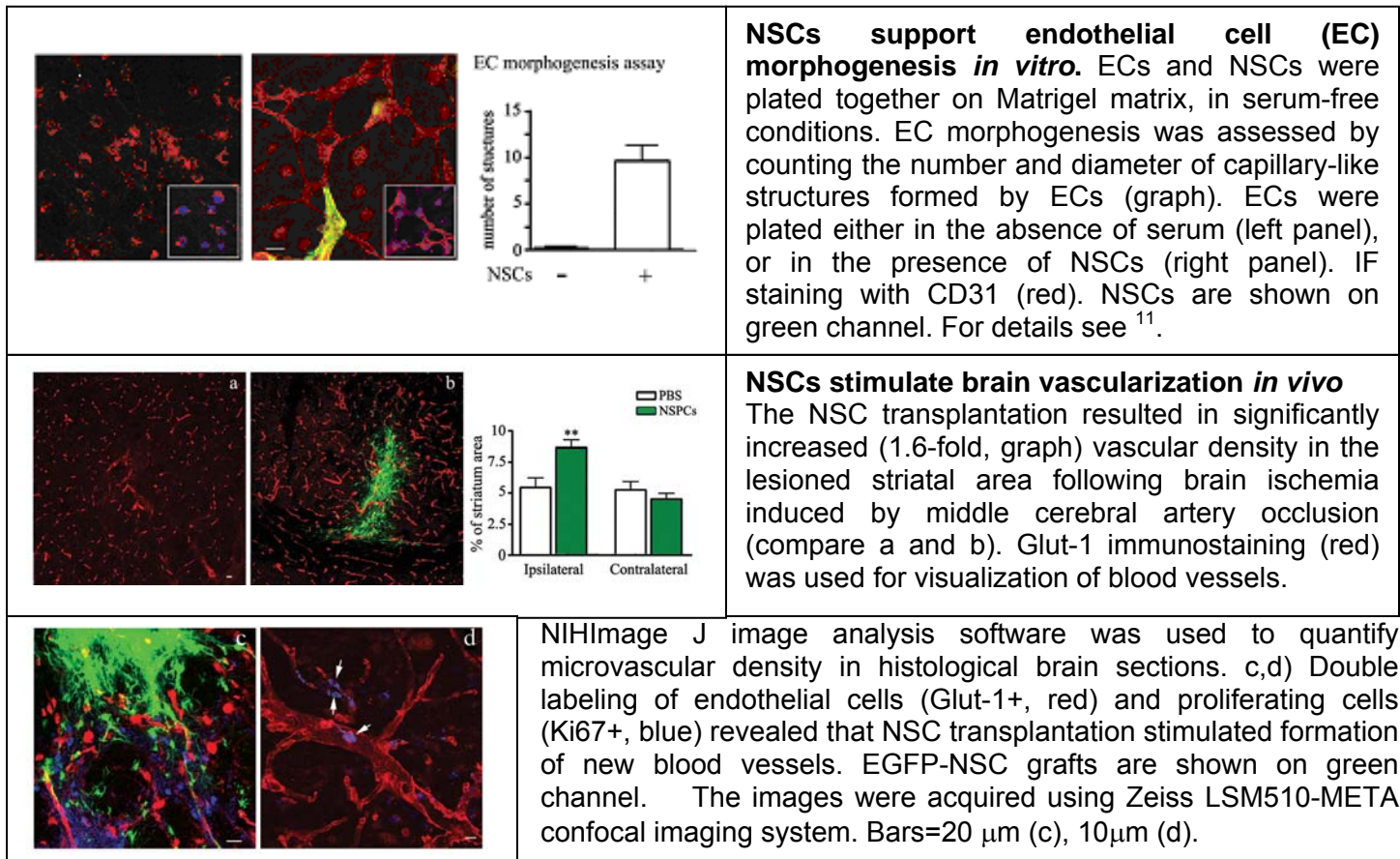


PRELIMINARY STUDIES

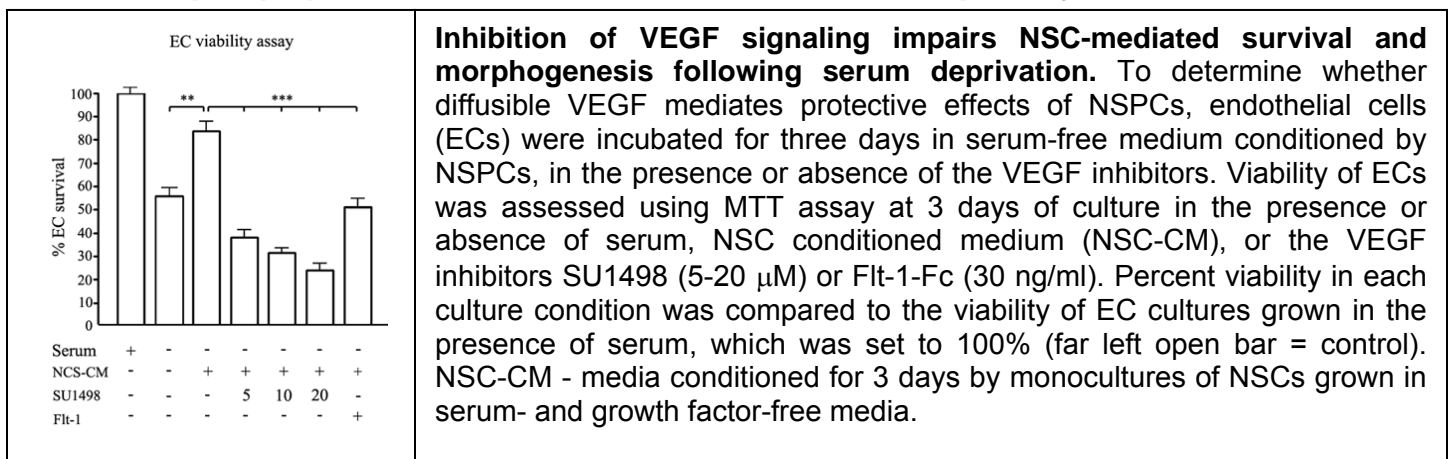
The following Preliminary data demonstrate our ability to: 1) study interaction between stem cells and endothelial cells *in vitro* using EC morphogenesis and MTT viability assays, 2) transplant stem cells into the mouse brain and assess the level of vascularization and angiogenesis, 3) knock down HIF-1 α expression in cultured stem cells using shRNAi lentivector technology, and 4) perform magnetic isolation of human BTSCs and their *in vitro* characterization.

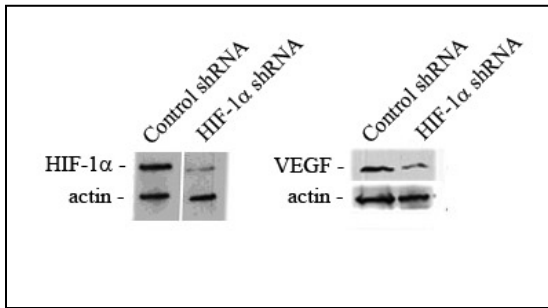
1. NSCs demonstrate vasculotropic properties *in vitro* and *in vivo*



Taken together, these data demonstrate the vasculotropic effect of NSCs: the neural stem cells support endothelial morphogenesis *in vitro* and induce angiogenesis *in vivo*. These results will be extended in the proposed experiments on BTSCs, to determine the role of BTSC-mediated support of tumor vascularization.

2. Vasculotropic properties of NSCs are mediated via HIF-1 α /VEGF pathway



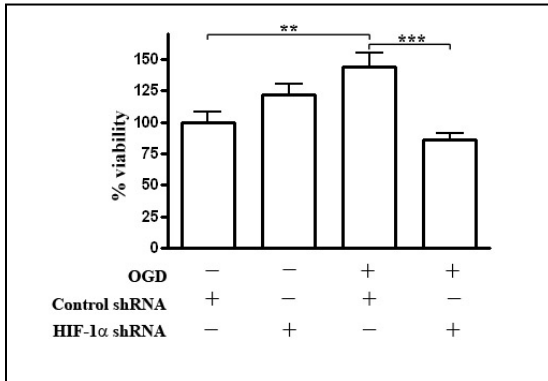


HIF-1 α knockdown attenuates VEGF expression.

Lentivector shRNAi (Mission TRC sh RNA Lentiviral particles, clone #TRCN0000054448) was used to knockdown HIF-1 α expression in cultured NSCs (HIF-1 α shRNA NSCs). The data demonstrate that the expression level of HIF-1 α is directly correlated with the expression level of VEGF in NSCs (85% and 78% reduction of HIF-1 α and VEGF protein expression, respectively).

These results show that NSC-induced support of EC survival and morphogenesis is mediated via HIF-1 α /VEGF signaling. These findings lead us to hypothesize that the similar signaling mechanism in BTSCs governs tumor angiogenesis. The experiments proposed in the Aims #1 and 2 will focus on the role of HIF-1 α in vasculotropic properties of BTSCs.

3. HIF-1 α knockdown decreases the resistance of NSCs to hypoxia.

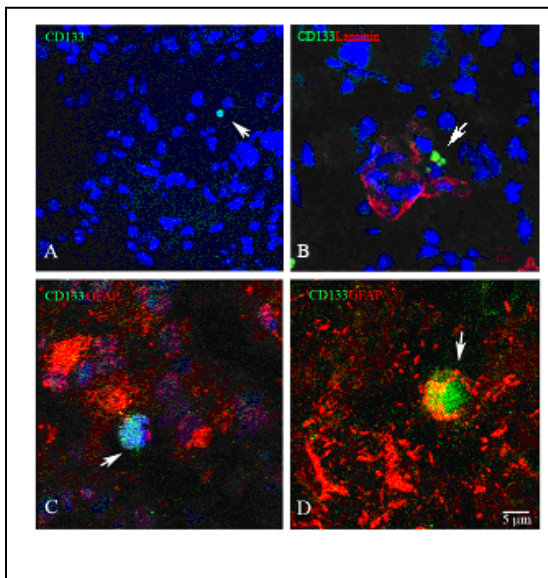


The viability of NSCs transduced with control (non-target) and Hif-1 α shRNAi was assessed with MTT assay at 2 days following 3h of oxygen-glucose deprivation (OGD). Cultures were placed in an anaerobic chamber containing a gas mixture of 5%H₂, 5%CO₂ and 85%N₂ to obtain <0.2% O₂. Normal culture medium was replaced with deoxygenated, glucose-free Earle's Balanced Salt Solution (EBSS), and cells were exposed to glucose-free anaerobic conditions for 3 or 5 h at 37°C. One-way Anova and Tukey post-test; ** p<0.01, *** p<0.001; n=24.

The results suggest that HIF-1 α plays an important role in NSC viability. We hypothesize that HIF-1 α inhibition in BTSCs will decrease not only the vasculotropic properties of the BTSCs, but also impair their own viability.

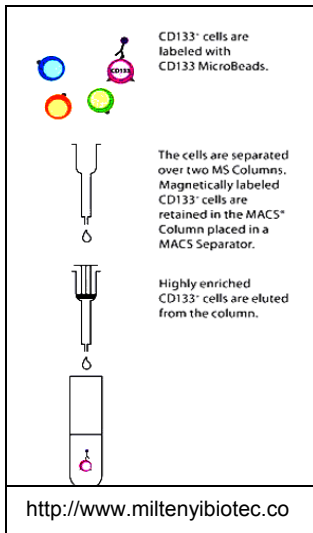
4. Identification and characterization of human CD133+ BTSCs

In order to check whether CD133+ cells isolated from brain tumor tissue exhibit characteristics of BTSCs, they were tested for self-renewal (ability to proliferate and form the neural cell specific structures neurospheres) and multipotency (ability to differentiate into the cells of different neural lineages), *in vivo* and *in vitro* expression of stem cell markers such as CD133 and nestin, and the capacity to induce tumor formation after injection into the mouse brain¹.

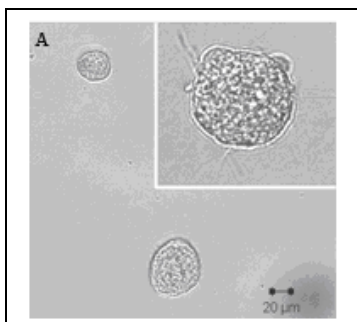


a) Identification of CD133+ cells in the brain tumor tissue:

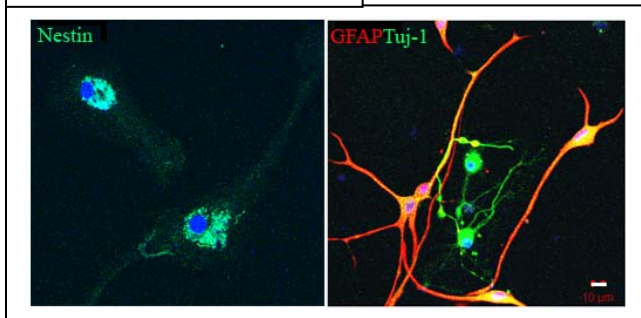
Human glioblastoma tissue was post-fixed overnight, cryoprotected in 30% sucrose and sectioned at 30 μ m thickness in the coronal plane. The histological sections were immunostained with the antibodies against CD133 (1:10, Miltenyi Biotech, green) and DAPI (blue). The blood vessels on panel B were visualized with anti-laminin antibody (1:25, Sigma, red) and the astrocytic marker GFAP was detected with anti-GFAP (1:500; Accurate, red). CD133+ BTSCs (arrow) were identified as rare population of cells, often found in the vicinity of the blood vessels (B). BTSCs were mostly undifferentiated (small round cells with a large nucleus and limited cytoplasm, negative to GFAP, C) However, some of them expressed astrocytic marker GFAP (D). 3-dimensional confocal image projections were acquired using Zeiss LSM510-META confocal imaging system. Bar: 5 μ m.



b) BTSC isolation: Tumor samples were obtained from Dr. H. Yonas (UNM Department of Neurosurgery), categorized according to diagnosis (medulloblastoma, glioblastoma and ependymoma) and processed separately. BTSCs were isolated from the human brain tumors and expanded in a specialized medium (Methods). Since BTSCs express the cell surface marker CD133, we utilized MACS® human CD133 Microbead Kit. Within 3 days of primary culture, the CD133⁺ were isolated, using magnetic cell separation technique and anti-human CD133 antibody-conjugated microbeads (MACS® Technology, Miltenyi Biotech, Auburn, CA), according to the manufacturer's recommendations. MACS® Technology is based on MACS MicroBeads, manual MACS Separators, and MACS Columns. The cells are magnetically labeled with microbeads, conjugated with the antibody against a specific self surface marker. The magnetically labeled cells are separated over a MACS Column placed in a MACS Separator. They are retained on the column, while unlabeled cells pass through. These cells can be collected as the unlabeled fraction. The retained cells are eluted from the MACS column after removal from the magnet. The isolated CD133-positive BTSCs were plated and grown in the BTSC medium (Methods) and used for the further characterization.

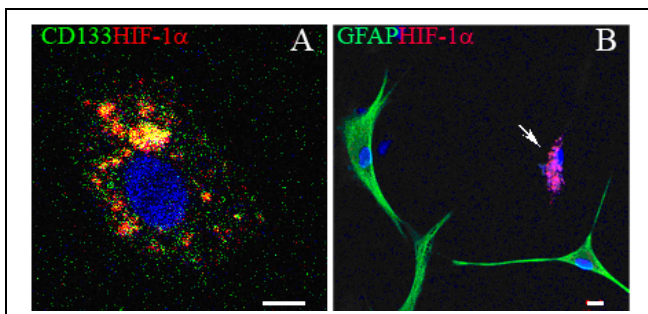


c) Self-renewal capacity of BTSCs was assessed using standard neurosphere assay, widely used for the characterization of the neural stem cells, based on the ability of the stem cells (cultured in clonal density) to form free-floating spherical clusters neurospheres, continuously through the multiple passages. BTSCs were plated at 8 cells/μl density in uncoated cell culture dishes, in defined BTSC medium. Fresh growth factors were added every three days. A) The isolated BTSCs plated in clonal density formed primary neurospheres. Neurosphere cultures are then passaged every 7 days. During each passage, spheres are enzymatically (trypsin/enzyme solution) and mechanically dissociated, and the cells are re-plated at density 8-10 cell/μl.



neuron-specific Tuj-1 (right panel, green). Thus, the isolated cells demonstrated multipotency: they differentiated into neurons and astrocytes. Taken together, the results suggest that the isolated CD133⁺ cells demonstrate all characteristic features of stem cells: the ability to proliferate, self-renew and differentiate into the cells of different lineages.

d) Differentiation potential of the BTSCs: The isolated BTSCs were expanded in growth factor (EGF/FGF)-containing media, supporting their undifferentiated state. The undifferentiated BTSCs expressed stem cell marker nestin (left panel, green). Following expansion, BTSCs were plated in serum-free, growth factor-free medium and allowed to differentiate for 7-8 days. Differentiation was assessed by immunofluorescence microscopy to identify the cells expressing cell-type specific antigens, including astrocyte-specific GFAP (right panel, red), and immature



5. The isolated CD133⁺ BTSCs express HIF-1α

A: Undifferentiated BTSCs were subjected to dual immunostaining with the antibodies against CD133 (1:10, Sigma, green) and HIF-1α (1:30, R&D, red). The results show that CD133⁺ cells express HIF-1α and that the two proteins often co-localize within perinuclear and probably Golgi-associated large vesicles (yellow). B: Differentiated BTSCs (7 days following growth factor withdrawal) were immunostained with the antibodies against GFAP (1:1000, Accurate, green) and HIF-1α (red). Hif-1α was expressed only in undifferentiated cells (arrow). The results suggest

that, similar to NSCs, BTSCs express Hif-1α, and that this expression decreases upon BTSC differentiation. Taken together, our preliminary results are in agreement with our overarching hypothesis that the brain tumor angiogenesis may be initiated and supported by BTSCs, via HIF-1α / VEGF signaling pathway.

GENERAL METHODS

Isolation of CD133-positive BTSCs.

Immediately after surgical resection, solid brain tumor masses (medulloblastoma, glioblastoma or ependymoma) will be placed on ice, in cold HBSS media, and subsequently (as soon as possible) processed for cell isolation. Tumor samples will be washed, acutely dissociated in oxygenated artificial cerebrospinal fluid, subject to enzymatic dissociation (37°C, 15 min in 1.3 mg/ml trypsin + 0.01% papain, and 0.01% DNase I), resuspended in the specialized BTSC media (chemically defined serum-free neural stem cell medium, with addition of human recombinant EGF (20 ng/ml), bFGF (20ng/ml), leukemia inhibitory factor (10 ng/ml), Neuronal Survival Factor (1x) and N-acetylcysteine (60 mg/ml) and plated at a density of 3×10^6 cells/60-mm plate. Within 3 days of primary culture, the CD133+ cells will be isolated using magnetic cell separation technique and anti-human CD133 antibody-conjugated microbeads (MACS® Technology, Miltenyi Biotec). Up to 10^6 will be used for each magnetic separation. A single cell suspension will be obtained by passing the cells through 30 μ m nylon mesh. The cells will be labeled by adding 100 μ l CD133 MicroBeads (final volume final volume 500 μ l per 10^6 total cells), resuspended and subjected to magnetic separation using MS Columns and **MiniMACS™ Separator** provided by Miltenyi Biotec.

Immunohistochemistry of the tumor sections will be performed as described in ¹¹. Brain tumor tissue will be post-fixed overnight, cryoprotected in 30% sucrose and sectioned at 30 μ m thickness in the coronal plane. Sections will be permeabilized with 0.1% (v/v) Triton X-100, blocked with 5 % donkey serum, and incubated with primary antibodies overnight at 4°C. The following antibodies will be used: anti-nestin (1:1000, BD Pharmingen), anti-CD133 (1:10, Miltenyi Biotec), anti-GFAP (1:500; Accurate Chem. & Sci. Corp.), anti-doublecortin (1:300, Santa Cruz Biotech), anti- β III tubulin/Tuj1 (1:300, Promega), anti-HIF-1 α (1:300, Chemicon), anti-VEGF (1:200; Abcam), anti-GLUT-1 (1:200; Chemicon), anti-Laminin (1:25, Sigma) and anti-CD59 (1:200; Millipore). Secondary antibodies will be used at a common dilution of 1:200. Samples will be imaged on a Zeiss LSM510 or Zeiss LSM510-META confocal imaging systems in the UNM CRTS Fluorescence Microscopy Facility.

Transplantation of BTSCs: To determine whether the isolated CD133-positive BTSCs have the capacity to initiate tumor *in vivo*, BTSCs and shRNA-BTSCs cells will be transplanted into cerebral cortex of the six week-old NOD-SCID mice. As few as 100 CD133⁺ cells were shown to be sufficient for the transplantation and tumor formation ¹. However, for the optimal results, we will transplant different amount of the cells ranging from 100 to 10 000 cells. Mice will receive stereotaxic injections using a Hamilton microsyringe with a 26-gauge blunt needle. Each animal will receive an injection of 2 μ l of cell suspension (25 000 cells/ μ l in PBS) at the rate of 1 μ l per minute at the following stereotactic coordinates (on coronal suture, from bregma: L+2.0 mm, V-3 mm). At 10 days post-transplantation, 6 mice (consisting of the mice with BTSC and shRNA-BTSCs transplants, n=3 mice/group) will be sacrificed and subjected to histological analysis that was used by the UNM Surgical Pathology laboratories for the initial diagnosis of the brain tissue. Both the initial tumor samples and xenografts will be immunostained with the antibodies against nestin, vimentin and β III tubulin – the markers highly expressed in the brain tumors ¹. To distinguish between transplanted and host cells within the xenografts, the samples will be probed for human-specific antigen CD59. Six remaining mice (with BTSC and shRNA BTSC transplants) will be used for the PET imaging of the tumor vasculature.

VEGF immunoassay. Quantikine mouse VEGF immunoassay (R&D systems) will be used to quantitate the concentration of VEGF released by BTSCs following 3 days in culture. Cell culture conditioned media will be collected, filtered and concentrated using iCON™ concentrator with 9K molecular weight cut-off (Pierce). Assay will be performed according to manufacturer's recommendations.

HIF-1 α immunoassay. After removal of conditioned medium used in VEGF immunoassay, BTSCs will be harvested and analyzed using Surveyor TMIC HIF-1 α immunoassay (R&D systems), according to manufacturer's recommendations.

HIF-1 α gene silencing *in vitro*. Lentivector shRNAi will be used to knockdown HIF-1 α expression in cultured BTSCs, expanded and passaged 3 times prior to transduction with shRNAi Lentiviral particles. Mouse HIF-1 α Mission TRC Lentivector shRNAi (Mission TRC sh RNA Lentiviral particles, clone #TRCN0000054448) will be purchased from Sigma. The protocol provides a system for long-term silencing of HIF-1 α and a stable cell selection using puromycin (PM) resistance. 60-70% confluent BTSCs will be incubated with Lentivector shRNAi (concentration 10 μ l/100 μ l medium) for 20h, in the presence of 8 μ g/ml Hexadimethrine Bromide. The puromycin titration experiments showed a high sensitivity on NSCs to PM, therefore the PM-resistant cell selection would be effective at the PM concentration as low as 0.25 μ g/ml. Five days following selection, the PM-resistant colonies will be passaged, expanded and subjected to Western blot analysis.

Western Blot analyses will be performed as described in ¹¹. Mouse monoclonal anti-VEGF (1:100, Abcam), rabbit polyclonal anti-HIF-1 α (1:500, Chemicon) and rabbit polyclonal anti-actin (1:1000, Sigma) will be used.

Endothelial cell morphogenesis assay. When plated on collagen or Matrigel matrix, endothelial cells form cellular networks and capillary tubes. Endothelial cell morphogenesis will be assessed under an inverted phase-contrast microscope, and images will be taken using LSM-META confocal imaging system. 10-15 images per each coverslip will be taken systematically across the coverslips. The number and diameter of capillary-like structures will be counted using LSM Image Browser. Statistical analysis will be performed using GraphPad Prism software.

Immunofluorescence Microscopy of BTSCs. IF staining will be performed as described in ¹¹. The following antibodies will be used: anti-HIF-1 α (1:300, Chemicon), anti-VEGF (1:200, Abcam), hCD59 -anti-human CD59, (Becton Dickinson,1:200), anti β -catenin and anti-N-cadherin (1:200, Transduction Labs), anti-mouse CD106/VE-cadherin (1:300, BioLegend), monoclonal anti-mouse CD31/PECAM-1 (1:300, BD Biosciences). Secondary antibodies (Jackson Laboratories) will be used at a common dilution of 1:200.

DOTA conjugation: The methods for PET imaging procedures was adopted from ¹³.

VEGF₁₂₁ will be purchased from SibTech Inc (Brookfield, CT). DOTA will be activated by 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) and N-hydroxysulfonosuccinimide (SNHS) at pH 5.5 for 30 min with a molar ratio of DOTA:EDC:SNHS at 10:5:4. The reaction mixture will be adjusted to pH 8.5 with 0.1 N NaOH solution and allowed to incubate overnight at 4°C. The DOTA-VEGF₁₂₁ conjugate will be then purified using PD-10 column (GE Healthcare) and concentrated by Centricon filter with 10-kDa molecular-weight cut-off (MWCO) (Millipore). The final concentration of DOTA-VEGF₁₂₁ will be measured based on UV absorbance at 280 nm using unconjugated VEGF₁₂₁ of known concentrations as the standard.

Cell-binding assay of VEGF₁₂₁ and DOTA-VEGF₁₂₁

DOTA conjugation at the amino groups in VEGF₁₂₁ protein may affect the VEGFR-binding affinity. Therefore cell-binding assays are carried out using both VEGF₁₂₁ and DOTA-VEGF₁₂₁ before *in vivo* imaging studies. VEGFR-1- and VEGFR-2-binding affinity of VEGF₁₂₁ and DOTA-VEGF₁₂₁ can be evaluated by PAE/VEGFR-1 (porcine aorta endothelial cells transfected with VEGFR-1) and PAE/VEGFR-2 (PAE cells transfected with VEGFR-2) cell-binding assays using ¹²⁵I-VEGF₁₆₅ (GE Healthcare) as the radio-ligand. The best-fit IC₅₀ values can be calculated by fitting the data by nonlinear regression using GraphPad Prism (GraphPad Software, Inc.).

⁶⁴Cu-labeling of DOTA-VEGF₁₂₁

Water and all buffers will be treated with Chelex 100 resin (Sigma) before use in radio-labeling procedures to ensure heavy metal-free conditions. Two μ Ci of ⁶⁴CuCl₂ were added to a solution of DOTA-VEGF₁₂₁ in 300 ml of 0.1 M sodium acetate buffer (pH $\frac{1}{4}$ 6.5; 10 mg of DOTA-VEGF₁₂₁ per mCi of ⁶⁴Cu). The reaction mixture will be incubated for 1 h at 40°C with constant shaking. The ⁶⁴Cu-DOTA-VEGF₁₂₁ conjugate can then be purified by PD-10 column using PBS as the mobile phase. The radioactive fractions (typically between 3.5 and 4.5 ml, total of 1 ml) containing ⁶⁴Cu-DOTA-VEGF₁₂₁ will be collected for further *in vivo* experiments.

Micro-PET imaging (PET imaging for small animals). Mice will be anesthetized with 2% isoflurane in oxygen the mice will be injected with 200 to 300 μ Ci of ⁶⁴Cu-DOTA-VEGF₁₂₁ (about 2 to 3 mg of VEGF₁₂₁) via the tail vein. Typically, 3- to 10-min static scans will be performed depending on the radioactivity left in each mouse. The images will be reconstructed by a two-dimensional, ordered-subsets expectation maximum (OSEM) algorithm, with no attenuation or scatter correction. To quantify the tracer uptake in the tumor and brain, a conversion factor should be determined using a 50-ml centrifuge tube filled with ⁶⁴CuCl₂ as a mouse phantom. For each micro-PET scan, three-dimensional ROIs will be drawn over the tumor and brain by using the vendor software on decay-corrected brain coronal images. Values in several adjacent slices (depending on the size of the tumor) will be averaged to obtain a reproducible value of activity concentration in the ROI drawn.

Biodistribution studies: After the last micro-PET scans, the mice will be euthanized for biodistribution studies to confirm the *in vivo* imaging results. The blood samples, tumor, major organs and tissues will be collected and weighted. The radioactivity in each tissue will be measured using a γ -counter and the results will be calculated as % ID/g. For each mouse, the radioactivity in the tissue sample will be calibrated against a known aliquot of the injectate and normalized to a body weight of 20 g. **Immunofluorescence staining:** To further validate the VEGFR expression level in the tumor tissue, we will perform immunofluorescence staining (see above) or tumor tissue slices or western blot analysis of tumor tissue lysates using VEGFR-1 and VEGFR-2 antibodies (Jackson Immuno Research).