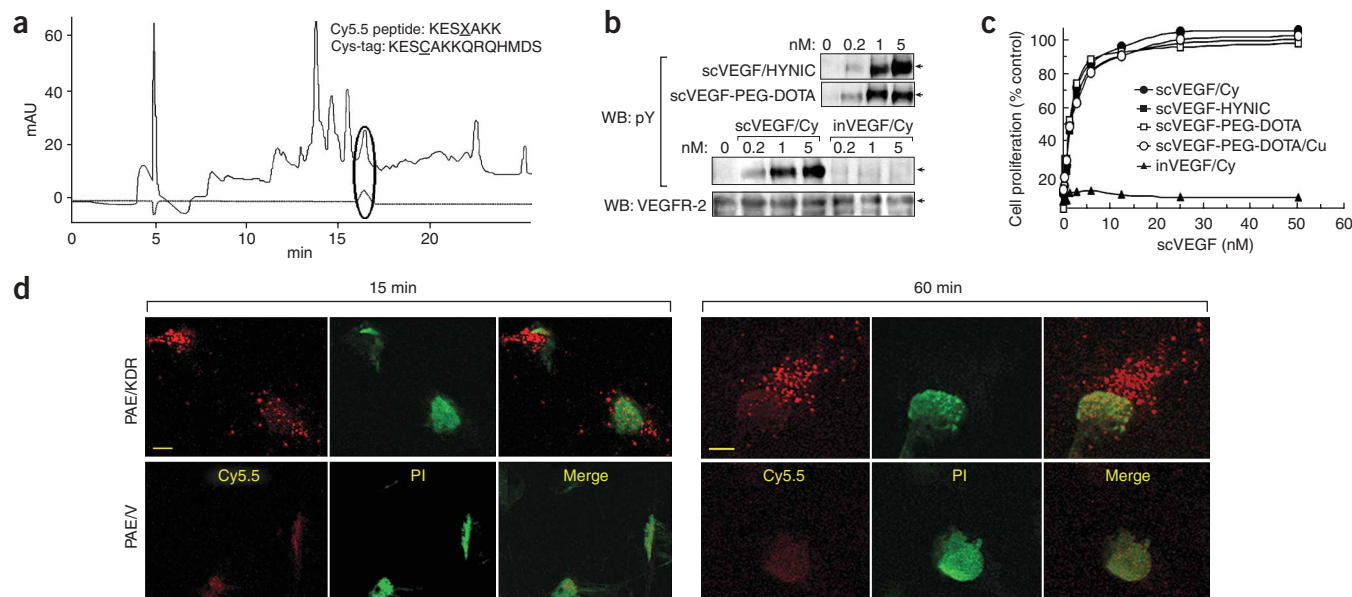
### Site-specific modification of scVEGF with contrast agents

When we recovered bacterially expressed scVEGF from inclusion bodies and refolded it in a glutathione-containing buffer, a single reactive cysteine appeared only after mild treatment with dithiothreitol (DTT)<sup>19</sup>. To assign this cysteine, we modified DTT-treated scVEGF with Cy5.5-maleimide and cleaved it with BrCN. Fractionation of cleavage products using reverse-phase high-performance liquid chromatography (RP-HPLC) revealed a single Cy5.5-labeled peptide (Fig. 1a, circled peak). The N-terminal amino-acid sequence of this peptide (Fig. 1a) corresponded to that of Cys-tag. Thus, in spite of the presence of 16 'native' cysteines in scVEGF, mild DTT treatment liberated only the Cys-tag thiol group and made it available for site-specific modification.

We labeled Cys-tag in scVEGF with three agents: a Cy5.5 dye for NIRF imaging, yielding a scVEGF/Cy probe; the <sup>99m</sup>Tc chelator hydraziniumpyridine (HYNIC) for SPECT imaging; and a *de novo*-synthesized <sup>64</sup>Cu chelator, PEG-DOTA, for PET imaging. The labeled conjugates showed activities similar to those of unmodified scVEGF in two tissue culture assays<sup>17–20</sup>: tyrosine phosphorylation of VEGFR-2, and competition with the chimeric toxin SLT-VEGF for binding to VEGFR-2 (Fig. 1b,c). Incubation of scVEGF-PEG-DOTA/<sup>64</sup>Cu at elevated temperatures (55 °C for 1 h or 95 °C for 20 min), which is essential for chelation of PET radionuclides, did not affect its ability to compete with SLT-VEGF (Fig. 1c, for 95 °C treatment). To obtain appropriate controls for nonspecific probe uptake, we inactivated each labeled scVEGF by conjugation of 7 or 8 biotins using *N*-hydroxy-succinimide (NHS) chemistry as described<sup>18</sup>. As expected, biotinylated

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**Figure 1** Site-specific modifications do not affect binding and internalization of scVEGF. **(a)** RP-HPLC separation of scVEGF/Cy cleavage products with detection at 280 nm (solid line) and 598 nm (dotted line). Inset: N-terminal sequencing of Cy5.5-labeled peptide circled on the HPLC profile. Peaks around 5 min are of systemic origin. **(b)** Stimulation of VEGFR-2 tyrosine autophosphorylation in 293/KDR cells. Lower panel: blot was stripped and re-probed with antibody for VEGFR-2. InVEGF/Cy, inactivated scVEGF/Cy. WB, western blot. **(c)** Competition with SLT-VEGF in 293/KDR cells. scVEGF conjugated to DOTA was tested before (scVEGF-PEG-DOTA) and after (scVEGF-PEG-DOTA/Cu) incubation at 95 °C for 20 min. **(d)** VEGFR-2 expressing endothelial cells rapidly internalize scVEGF/Cy. Cy5.5, red; propidium iodide (PI), green. Scale bars, 20  $\mu$ m.

conjugates did not bind to VEGFR-2 (**Fig. 1b,c** for inactivated scVEGF/Cy, called inVEGF/Cy). We further characterized binding of scVEGF-PEG-DOTA/ $^{64}\text{Cu}$  to 293/KDR cells by Scatchard analysis, which yielded a  $K_d$  of 2.8 nM with  $1.3 \times 10^6$  binding sites per cell (**Supplementary Fig. 1** online). This value is similar to the corresponding values reported for binding of a  $^{125}\text{I}$ -VEGF $_{121}$  fusion protein to the same cells<sup>21</sup>. Finally, we tracked the internalization of scVEGF/Cy using confocal microscopy of live porcine aortic endothelial (PAE) cells, either overexpressing VEGFR-2 (PAE/KDR) or lacking the receptor (PAE/V). Only PAE/KDR cells accumulated Cy5.5 in a time-dependent manner, as distinct specks in the perinuclear space (**Fig. 1d**), indicating that labeling of Cys-tag did not prevent VEGFR-2-mediated endocytosis of scVEGF. Together, these data indicate that the functional characteristics of scVEGF were not affected by site-specific modification of Cys-tag.

### NIRF imaging with scVEGF/Cy

For NIRF imaging we used luciferase-expressing derivatives of two widely used mammary adenocarcinoma cell lines, mouse 4T1 (ref. 22) and human MDA-MB-231, grown orthotopically in Balb/c and SCID/NCr mice, respectively. The borders of these tumors were readily defined by bioluminescence imaging (BLI), providing a framework for assignment of NIRF images obtained with scVEGF/Cy.

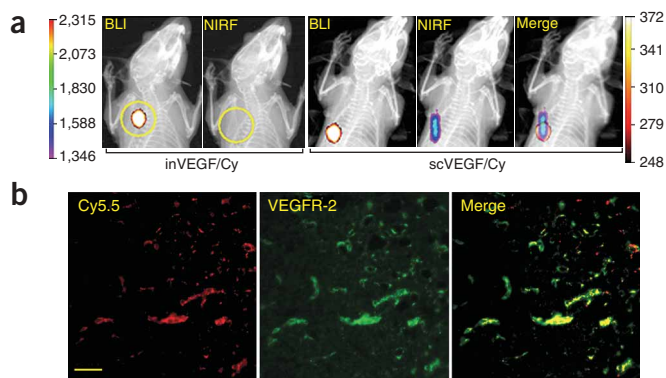
To confirm the selectivity of scVEGF/Cy binding *in vivo*, we first tested functionally inactive inVEGF/Cy, and found no evidence of its uptake in the tumor areas defined by BLI (**Fig. 2a**). By contrast, functionally active scVEGF/Cy selectively accumulated in BLI-defined areas, indicating that binding to VEGF receptors was crucial for imaging (**Fig. 2a**, scVEGF/Cy panels). Selective accumulation of scVEGF/Cy could be detected in barely palpable tumors, indicating that imaging of VEGF receptors might be particularly useful for detecting small primary tumors and metastatic lesions. The pattern

of scVEGF/Cy accumulation was highly heterogeneous, as has been reported for all molecular imaging probes that target endothelial cell markers<sup>1–3,11–17</sup>. Injection of scVEGF/Cy together with a tenfold excess of unlabeled scVEGF resulted in a markedly weaker NIRF image (data not shown). The intensity of NIRF images persisted for several hours after scVEGF/Cy injection and remained nearly constant for at least 7 d after injection, whereas the tumors continued to grow over the area of the probe uptake (**Supplementary Figs. 2 and 3** online).

As clearance of radiolabeled VEGF-based probes occurs within around 1 h (refs. 16,18), persistent Cy5.5 fluorescence within the tumor area could be best explained by internalization of scVEGF/Cy by receptor-mediated endocytosis and entrapment of highly charged Cy5.5 inside cells. For both tumor models, Cy5.5 fluorescence co-localized with immunofluorescence staining for VEGFR-2, (**Fig. 2b**, for 4T1luc tumors), which, in turn, co-localized with a pan-endothelial marker, CD31 (data not shown). Some areas had low Cy5.5 fluorescence, however, reflecting the heterogeneity of scVEGF/Cy accumulation, in spite of the presence of VEGFR-2 (**Fig. 2c**).

Hypothetically, scVEGF/Cy could accumulate as a result of enhanced endocytic activity of tumor endothelial cells. We therefore tested whether an unrelated growth factor can target Cy5.5 to the tumor endothelium. For these experiments, we engineered a Cys-tagged epidermal growth factor, site-specifically labeled it with Cy5.5-maleimide and tested it in the 4T1 tumor model. Although the label accumulated in the tumor, Cy5.5 fluorescence did not co-localize with CD31 (**Supplementary Fig. 4** online).

Our NIRF experiments showed that the areas of enhanced scVEGF/Cy uptake were consistently extended beyond BLI-defined tumor areas in both the 4T1luc and MDA-231luc models (**Fig. 2b**, **Supplementary Figs. 2 and 3** online). As BLI depends on the uptake and metabolism of luciferin and does not necessarily mark the exact histological margins of the tumor, this effect might be an artifact. As tumors are

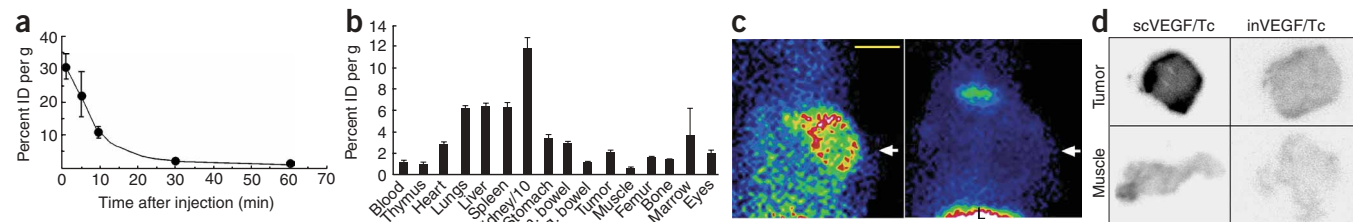
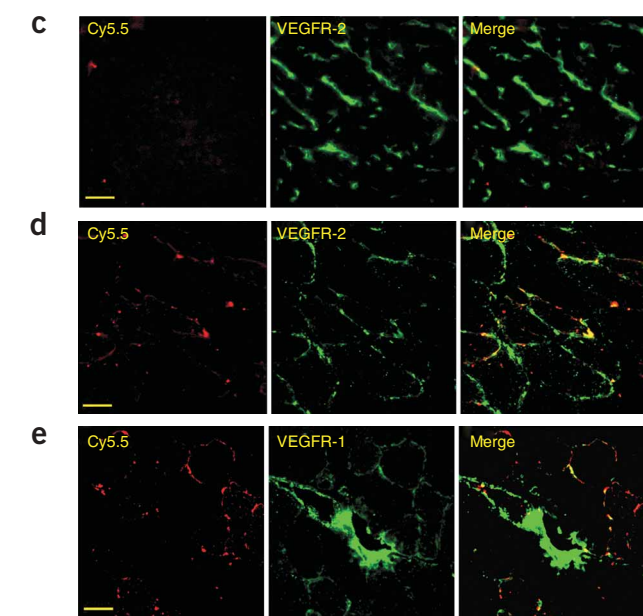


**Figure 2** NIRF imaging with scVEGF/Cy and localization of Cy5.5 on histological sections. **(a)** Sequential NIRF imaging and BLI of the same 4T1 luc-tumor-bearing mouse injected intravenously with scVEGF/Cy or inactive control inVEGF/Cy. Representative images taken from mouse in the same position are shown. **(b,c)** Co-localization of Cy5.5 fluorescence and immunofluorescence staining for VEGFR-2 on 4T1 luc tumor cryosections. Cy5.5, red; VEGFR-2, green. Scale bars, 20 μm. **(d,e)** Co-localization of Cy5.5 and VEGF receptors in muscle tissue contiguous to MDA-231 luc tumor. Cy5.5, red; VEGFR-2 and VEGFR-1, green. Scale bars, 20 μm.

known to induce changes in the surrounding host vasculature<sup>23</sup>, however, we hypothesized that such tumor-host interactions might be responsible for the accumulation of scVEGF beyond the margins of the tumors. Co-localization analysis of serial cryosections containing both tumor and surrounding muscle tissue revealed Cy5.5 fluorescence in endothelial capillary cells between large, well-defined muscle cells (Fig. 2d,e). In these areas, Cy5.5 fluorescence co-localized with immunofluorescence staining for both VEGFR-2 and VEGFR-1, indicating that both receptors might be responsible for the uptake of scVEGF/Cy in tumor-activated host vasculature.

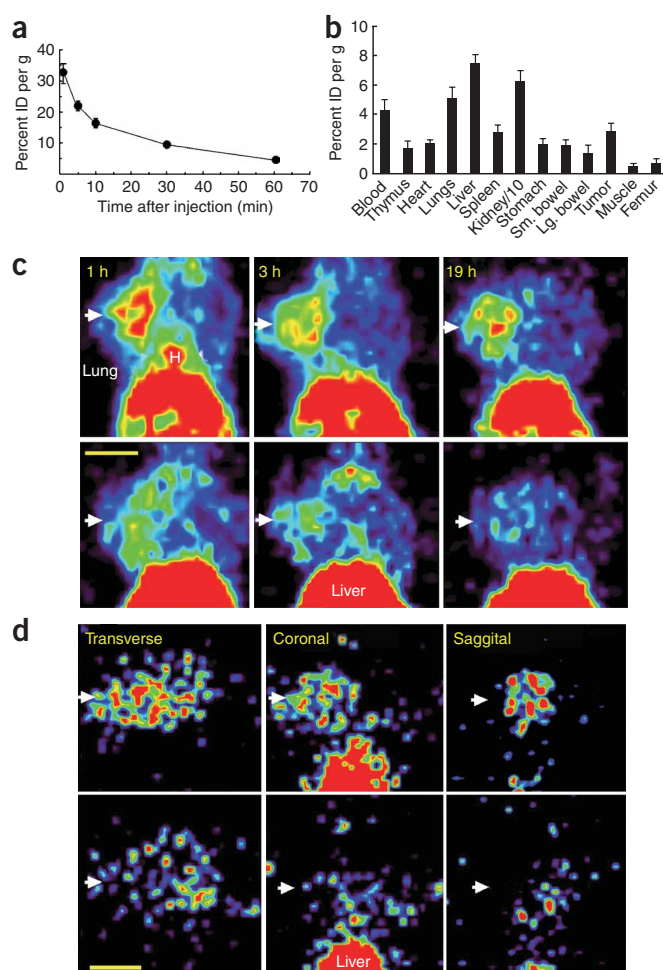
### SPECT imaging with scVEGF/Tc

We used <sup>99m</sup>Tc to radiolabel scVEGF-HYNIC and its inactivated analog, yielding scVEGF/Tc and inVEGF/Tc, respectively. Both tracers were >95% cleared from the circulation within 50 min of intravenous injection (Fig. 3a, for scVEGF/Tc). To characterize the stability of scVEGF/Tc in the circulation, we obtained plasma from blood collected 3 and 50 min after the injection and precipitated scVEGF/Tc with the soluble VEGF receptor KDR-Fc from plasma aliquots containing the same amounts of radioactivity. Western blot analysis showed that similar amounts of scVEGF were collected from both



**Figure 3** Blood clearance, biodistribution and SPECT imaging of scVEGF/Tc. **(a,b)** Blood clearance **(a)** and biodistribution **(b)** of radioactivity 1 h after intravenous injection of scVEGF/Tc. Sm. and Lg. bowel, small and large bowel, respectively. ID, injected dose. **(c)** SPECT imaging. Serial frontal images of two separate 4T1 luc-tumor-bearing mice taken 1 h after injection of scVEGF/Tc (left) or inVEGF/Tc (right). Arrows indicate tumor positions. L, liver rim; white is the highest activity in false color images. Scale bar, 1 cm. **(d)** Autoradiographs of cryosections of tumors and normal pectoralis muscle from the contralateral chest wall removed immediately after *in vivo* imaging.





**Figure 4** Blood clearance, biodistribution and PET imaging of scVEGF/Cu. (a,b) Blood clearance (a) and biodistribution (b) of radioactivity 2 h after injection with scVEGF/Cu. (c) PET imaging. Serial frontal images of two mice bearing 4T1luc tumors in the left axillary fat pads 1, 3 and 19 h after injection of either 100  $\mu$ Ci scVEGF/Cu (top row) or equivalent amounts of inVEGF/Cu (bottom row). White arrows mark left mammary fat pad tumor in each image. H, heart or blood pool activity observed at 1 h. Scale bar, 1 cm. (d) Representative transverse, coronal and sagittal tomographic slices through the left mouse mammary fat pad tumor taken 2 h after injection of 6.25  $\mu$ Ci of free (upper row) or KDR-Fc-bound scVEGF/Cu (lower row). White arrows mark the left mammary fat pad tumor. Scale bar, 1 cm.

difference was greatest after 19 h (Fig. 4c). To confirm that accumulation of scVEGF/Cu was receptor mediated, we preincubated scVEGF/Cu with KDR-Fc before injection, and used the same amounts of KDR-Fc-bound and free scVEGF/Cu for PET imaging. Preincubation with KDR-Fc greatly reduced the accumulation of scVEGF/Cu in the tumor area (Fig. 4d).

## DISCUSSION

Our data indicate that scVEGF is a robust targeting protein that allows the development of molecular imaging agents for different imaging modalities through facile thiol-directed chemistries. The use of VEGF-based imaging might raise concerns about a potential pro-angiogenic outcome. Although a definitive answer to these concerns requires extensive experimentation, we expect that injection of small amounts of the protein for diagnostic imaging will cause no adverse effects<sup>26</sup>. In addition, it has been reported that endogenous expression of a VEGF fragment containing amino acids 1–113 does not stimulate tumor growth and, in fact, can inhibit angiogenesis<sup>27</sup>. In view of these data, we expect that scVEGF, which combines two fragments consisting of amino acids 3–112 of the protein, would be unlikely to induce angiogenesis or secondary tumors, especially at the tracer dosages needed for imaging.

Imaging by NIRF, SPECT and PET shows that scVEGF-based agents bind to and are internalized by VEGF receptors in the tumor area. Nevertheless, low but detectable accumulation of inactivated or KDR-Fc-bound scVEGF/Cu probe in the tumor area indicates that nonspecific mechanisms might also contribute to accumulation within the tumor. Further experiments are required to establish whether nonspecific mechanisms also contribute to imaging with scVEGF-based probes, or whether they are completely superseded by receptor-mediated uptake.

Judging by co-registered NIRF and BLI images, the scVEGF/Cu uptake area includes contiguous host vasculature, where sequestered Cy5.5 is found in host capillary endothelial cells. As activation of host vasculature is induced by very small growing tumor lesions<sup>25</sup>, investigation is warranted to establish whether diagnostic imaging with scVEGF-based probes could improve detection of such lesions.

The accumulation and retention of all scVEGF-based probes in the tumor area is highly heterogeneous, and several mechanisms (for example, non-uniform perfusion of tumor vasculature, differential receptor occupancy by host VEGF or differential accessibility of VEGF receptors on luminal and subluminal surfaces of the endothelium) might be responsible for this effect. As the clinical value of scVEGF-based imaging agents will depend to a great extent on their ability to characterize the prevalence of VEGF receptors, this issue should be addressed in future research.

In summary, angiogenesis is and will remain an important therapeutic target that is suitable for complex and personalized treatment regimens<sup>28–31</sup>. We expect that molecular imaging of VEGF receptors with scVEGF-based agents will facilitate progress in this area.

## PET imaging with scVEGF/Cu

We used <sup>64</sup>Cu to label scVEGF-PEG-DOTA and its inactivated analog, yielding scVEGF/Cu and inVEGF/Cu, respectively. As expected, because of incorporation of the PEG linker between DOTA and scVEGF, scVEGF/Cu showed considerably prolonged blood clearance (Fig. 3a), as compared with scVEGF directly conjugated to DOTA (data not shown) or scVEGF/Tc (Fig. 4a). The stability in the circulation tested by KDR-Fc precipitation as described above (Supplementary Fig. 5 online) indicated that scVEGF/Cu was stable and intact until blood clearance of at least 95% of the activity. We tested the stability of scVEGF/Cu in plasma *ex vivo* for times beyond 1 h (Supplementary Fig. 7 online) and found that less than 5% of <sup>64</sup>Cu was released from protein complexes even after 5 h of incubation at 37 °C, either in pooled mouse plasma or in radiolabeling buffer.

The biodistribution of scVEGF/Cu showed half as much renal accumulation of the tracer as compared with scVEGF/Tc (Fig. 4b, compare with Fig. 3b), a distinct advantage with respect to the relative radiation dose to the kidneys. More importantly, scVEGF/Cu also delivered more tracer to the tumor (Fig. 4b, compare with Fig. 3b). As our NIRF imaging results indicated that scVEGF-based probes were internalized and that the imaging agent remained sequestered for a long time after clearance, we performed serial PET imaging of mice bearing 4T1luc tumors 1, 3 and 19 h after injection of either scVEGF/Cu (Fig. 4c, top row) or inVEGF/Cu (Fig. 4c, lower row). Persistent focal accumulation of scVEGF/Cu in the tumor area was substantially higher and more heterogeneous than that of inVEGF/Cu, and the

## METHODS

**Tissue culture.** We stably transfected 4T1 (ATCC no. CRL-2539) and MDA-MB-231 (ATCC no. HTB-26) cells with the pGL3 (Promega) and pcDNA/Zeo (Invitrogen) plasmids and clonally selected them on zeocin ( $0.5 \text{ mg ml}^{-1}$ ). We designated the clones with the highest luciferase activity 4T1luc and MDA-231luc, respectively. 293/KDR and PAE/KDR cells expressing full-length human VEGFR-2, 293/KDR-Fc cells expressing soluble VEGFR-2 (KDR-Fc), and PAE/V cells have been described previously<sup>21</sup>. We performed VEGFR-2 autophosphorylation and competition with SLT-VEGF as described<sup>17–20</sup>.

**Cell microscopy.** We incubated PAE/KDR and PAE/V cells with  $10 \text{ nM}$  scVEGF/Cy at  $37^\circ\text{C}$ , washed them extensively with PBS and then with  $0.5 \text{ M}$  NaCl in PBS, mounted them in culture medium with  $2 \mu\text{M}$  propidium iodide (Sigma) and imaged them immediately using a confocal laser scanning microscope (Olympus FV1000; Olympus America).

**Animals.** We gave BALB/c and SCID/NCr (BALB/c background) mice (5–6 weeks old, Charles River Laboratories) 4T1luc cells ( $5 \times 10^3$  cells per mouse) and MDA-231luc cells ( $5 \times 10^6$  cells per mouse), respectively, in the fat pad regions. The protocol for the animal studies was approved by the Institutional Animal Care and Use Committees at the University of Connecticut Health Center and at Stanford University.

**NIRF imaging.** We treated scVEGF with DTT as described<sup>19</sup>, mixed it with a fivefold molar excess of Cy5.5-maleimide (GE Healthcare), incubated it for  $1.5 \text{ h}$  and desalted it. We separated free scVEGF on a Q-column (GE Healthcare) and calculated the extent of modification from RP-HPLC peak intensities (C4 Alltech Macrosphere) at  $216 \text{ nm}$  for protein and  $598 \text{ nm}$  for Cy5.5. For NIRF and BLI, we gave mice scVEGF/Cy ( $10 \mu\text{g}$  per mouse) intravenously;  $5 \text{ min}$  later, luciferin ( $0.5 \text{ mg}$  per mouse) intraperitoneally; and  $5 \text{ min}$  later, a mixture of ketamine ( $120 \text{ mg kg}^{-1}$ ) and xylazine ( $8 \text{ mg kg}^{-1}$ ) intramuscularly. We obtained images on a Kodak Image Station 4000 Multi-Modal Imaging System (IS4000MM) equipped with an X-ray unit and on a Kodak Image Station 2000.

**N-terminal sequencing.** scVEGF conjugates were completely denatured with a 100-fold molar excess of Tris(2-carboxyethyl)phosphine (Pierce) at  $65^\circ\text{C}$  for  $1 \text{ h}$  followed by a 30-min incubation with a 1,000-fold excess of iodoacetamide, then digested with BrCN (60-fold molar excess) for  $16 \text{ h}$  in 70% formic acid. We analyzed peptide fragments separated by RP-HPLC (C4 column) by N-terminal sequencing at Yale Cancer Center Mass Spectrometry Resources at the W. M. Keck Foundation Biotechnology Resource Laboratory.

**Immunofluorescence.** We fixed tumor  $7\text{-}\mu\text{m}$ -thick cryosections with 1% formaldehyde (Polysciences) and blocked them with an avidin-biotin blocking kit (Molecular Probes) followed by blocking with 5% appropriate normal serum. Antibodies for VEGFR-2(KDR/Flk-1) and CD31(PECAM) were from Pharmingen; antibody for VEGFR-1(Flt-1) was from NeoMarkers. We coupled biotinylated second antibodies to streptavidin-peroxidase conjugate and visualized them by AlexaFluor-488 using tyramine amplification technique (TSA HRP-streptavidin Kit, Molecular Probes). We observed and digitized images on a Zeiss LSM 510 microscope using a  $\times 43$  oil objective.

**SPECT imaging.** We conjugated HYNIC-maleimide to scVEGF and radiolabeled it with  $^{99\text{m}}\text{Tc}$  as described<sup>16,18</sup> to an activity of  $25 \mu\text{Ci}$  per  $\mu\text{g}$  protein for clearance and biodistribution ( $0.1 \text{ mCi}$  per mouse,  $n = 4$ ) and  $160 \mu\text{Ci}$  per  $\mu\text{g}$  for SPECT imaging ( $1\text{--}1.2 \text{ mCi}$  per mouse,  $n = 5$ ). We performed blood clearance, biodistribution and SPECT imaging as described<sup>16,18</sup>.

**PET imaging.** We mixed NHS-PEG-maleimide (Nektar Therapeutics) with a fivefold molar excess of 2-(4-aminobenzyl)-DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, Macrocyclic) in carbonate buffer pH 8.0 and incubated it for  $1 \text{ h}$ . After quenching of unreacted NHS ( $0.1 \text{ M}$  Tris-HCl, pH 8.0,  $30 \text{ min}$ ), we added deprotected scVEGF to a final protein-to-DOTA molar ratio of 1:10, incubated for  $1 \text{ h}$ , and then carried out RP-HPLC purification on a preparative C4 Vydac column. We incubated  $20 \mu\text{g}$  of conjugate with  $400\text{--}500 \mu\text{Ci}$  of  $^{64}\text{Cu}$  (Washington University Medical School) in  $0.1 \text{ M}$  sodium acetate (pH 5.5) for  $1 \text{ h}$  at  $55^\circ\text{C}$ , sequestered residual  $^{64}\text{Cu}$  by  $1 \text{ mM}$  EDTA, and removed it by desalting, yielding 40% efficiency of scVEGF-PEG-DOTA

radiolabeling. We gave two groups of tumor-bearing mice ( $n = 6$ )  $2\text{--}5 \mu\text{Ci}$  per mouse intravenously for biodistribution studies or  $100 \mu\text{Ci}$  per mouse intravenously for PET imaging. To sequester scVEGF, we mixed scVEGF/Cu ( $6.25 \mu\text{Ci}$ ,  $1.25 \mu\text{g}$ ) with a fivefold molar excess of recombinant human soluble VEGFR-2/KDR-Fc (R&D Systems) in  $0.1 \text{ M}$  Tris-HCl, pH 8.0,  $0.15 \text{ M}$  NaCl and  $0.1\%$  BSA, and incubated it for  $2 \text{ h}$  at  $4^\circ\text{C}$  before injection. We incubated control scVEGF/Cu ( $6.25 \mu\text{Ci}$ ,  $1.25 \mu\text{g}$ ) under the same conditions without soluble VEGFR-2. We obtained 10-min static scans of mice in a prone position on a microPET R4 rodent model scanner (Concorde Microsystems) equipped with computer-controlled vertical and horizontal bed motion, with an effective axial field of view (FOV) of  $7.8 \text{ cm}$  and a transaxial FOV of  $10 \text{ cm}$ . We placed mice anesthetized with 2% isoflurane near the center of the FOV to ensure the highest image resolution and sensitivity. We reconstructed the images by a two-dimensional, ordered-subsets expectation maximum algorithm. No correction was necessary for attenuation and scattering. We obtained relative tumor or organ radioactivity concentrations from mean pixel values within the multiple ROI volumes.

*Note: Supplementary information is available on the Nature Medicine website.*

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## AUTHOR CONTRIBUTIONS

M.V.B. and Z.L. contributed equally to this manuscript. M.V.B., F.G.B. and J.M.B. conceived of and initiated the project, coordinated discovery research and wrote the manuscript; M.V.B. designed imaging conjugates, validated all probes in tissue culture and performed colocalization studies; M.V.B. and J.M.B. designed and conducted optical imaging experiments; Z.L. and F.G.B. prepared radiolabeled SPECT and PET probes and performed all experiments with these probes; V.P. and B.T.G. made scVEGF-based conjugates; and K.C. performed confocal microscopy experiments. All authors discussed and commented on the manuscript.

## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at [www.nature.com/naturemedicine/](http://www.nature.com/naturemedicine/).

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1. Miller, J.C. *et al.* Imaging angiogenesis: applications and potential for drug development. *J. Natl. Cancer Inst.* **97**, 172–187 (2005).
2. Haubner, R. & Wester, H.J. Radiolabeled tracers for imaging of tumor angiogenesis and evaluation of anti-angiogenic therapies. *Curr. Pharm. Des.* **10**, 1439–1455 (2004).
3. Wu, Y. *et al.* microPET imaging of glioma integrin  $\alpha_3\beta_3$  expression using (64)Cu-labeled tetrameric RGD peptide. *J. Nucl. Med.* **46**, 1707–1718 (2005).
4. Ferrara, N., Gerber, H.P. & LeCouter, J. The biology of VEGF and its receptors. *Nat. Med.* **9**, 669–676 (2003).
5. Manley, P.W. *et al.* Advances in the structural biology, design and clinical development of VEGF-R kinase inhibitors for the treatment of angiogenesis. *Biochim. Biophys. Acta* **1697**, 17–27 (2004).
6. Couffinhal, T. *et al.* Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in normal and arteriosclerotic human arteries. *Am. J. Pathol.* **150**, 1673–1685 (1997).
7. Koukourakis, M.I. *et al.* Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res.* **60**, 3088–3095 (2000).
8. Witmer, A.N. *et al.* Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia. *J. Histochem. Cytochem.* **50**, 767–777 (2002).
9. Vajkoczy, P. *et al.* Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J. Clin. Invest.* **109**, 777–785 (2002).
10. Bikfalvi, A. *et al.* Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: binding, internalization, degradation, and biological effect. *J. Cell. Physiol.* **149**, 50–59 (1991).

11. Li, S. *et al.* Characterization of  $^{123}\text{I}$ -vascular endothelial growth factor-binding sites expressed on human tumour cells: possible implication for tumour scintigraphy. *Int. J. Cancer* **91**, 789–796 (2001).
12. Li, S. *et al.* Imaging gastrointestinal tumours using vascular endothelial growth factor-165 (VEGF165) receptor scintigraphy. *Annals Oncol.* **14**, 1274–1277 (2003).
13. Li, S. *et al.* Iodine-123-vascular endothelial growth factor-165 ( $^{123}\text{I}$ -VEGF165). Biodistribution, safety and radiation dosimetry in patients with pancreatic carcinoma. *Q. J. Nucl. Med. Mol. Imag.* **48**, 198–206 (2004).
14. Lu, E. *et al.* Targeted *in vivo* labeling of receptors for vascular endothelial growth factor: approach to identification of ischemic tissue. *Circulation* **108**, 97–103 (2003).
15. Chan, C. *et al.* A human transferrin(hnTf)-VEGF fusion protein containing an integrated binding site for  $^{111}\text{In}$  for imaging tumor angiogenesis. *J. Nucl. Med.* **46**, 1745–1752 (2005).
16. Blankenberg, F.G. *et al.* Tumor imaging using a standardized radiolabeled adapter protein docked to vascular endothelial growth factor (VEGF). *J. Nucl. Med.* **45**, 1373–1380 (2004).
17. Backer, M.V. *et al.* Vascular endothelial growth factor selectively targets boronated dendrimers to tumor vasculature. *Mol. Cancer Ther.* **4**, 1423–1429 (2005).
18. Blankenberg, F.G. *et al.* *In vivo* tumor angiogenesis imaging with site-specific labeled  $^{99\text{m}}\text{Tc}$ -HYNIC-VEGF. *Eur. J. Nucl. Med. Mol. Imag.* **33**, 841–848 (2006).
19. Backer, M.V., Patel, V., Jehning, B.T., Claffey, K. & Backer, J.M. Surface immobilization of active vascular endothelial growth factor via a cysteine-containing tag. *Biomaterials* **27**, 5452–5458 (2006).
20. Backer, M.V. & Backer, J.M. Targeting endothelial cells overexpressing VEGFR-2: selective toxicity of shiga-like toxin-VEGF fusion proteins. *Bioconjugate Chem.* **12**, 1066–1073 (2001).
21. Backer, M.V. & Backer, J.M. Functionally active VEGF fusion proteins. *Prot. Exp. Purif.* **23**, 1–7 (2001).
22. Heppner, G.H. *et al.* Nontransgenic models of breast cancer. *Breast Cancer Res.* **2**, 331–334 (2000).
23. Li, C.Y. *et al.* Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. *J. Natl. Cancer Inst.* **92**, 143–147 (2000).
24. Ono, M. *et al.* Intracellular metabolic fate of radioactivity after injection of technetium-99m-labeled hydrazino nicotinamide derivatized proteins. *Bioconjugate Chem.* **10**, 386–394 (1999).
25. Ono, M. *et al.* Control of radioactivity pharmacokinetics of  $^{99\text{m}}\text{Tc}$ -HYNIC-labeled polypeptides derivatized with ternary ligand complexes. *Bioconjugate Chem.* **13**, 491–501 (2002).
26. Zhang, W. *et al.* A monoclonal antibody that blocks VEGF binding to VEGFR2 (KDR/Flk-1) inhibits vascular expression of Flk-1 and tumor growth in an orthotopic human breast cancer model. *Angiogenesis* **5**, 35–44 (2002).
27. Lee, S. *et al.* Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J. Cell Biol.* **169**, 681–691 (2005).
28. Jain, R.K. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* **307**, 58–62 (2005).
29. McCarty, M.F. *et al.* Promises and pitfalls of anti-angiogenic therapy in clinical trials. *Trends Mol. Med.* **9**, 53–58 (2003).
30. Kerbel, R.S. & Kamen, B.A. The anti-angiogenic basis of metronomic chemotherapy. *Nat. Rev. Cancer* **4**, 423–436 (2004).
31. Jain, R.K. *et al.* Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nature Clin. Practice Onc* **3**, 24–40 (2006).