Published in final edited form as:

NMR Biomed. 2012 May ; 25(5): 726–736. doi:10.1002/nbm.1786.

Phase Contrast MRI is an Early Marker of Micrometastatic Breast Cancer Development in the Rat Brain

Matthew D Budde, PhD^{1,*}, Eric Gold¹, E. Kay Jordan, DVM¹, Melissa Smith-Brown¹, and Joseph A Frank, MS, MD^{1,2}

¹Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Bethesda, MD

²National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD

Abstract

The early growth of micrometastatic breast cancer in the brain often occurs through vessel co-option and is independent of angiogenesis. Remodeling of the existing vasculature is an important step in the evolution of co-opting micrometastases into angiogenesis-dependent solid tumor masses. The purpose of this study was to determine if phase contrast MRI, an intrinsic source of contrast exquisitely sensitive to the magnetic susceptibility properties of deoxygenated hemoglobin, could detect vascular changes occurring independent of angiogenesis in a rat model of breast cancer metastases to the brain. Twelve nude rats were administered with 10⁶ MDA-MB-231BRL “brain seeking” breast cancer cells through intracardiac injection. Serial, multiparametric MRI of the brain was performed weekly until metastatic disease was detected. The results demonstrate that images of the signal phase were more sensitive to metastatic brain lesions (area under receiver operating characteristic curve, AUC = 0.97) compared to T₂* gradient echo magnitude images, (AUC = 0.73). The difference between the two techniques was likely the result of the confounding effects of edema on the magnitude signal. A region of interest analysis revealed that vascular abnormalities detected with phase contrast MRI preceded tumor permeability as measured with contrast-enhanced MRI by 1 to 2 weeks. Tumor size was correlated with permeability ($R^2 = 0.23$, $p < 0.01$), but phase contrast was independent of tumor size ($R^2 = 0.03$). Histopathological analysis demonstrated that capillary endothelial cells coopted by tumor cells were significantly enlarged, but less dense, compared to the normal brain vasculature. Whereas co-opted vessels were VEGF-negative, vessels within larger tumor masses were VEGF-positive. In conclusion, phase contrast MRI is believed to be sensitive to vascular remodeling in co-opting brain tumor metastases independent of sprouting angiogenesis and may therefore aid in pre-clinical studies of angiogenic-independent tumors or monitoring continued tumor growth following anti-angiogenic therapy.

Keywords

magnetic resonance imaging; phase contrast; vascular remodeling; brain metastasis; vessel co-option; ferumoxides

Introduction

Metastatic brain tumors are the most common type of intracranial tumor (1). Among women, primary tumors of the breast are the most frequent cause of brain metastases and are

*Corresponding author: 9000 Rockville Pike, Building 10, B1N256, Bethesda, MD 20892, budde@mail.nih.gov.

associated with poor prognosis. The mean survival from diagnosis of central nervous system metastasis is 2–16 months with a 1-year survival of about 20%. (2–5). There are currently few effective therapies for brain metastases. One factor that contributes to the lack of treatment response is the inability to detect micrometastases noninvasively at an early stage combined with an inadequate understanding of the mechanism of tumor growth and development (6).

Metastatic breast cancer cells encounter a different microenvironment in the brain as compared to primary mammary tumor bed or other metastatic sites (7). Angiogenesis is regarded as a prerequisite for significant tumor growth and progression, and vascular endothelial growth factor (VEGF) is a potent pro-angiogenic factor (8). Early studies of solid tumors demonstrated that rapidly dividing tumor cells outgrow the available oxygen supply and become hypoxic, leading to the accumulation of hypoxia-inducible factor 1 (HIF-1 α) and other factors in the cell nucleus (9). HIF-1 α enables a cascade of effects that promote neovascularization through the VEGF pathway (10,11). In contrast to this well-accepted paradigm, Holash et al, demonstrated that infiltrative tumors can grow by co-opting the existing vasculature (12). Importantly, tumor proliferation through co-option occurs through an angiogenesis independent mechanism with low levels of VEGF expression (13) and can continue uninhibited despite the lack of angiogenesis (14). In metastatic breast cancer to the brain, the development of a tumor depends on several necessary steps including: circulating tumor cells arresting in the vasculature, extravasating across the endothelial cell layer, and proliferating to form micrometastases that may grow into macrometastases (15). In experimental studies of brain metastases that initially proliferated by co-opting vessels, remodeling of the existing vasculature was a necessary step for the continuous growth of micrometastases (15). It is believed that co-opted growth impairs local perfusion and diminishes oxygen availability (8). This, in turn, initiates the switch to angiogenesis-dependent growth, and VEGF-dependent modulation of the preexisting vasculature or initiation of sprouting angiogenesis (11).

Magnetic resonance imaging (MRI) is particularly well suited for monitoring and visualizing tumor physiology noninvasively. Vessels contributing to the neovascularization of tumors are highly permeable to gadolinium chelates and can be monitored with T₁-weighted MRI. By comparison, T₂*-weighted MRI is sensitive to the magnetic susceptibility properties of oxygenated versus deoxygenated hemoglobin, also known as blood oxygen level dependent (BOLD) contrast, and is therefore a possible surrogate marker of the oxygenation status of tumors (16). Thus, the spatial and temporal relationship between angiogenesis and hypoxia can be examined noninvasively with MRI. Tumors with decreased oxygenation (hypoxic) appear hypointense on T₂*-weighted MR images and are more aggressive (17), have a worse prognosis than those that are oxygenated (16), and have a poor response to therapy (18). Whereas T₂*-weighted images are created from the magnitude of the underlying MR signal, images depicting the phase component of the signal can likewise be examined. Both T₂* magnitude and phase contrast images are exquisitely sensitive to the magnetic susceptibility properties of tissues (19,20), but phase images have revealed normal and pathological features of tissues not apparent with conventional T₂* magnitude images (21). In the context of tumor physiology, these phase changes are primarily caused by deoxyhemoglobin, which is paramagnetic and therefore induces a change in the resonance frequency of nearby protons relative to the surrounding tissue. It is unknown whether phase contrast MRI has improved sensitivity in detecting the vascular changes associated with hypoxia compared to conventional T₂* magnitude images.

In the current work, we used a rodent model of metastatic breast cancer to the brain (22,23) to test the hypothesis that changes in the oxygenation status of developing metastasis could be detected with phase contrast MRI. It was expected that these changes would be

coincident with or precede the switch to angiogenesis-dependent growth monitored with contrast enhanced MRI. The results support the notion that phase contrast MRI is sensitive to vascular remodeling or changes associated with hypoxia and that micrometastatic tumor formation can be detected noninvasively prior to the development of angiogenesis.

Materials and Methods

Tumor Cell Line

MDA-MB-231-BRL (231BRL) cells (gift from Dr. Diane Palmieri) (6) were cultured with DMEM growth media (Invitrogen, Carlsbad, CA) containing 10 % fetal bovine serum and 1 % penicillin streptomycin antibiotics at 37° C in a 5% CO₂ conditioned incubator (23). The 231BRL cells were labeled with the commercially available superparamagnetic iron oxide nanoparticles (SPION) ferumoxides (Feridex IV[®], Berlex Laboratories, Wayne, NJ) contrast agent complexed to preservative free protamine sulfate (10 mg/ml, American Pharmaceuticals Partner, Schaumburg, IL) as previously described (24). Ferumoxides (Fe) and protamine sulfate (Pro) were mixed in fresh serum-free RPMI 1640 medium (Biosource, Camarillo, Ca) containing 25 mM HEPES, MEM non-essential amino acids, sodium pyruvate, and L-glutamine at concentration ratio of Fe:Pro of 100 µg/ml:6 µg/ml of media and mixed for less than 30 seconds to form the FePro complex. The FePro suspension was added directly to cultured cells and incubated for two hours. An equal volume of serum complete media was added back to the flask and cells were incubated overnight. Cells were washed 3 times with 10 units/ml of heparinized PBS, trypsinized, and filtered with a 40 µm nylon cell strainer. Cells were suspended in sterile heparinized PBS at 10⁶ cells/200 µl for injections.

Animal Procedures

All studies were approved by the Animal Care and Use Committee at our institution. Twelve 6 to 8 week old NIH-*rmu* rats (Charles River, Wilmington, MA) received 10⁶ 231BRL cells by intracardiac (IC) injection. Rats were anesthetized with 2–3% isoflurane in 100 % O₂. A 200 µl suspension of tumor cells in 10 units/ml heparin in PBS was delivered into the left ventricle through a 27-gauge needle under real time ultrasound guidance using a 14 MHz linear probe (Acuson Sequoia C256, Siemens Medical Solutions, Malvern, PA). Eight rats were used for serial analysis and underwent MRI prior to injection, 1 day post-injection, and weekly thereafter for 5 weeks. Of these eight rats, four received 231BRL cells labeled with FePro, and four received unlabeled cells. An additional four rats received FePro-labeled cells, but underwent imaging only at weeks 4 and 5 and were used for the analysis at the terminal endpoint. All animals were euthanized at week 5 or earlier if they exhibited any evidence of significant weight loss (> 20 %), neurological changes such as paraplegia, or apparent pain or weakness in limbs. Carbogen (95/5 % O₂/CO₂) was used as the inhalation gas during all MRI sessions to minimize the magnetic susceptibility effects of vessels in the normal brain (25). Rats were anesthetized with 2–3% isoflurane for induction and 1.5 % isoflurane in carbogen was delivered through a nosecone. Body temperature was monitored with a rectal probe and maintained at 37°C using a warm water pad.

Magnetic Resonance Imaging

In vivo MRI was performed on a 7 T Bruker Biospec (Billirica, Ma) using a 40 mm i.d. quadrature volume coil (Doty Scientific, Columbia, SC). Twenty axial slices at a thickness of 0.5 mm were acquired to cover the entire brain using a multiple gradient echo (MGE) sequence with the following parameters: TR = 3500 ms, TE = 3.5 ms (first-echo) 4.25 ms echo spacing, 12 echoes, 90° flip angle, 2 averages. A 3×3 cm² field-of-view and a 256×256 matrix size yielded an in-plane resolution of 117×117 µm². A T₂-weighted RARE sequence was collected with the identical spatial resolution and the following parameters: TR = 5000

ms, TE = 50 ms, 8 echoes, 8 averages. T₁-weighted gradient echo images were acquired before and after the injection of 0.3 mmol/kg Gadolinium-dimeglumine (Gd-DTPA, Magnevist, Bayer-Schering Pharma, NJ) through the lateral tail-vein with the following parameters: TR = 600 ms, TE = 10 ms, 2 averages, 60 flip angle. Ex vivo MRI was performed on a 7 T Bruker vertical bore magnet. Brains were immersed in susceptibility matching fluid (Fomblin, Solvay Solexis, Inc, West Deptford, NJ) and imaged with a 3D gradient echo sequence: TR = 500 ms, TE = 10 ms, 3.0×1.5×1.25 cm³ FOV, 512×256×128 matrix, 4 averages, 30° flip angle; and a 3D RARE sequence with the identical spatial resolution and TR = 2000, TE = 12 ms, 8 echoes, 4 averages.

Data Analysis

Images were reconstructed using custom software written in Matlab (Mathworks, Natick, MA). Each echo of the MGE sequence was reconstructed separately to create both magnitude and phase images. The magnitude images from all echoes were summed to generate T₂*-weighted images with an effective TE of 27 ms, resulting in images with a high signal-to-noise ratio and excellent T₂* contrast (26). T₂* maps were created by fitting magnitude images to a single exponential on a pixel-by-pixel basis. Phase images were unwrapped to remove low frequency phase variations attributed to B₀ inhomogeneities using 2D homodyne filtering (27) with a 2D Hamming filter (FWHM = 64 pixels). Since the later echoes were prone to decreased SNR and therefore noisy phase images, the unwrapped phase images from only the first six echoes were averaged to create a mean, unwrapped phase image with an effective TE of 16 ms. T₂-weighted images were reconstructed in magnitude format. All images were registered to a common coordinate frame using a 7 parameter 3D model (rigid-body and global scaling) in AIR 5.2.5 (28).

For the serial analysis, 52 tumors were analyzed in 8 animals. An additional 22 tumors were analyzed in the 4 animals used for the terminal endpoint only. Tumors were manually circumscribed as hyperintense regions on the T₂-weighted images at week 5. Tumors in the cerebrum were included and those of the hippocampus, cerebellum, and brainstem were excluded in subsequent analysis due to the large-scale magnetic field inhomogeneities in these regions. For each tumor included, corresponding regions of interest (ROIs) were placed in the contralateral hemisphere provided these regions were devoid of tumors. ROIs were applied to the spatially registered images to determine the serial changes in phase and T₂*. The mean and standard deviation of the phase images, as well as the mean T₂* and T₂-weighted signal intensity were derived for each of the time-points.

Histopathology

Perfusion-fixed brains were excised, immersed in 4 % paraformaldehyde overnight, cryoprotected in a sucrose gradient, and frozen in liquid nitrogen. Cryosections were cut at a thickness of eight microns, and each section underwent hematoxylin and eosin (H&E) staining, immunohistochemistry or immunofluorescence. Primary antibodies for immunostaining included anti-human CD44 (Abcam), anti-human beta1-integrin (Abcam), anti-human Matrix Metalloproteinase 9 (MMP9; Cell Signaling Technology), anti-human VEGF (Zymed), anti-human HIF-1 α (Chemicon), and anti-rat endothelial (Tusc5; Genway). Briefly, slides were rinsed with phosphate buffered saline (PBS), incubated with blocking serum for 10 min, and incubated with the primary antibody (1:200 dilution) at 4°C overnight. Sections were washed 3× with PBS and incubated with the secondary antibody (1:200 dilution) for 30 minutes at room temperature and rinsed 3× in PBS. Immunofluorescent sections were cover-slipped with VectaShield with DAPI and imaged using a laser scanning confocal microscope (Zeiss LSM, Germany). Immunohistochemistry sections underwent streptavidin-biotin horseradish peroxidase detection, 3,3'-diaminobenzidine solution (DAB) colorization, and hematoxylin counterstaining. To

quantify vascular changes, sections stained for endothelial cells were digitized and thresholded. Vascular density and area were derived using the Analyze Particles tool of ImageJ. A subset (n=4) of the animals from the MRI analysis were used for histological analysis. Four separate tumors and the adjacent normal brain were analyzed for each animal.

Statistical Analysis

For statistical analysis of serial MRI data, a linear mixed model was used, with time (week) as a repeated measure and the region (tumor vs. contralateral) as within-subject factor. If the overall model was significant, post-hoc comparisons were performed between tumor and contralateral values for each week, or each region was compared to its pre-injection value (week -1). Week 2 was considered the baseline value for contrast enhancement since these images were not performed pre-injection, and the tumor and contralateral values were equivalent to one another at this time point. T₂-weighted signal intensities were normalized to the contralateral hemisphere, so only the effect of time was included in the model. For analysis of the terminal endpoint alone, a Pearson's correlation was performed to relate MRI parameters with tumor volume. A receiver operating characteristic curve (AUC) analysis was performed to determine the performance of each MRI parameter in discriminating tumors from contralateral regions. Histology-derived vessel density and size was compared between tumors and normal brain using paired t-tests. A p-value of 0.05 was considered significant for all tests. Statistical analysis was performed using JMP 7.0 (SAS Institute Inc, Cary, NC).

Results

Breast cancer metastases in the brain manifested as edematous masses on T₂-weighted MRI (Fig. 1A) at 5 weeks post-injection of MB231-BR cells. Tumors as small as 300 μ m could readily be detected on T₂-weighted images. In addition, the edema also caused most tumors to appear iso-, or even hyper-intense on T₂*-weighted magnitude images (Fig. 1B). By comparison, tumors were highly visible on phase images (Fig. 1C) and were evident as focal variations in contrast. The decreased T₂* and increased phase contrast suggests a decrease in oxygenation, or hypoxic environment, in developing tumors although the spatial phase pattern was heterogeneous across different lesions. The susceptibility-induced changes were evident when the tumor diameter was less than 500 μ m. However, phase changes associated with large vessels, ventricles, and magnetic field inhomogeneities were also prominent, and therefore, phase images alone were not sufficient to uniquely identify metastatic lesions. BBB permeability, as visualized by leakage of Gd-DTPA, was also heterogeneous between different lesions (Fig. 1D). The source of the in vivo phase contrast in metastatic brain tumors was likely caused by deoxygenated hemoglobin in the vasculature, since the phase changes were not maintained on the *ex vivo* MRI phase images on tissues prepared by perfusion fixation (Fig. 2). The accumulation of fluid in the extracellular space surrounding the metastatic lesions as the source of edema was apparent on the corresponding H&E stained-sections. The tumors displayed a highly infiltrative morphology with poorly demarcated borders.

Serial changes were assessed in 8 animals that underwent weekly MRI examinations beginning with a pre-injection baseline scan. A retrospective analysis was used by manually circumscribing tumors (n=52) on T₂-weighted images at the terminal endpoint and applying these regions to the other parametric maps and images (Fig. 3). Tumors were identified in nearly all regions of the brain and brainstem, particularly along white and gray matter interfaces, but only cerebral tumors were included in the analysis. Baseline images (week -1; Fig. 3) were essential to differentiate labeled cells and developing tumor vasculature from the preexisting vasculature (Fig. 3, arrows) in subsequent images. Vessels had decreased T₂*-weighted signal intensity and a phase contrast pattern typical of vessels

oriented perpendicular to the main magnetic (B_0) field (29) and could easily be identified on registered serial MRI studies. At 1 day post injection of FePro labeled 231BRL cells, numerous T_2^* hypointensities were detected throughout the brain consistent with clusters of tumor cells arrested in the vasculature. The corresponding phase images depict a dipole phase pattern characteristic of punctate iron sources (30). Since tumors induced spatially varying positive and negative phase contrast, both the first order (mean) and second order (standard deviation) moments of the pixels encompassed by the tumor margins were quantified for phase images (Fig. 3). By week 1, the hypointense voxels had decreased and were no longer apparent on subsequent MRI studies consistent with previous reports. A small metastatic lesion was visible at week 3, but this tumor did not appear to be associated with the locations of labeled cells at day 1 post-injection. Figure 4 depicts a single tumor from a different animal that received labeled cells with evidence of changes in phase, T_2 , and T_2^* occurring at week 4, whereas the tumor did not have significant BBB permeability at week 4.

Across all animals, the mean phase within a ROI did not significantly differ at any stage of tumor progression (Fig. 5), nor did this parameter change with the presence of iron labeled breast cancer cells within the brain. By comparison, the phase standard deviation within the eventual tumor margin was sensitive to vascular changes accompanying tumor development. At day 1 after injection of FePro labeled 231BRL cells, the phase standard deviation was significantly increased compared to the baseline values in both the regions that eventually developed metastases ($p < 0.0001$) and compared to parenchyma without lesions ($p < 0.0001$), but there was no significant difference between the two (paired t-test, $p = 0.84$). The hypointense voxels seen on T_2^* -weighted images as a result of FePro labeled cells within the vasculature at one day following IC injection does not predict margination into the parenchyma and metastatic growth. On the other hand, the dispersion of phase within developing metastases had a non-significant increase at week 3 compared to the contralateral cortex ($p = 0.11$) and was significantly greater than both the contralateral hemisphere (Table 1) and pre-injection values at weeks 4 ($p = 0.025$) and 5 ($p = 0.005$). The phase changes preceded the significant increases in T_2 -weighted signal intensity ($p = 0.003$) and the percentage of Gd-DTPA contrast enhancement ($p = 0.013$) occurring at week 5 compared to baseline values. T_2^* values were not significant at any time-point. A subsequent concern was whether the FePro labeling had any effect on the development of phase contrast changes in developing tumors. At week 4, the phase dispersion in tumors from animals that received FePro-labeled cells ($n=4$) was not significantly different than the tumors in animals that received unlabeled cells ($n=4$, two-sample t-test, $p = 0.42$). As further evidence that the phase changes observed in the developing tumors was not associated with FePro labeling, both animals shown in figures 1 and 2 received unlabeled cells.

At the terminal endpoint (week 5), the performance of each contrast mechanism in detecting tumors was evaluated with a receiver operating characteristic analysis (ROC). At this timepoint, 6.2 ± 4.7 (mean $\pm$ s.d) tumors were identified per animal ($n=12$), yielding a mean tumor volume of 0.44 ± 0.23 mm³ per tumor. Using T_2 -weighted images as the standard, the phase dispersion was better at distinguishing tumors from the normal brain tissue (area under the ROC curve; AUC = 0.97) than the mean phase (AUC = 0.63), T_2^* (AUC = 0.73), or the signal change following Gd-DTPA leakage (Enhancement; AUC = 0.92). Tumor volume at the terminal endpoint was significantly correlated with the degree of Gd-DTPA enhancement ($R^2 = 0.23$; $p = 0.01$) but not with the phase mean ($R^2 = 0.008$), phase s.d. ($R^2 = 0.03$), T_2 -weighted signal intensity ($R^2 = 0.04$), or T_2^* ($R^2 = 0.04$).

The vascular abnormalities associated with 231BRL brain metastases are shown in figure 5. Tumor growth and progression occurred largely through co-option of the existing vasculature, as nearly all clusters of CD44-positive tumor cells surrounded a central

endothelial cell (Fig. 6A). Compared to the vasculature in the normal cortex, the vessels co-opted by metastases were significantly enlarged ($p = 0.0005$) and less dense ($p = 0.0037$) (Fig. 6C & D). These vascular structures were also tortuous and convoluted (Fig. 6E & F) which suggested the presence of vessel remodeling without sprouting angiogenesis. Nuclear HIF-1 α staining was present in tumor cells (Fig. 7A & B) despite their proximity to the vasculature. The pro-invasive markers beta1-integrin (Fig. 7C) and Matrix Metalloproteinase (MMP9; Fig 5D) were indicative of the invasive quality of this brain seeking breast cancer. 231BRL metastases occasionally formed larger, solid tumor masses (Fig. 7E, arrows) containing VEGF-positive vessels, whereas smaller co-opted vessels adjacent to the tumor mass did not express VEGF (Fig. 7E, arrowheads). Reactive astrocytosis was evident in the proximity of both solid and co-opted metastatic tumors.

Discussion

Phase contrast MRI depicts the susceptibility changes of developing brain metastases following intracardiac injection of MDA-MB-231-BRL cells. Due to its decreased sensitivity to edema, phase contrast is more sensitive than T_2^* magnitude images or T_2^* maps, and it depicts early vascular changes in micrometastatic lesions before the disruption of the blood brain barrier as demonstrated by contrast enhanced MRI. 231BRL breast cancer micrometastases in the brain initially proliferate through vessel co-option (31). Phase contrast MRI, in combination with carbogen inhalation, reliably detected early metastatic tumors. The phase changes were presumed to be a result of increased deoxyhemoglobin associated with vascular remodeling that preceded angiogenesis. Phase contrast MRI has the potential to detect the early formation of micrometastases growing through vessel co-option and may reflect vascular remodeling independent of angiogenesis.

The development of metastases in the brain caused by circulating tumor cells depends on several necessary processes including: cell arrest in the capillaries, extravasation through the endothelial cell layer, close contact to vessels to promote micrometastatic development, and continuous growth (15). The arrest of FePro labeled 231BRL cells in the brain capillaries at day 1 following intracardiac injection can be seen as hypointensities on T_2^* weighted MR images and has been described elsewhere (22,23,32). Unlike the micron-sized iron oxide particles used by Heyn et al, a superparamagnetic iron oxide nanoparticle (i.e., ferumoxides) was employed in the current study since the agent will be diluted through cell division and/or be metabolized in vivo (33,34). The dilution of the magnetic label over time ensures that the iron label does not potentially obscure vascular-associated changes, since they are both detected by their magnetic susceptibility properties. Dormant, isolated ferumoxides-labeled breast cancer cells, although sparse, can consistently be found in the rat brain sections by Prussian blue stains (22) but were not investigated in this study. The effects of the superparamagnetic iron oxide nanoparticles within the cells were better appreciated on phase contrast MR images and were visualized as magnetic dipoles originating from punctate iron sources (30).

A necessary second step in metastatic tumor development is the extravasation of cells across the endothelial cell layer since cells arrested in the vasculature do not proliferate (15). Extravasation can occur within the first days following arrest and is complete by two weeks post-injection of circulating tumor cells (15,23). Identifying ferumoxides-labeled cells within the vasculature from those that have marginated into the brain is currently not possible with MRI. However, the rate-limiting steps for brain metastases formation are replication with growth, and not arrest or margination (35). Only a small fraction (3–5%) of the 231BRL cells within the brain parenchyma ultimately progress into macrometastases (22), whereas a larger fraction either die or remain dormant (23,32). In metastatic tumors in the brain whose initial growth was through vessel co-option, remodeling of the existing

vasculature was a necessary condition for continued growth (15). Specifically, in early metastatic brain tumor growth, formation of capillary loops (15) and vascular structures resembling glomeruli (36) in the absence of sprouting angiogenesis was associated with the development of macrometastases. Conversely, micrometastases that failed to induce remodeling ultimately regressed, and vascular remodeling alone was not sufficient to induce angiogenesis. Instead, vascular remodeling is also evident in tumors that resume growth following chronic antiangiogenic treatment (37). In the current study, phase contrast MRI was sensitive to vascular remodeling that occurred before the onset of angiogenesis as evidenced by contrast-enhanced MRI (Figs. 4 & 5). Vessels co-opted by tumor cells were enlarged compared to those of the normal brain and were more tortuous and convoluted (Fig. 6). It is believed that the increase in tortuosity of co-opted vessels as well as vessel regression impairs flow and perfusion (38,39). The mechanisms of remodeling are not entirely understood, although physical obstruction (8) and molecular signaling between the tumor and endothelial cells (40) are both possible. The current work used a retrospective analysis to examine macrometastases visible at week 5 in order to understand the biological evolution of the breast cancer metastases on MRI. Similar retrospective analysis has been used to unravel the serial evolution of MRI findings in other disorders (41). In addition to the phase changes in metastatic lesions, phase images were equally sensitive to the differences in magnetic susceptibility occurring at tissue and air interfaces. Thus, a prospective analysis identifying focal phase abnormalities that did or did not progress into metastases was not possible. Nonetheless, the ROI approach suggested a high degree of sensitivity and specificity for metastatic lesions compared to the normal brain.

The growth of tumors by co-opting the vasculature is substantially different than the growth of expansive, solid tumors (42). Melanoma and breast cancer metastases to the brain display a coopted growth pattern whereas lung and prostate metastasis in the brain develop as expansive masses. The longstanding view for solid tumors is that once a tumor grows beyond several hundred microns, the diffusion limit of oxygen (43), it becomes hypoxic and initiates the angiogenic cascade. Co-opting tumors in the brain, in contrast, proliferate along the basement membrane within close proximity to the vascular network(44). Although the vasculature allows sufficient access to the oxygen and nutrients, co-optive growth is mediated by molecular cues within the basement membrane, such as beta1-integrin (Fig. 7C), and is independent of oxygenation and angiogenesis (42). Co-opting metastatic brain tumors can ultimately progress to angiogenesis-dependent growth, resulting in VEGF-dependent modulation of the existing vasculature or inducing neovascularization (11). In the current study, metastatic foci that developed into larger, solid masses contained VEGF-positive vessels, whereas smaller, co-opted vessels remained VEGF-negative (Fig. 7E). Reports have shown that co-opted tumor vasculature regresses prior to angiogenesis formation and this event is mediated by angiopoietin-2 (40) and endothelial apoptosis (45). Hypoxia, and specifically HIF1 α , has been implicated in promoting tumor cell proliferation (46) and invasiveness (47). HIF1 α staining was evident in 231BRL cells, even those within a few cell layers of the endothelial cell layer. In addition to hypoxia, acidosis also increases VEGF expression (48) and can increase the invasiveness of MB231 cells (49). Thus, the interaction between the tumor cells and their microenvironment are of considerable interest and further investigation is warranted.

Phase contrast and T₂* magnitude images are both sensitive to the magnetic susceptibility properties of tissues. However, phase contrast MRI offers several distinct advantages over T₂*-weighted magnitude images or T₂* maps. Firstly, phase contrast images are routinely filtered to remove large-scale magnetic field (B₀) inhomogeneities and this post-processing step accentuates local changes. Secondly, phase changes that are vascular in origin are markedly easier to visualize since the paramagnetic properties of deoxyhemoglobin induce a spatially varying phase shift. We exploited this change in phase contrast non-uniformity by

measuring the dispersion of phase values within regions of interest. Certain other technical aspects can alter phase contrast (20), but the phase dispersion within identical regions of interest in the current study was shown to be stable over time using identical acquisition and reconstruction methods (Fig. 5). Thirdly, and perhaps most importantly, the contrast mechanism of T_2^* is a combination of both magnetic inhomogeneity (T_2') and spin-spin relaxation (T_2) effects ($1/T_2^* = 1/T_2 + 1/T_2'$). Therefore, edema associated with tumors increases T_2 and therefore counters the decrease in T_2' caused by deoxyhemoglobin (Fig. 1). Conversely, phase images are less sensitive to peritumoral edema (50).

In the current work, the origin of the in vivo phase contrast in metastatic lesions is likely vascular since it is not maintained following perfusion and fixation (Fig. 2), whereas the contrast between gray and white matter is maintained since it is largely independent of heme iron (29). It should also be noted that in addition to deoxyhemoglobin, other biological sources of magnetic susceptibility can occur, including calcification, myelination, and non-heme iron. It is unlikely that these are the source of changes in the current study since these would likely be maintained following perfusion and fixation. Although T_2^* -signal intensity, or BOLD contrast, is related to tumor oxygenation and perfusion (51), it is not an absolute measure of the partial pressure of oxygen in the blood or tissue (pO_2) (52). Specifically, both T_2^* and phase contrast are dependent on oxygenation, hematocrit, and blood volume (53), all of which could be abnormal in co-opting brain metastases. It remains to be seen whether phase contrast MRI is better correlated with pO_2 than T_2^* magnitude contrast. Often, the response of the tumor vasculature is measured by the change in T_2^* magnitude or phase following carbogen challenge compared to baseline measurements under air breathing (54,55), and the change seems to reflect the perfusion status of the tumor (18,56). Although we did not collect images during air breathing in the current study, it is likely that the co-opted vessels were less responsive to carbogen than those of the normal brain, by virtue of their contrast while under carbogen. Future studies using such an approach will provide additional insight into the physiology of co-opted metastatic lesions in the brain. Nonetheless, we have demonstrated that phase contrast MRI is more sensitive to vascular disruptions in co-opting brain metastases than T_2^* magnitude MRI.

Acknowledgments

We thank Dr. Diane Palmieri for providing the MDA-MB-231-BRL cells. This work was supported by the Intramural Research Program of the Clinical Center at the National Institutes of Health.

Abbreviations

AUC	Area Under the Curve
BBB	Blood Brain Barrier
BOLD	Blood Oxygen Level Dependent
FePro	Ferromoxides-Protamine
Gd-DTPA	Gadolinium
HIF	Hypoxia Inducible Factor
MRI	Magnetic Resonance Imaging
ROC	Receiver Operating Characteristic
ROI	Region of Interest
VEGF	Vascular Endothelial Growth Factor

References

1. Krumina G. Metastatic disease of the brain: parenchyma. *Eur Radiol.* 2005; 15(3):608–616. [PubMed: 15696291]
2. Weil RJ, Palmieri DC, Bronder JL, Stark AM, Steeg PS. Breast cancer metastasis to the central nervous system. *Am J Pathol.* 2005; 167(4):913–920. [PubMed: 16192626]
3. Altundag K, Bondy ML, Mirza NQ, Kau SW, Broglio K, Hortobagyi GN, Rivera E. Clinicopathologic characteristics and prognostic factors in 420 metastatic breast cancer patients with central nervous system metastasis. *Cancer.* 2007; 110(12):2640–2647. [PubMed: 17960791]
4. Cheng X, Hung MC. Breast cancer brain metastases. *Cancer metastasis reviews.* 2007; 26(3–4): 635–643. [PubMed: 17717635]
5. Gril B, Evans L, Palmieri D, Steeg PS. Translational research in brain metastasis is identifying molecular pathways that may lead to the development of new therapeutic strategies. *Eur J Cancer.* 2010; 46(7):1204–1210. [PubMed: 20303257]
6. Palmieri D, Chambers AF, Felding-Habermann B, Huang S, Steeg PS. The biology of metastasis to a sanctuary site. *Clin Cancer Res.* 2007; 13(6):1656–1662. [PubMed: 17363518]
7. Kang Y. New tricks against an old foe: molecular dissection of metastasis tissue tropism in breast cancer. *Breast disease.* 2006; 26:129–138. [PubMed: 17473371]
8. Jain RK, di Tomaso E, Duda DG, Loeffler JS, Sorensen AG, Batchelor TT. Angiogenesis in brain tumours. *Nat Rev Neurosci.* 2007; 8(8):610–622. [PubMed: 17643088]
9. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med.* 2003; 9(6):677–684. [PubMed: 12778166]
10. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* 1992; 359(6398):843–845. [PubMed: 1279431]
11. Kusters B, de Waal RM, Wesseling P, Verrijp K, Maass C, Heerschap A, Barentsz JO, Sweep F, Ruiter DJ, Leenders WP. Differential effects of vascular endothelial growth factor A isoforms in a mouse brain metastasis model of human melanoma. *Cancer Res.* 2003; 63(17):5408–5413. [PubMed: 14500375]
12. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* 1999; 284(5422):1994–1998. [PubMed: 10373119]
13. Leenders WP, Küsters B, Verrijp K, Maass C, Wesseling P, Heerschap A, Ruiter D, Ryan A, de Waal R. Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. *Clin Cancer Res.* 2004; 10(18 Pt 1):6222–6230. [PubMed: 15448011]
14. Claes A, Gambarota G, Hamans B, Van Tellingen O, Wesseling P, Maass C, Heerschap A, Leenders W. Magnetic resonance imaging-based detection of glial brain tumors in mice after antiangiogenic treatment. *Int J Cancer.* 2008; 122(9):1981–1986. [PubMed: 18081012]
15. Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, Winkler F. Real-time imaging reveals the single steps of brain metastasis formation. *Nat Med.* 2009
16. Tatum JL, Kelloff GJ, Gillies RJ, Arbeit JM, Brown JM, Chao KS, Chapman JD, Eckelman WC, Fyles AW, Giaccia AJ, Hill RP, Koch CJ, Krishna MC, Krohn KA, Lewis JS, Mason RP, Melillo G, Padhani AR, Powis G, Rajendran JG, Reba R, Robinson SP, Semenza GL, Swartz HM, Vaupel P, Yang D, Croft B, Hoffman J, Liu G, Stone H, Sullivan D. Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. *Int J Radiat Biol.* 2006; 82(10):699–757. [PubMed: 17118889]
17. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer metastasis reviews.* 2007; 26(2):225–239. [PubMed: 17440684]
18. Rodrigues L, Howe F, Griffiths J, Robinson S. Tumor R2* is a prognostic indicator of acute radiotherapeutic response in rodent tumors. *J Magn Reson Imaging.* 2004; 19(4):482–488. [PubMed: 15065173]
19. Mittal S, Wu Z, Neelavalli J, Haacke EM. Susceptibility-weighted imaging: technical aspects and clinical applications, part 2. *AJNR Am J Neuroradiol.* 2009; 30(2):232–252. [PubMed: 19131406]

20. Haacke EM, Mittal S, Wu Z, Neelavalli J, Cheng YC. Susceptibility-weighted imaging: technical aspects and clinical applications, part 1. *AJNR Am J Neuroradiol*. 2009; 30(1):19–30. [PubMed: 19039041]
21. Rauscher A, Sedlacik J, Barth M, Haacke EM, Reichenbach JR. Noninvasive assessment of vascular architecture and function during modulated blood oxygenation using susceptibility weighted magnetic resonance imaging. *Magn Reson Med*. 2005; 54(1):87–95. [PubMed: 15968657]
22. Song HT, Jordan EK, Lewis BK, Gold E, Liu W, Frank JA. Quantitative T(2)* imaging of metastatic human breast cancer to brain in the nude rat at 3 T. *NMR Biomed*.
23. Song HT, Jordan EK, Lewis BK, Liu W, Ganjei J, Klaunberg B, Despres D, Palmieri D, Frank JA. Rat model of metastatic breast cancer monitored by MRI at 3 tesla and bioluminescence imaging with histological correlation. *Journal of translational medicine*. 2009; 7:88. [PubMed: 19840404]
24. Arbab AS, Yocum GT, Kalish H, Jordan EK, Anderson SA, Khakoo AY, Read EJ, Frank JA. Efficient magnetic cell labeling with protamine sulfate complexed to ferumoxides for cellular MRI. *Blood*. 2004; 104(4):1217–1223. [PubMed: 15100158]
25. Himmelreich U, Weber R, Ramos-Cabrer P, Wegener S, Kandal K, Shapiro EM, Koretsky AP, Hoehn M. Improved stem cell MR detectability in animal models by modification of the inhalation gas. *Mol Imaging*. 2005; 4(2):104–109. [PubMed: 16105508]
26. Yang QX, Demeure RJ, Dardzinski BJ, Arnold BW, Smith MB. Multiple echo frequency-domain image contrast: improved signal-to-noise ratio and T2 (T2*) weighting. *Magn Reson Med*. 1999; 41(2):423–428. [PubMed: 10080294]
27. Noll DC, Nishimura DG, Macovski A. Homodyne detection in magnetic resonance imaging. *IEEE Trans Med Imaging*. 1991; 10(2):154–163. [PubMed: 18222812]
28. Woods RP, Grafton ST, Watson JD, Sicotte NL, Mazziotta JC. Automated image registration: II. Intersubject validation of linear and nonlinear models. *J Comput Assist Tomogr*. 1998; 22(1):153–165. [PubMed: 9448780]
29. Marques JP, Maddage R, Mlynarik V, Gruetter R. On the origin of the MR image phase contrast: an in vivo MR microscopy study of the rat brain at 14.1 T. *NeuroImage*. 2009; 46(2):345–352. [PubMed: 19254768]
30. McAuley G, Schrag M, Sipos P, Sun SW, Obenaus A, Neelavalli J, Haacke EM, Holshouser B, Madacsi R, Kirsch W. Quantification of punctate iron sources using magnetic resonance phase. *Magn Reson Med*. 2010; 63(1):106–115. [PubMed: 19953510]
31. Lorgier M, Felding-Habermann B. Capturing changes in the brain microenvironment during initial steps of breast cancer brain metastasis. *Am J Pathol*. 2010; 176(6):2958–2971. [PubMed: 20382702]
32. Heyn C, Ronald JA, Ramadan SS, Snir JA, Barry AM, MacKenzie LT, Mikulis DJ, Palmieri D, Bronder JL, Steeg PS, Yoneda T, MacDonald IC, Chambers AF, Rutt BK, Foster PJ. In vivo MRI of cancer cell fate at the single-cell level in a mouse model of breast cancer metastasis to the brain. *Magn Reson Med*. 2006; 56(5):1001–1010. [PubMed: 17029229]
33. Pawelczyk E, Arbab AS, Pandit S, Hu E, Frank JA. Expression of transferrin receptor and ferritin following ferumoxides-protamine sulfate labeling of cells: implications for cellular magnetic resonance imaging. *NMR in biomedicine*. 2006; 19(5):581–592. [PubMed: 16673357]
34. Arbab AS, Wilson LB, Ashari P, Jordan EK, Lewis BK, Frank JA. A model of lysosomal metabolism of dextran coated superparamagnetic iron oxide (SPIO) nanoparticles: implications for cellular magnetic resonance imaging. *NMR in biomedicine*. 2005; 18(6):383–389. [PubMed: 16013087]
35. Chambers AF, MacDonald IC, Schmidt EE, Morris VL, Groom AC. Clinical targets for anti-metastasis therapy. *Adv Cancer Res*. 2000; 79:91–121. [PubMed: 10818678]
36. Dome B, Timar J, Paku S. A novel concept of glomeruloid body formation in experimental cerebral metastases. *J Neuropathol Exp Neurol*. 2003; 62(6):655–661. [PubMed: 12834110]
37. Huang J, Soffer SZ, Kim ES, McCrudden KW, New T, Manley CA, Middlesworth W, O'Toole K, Yamashiro DJ, Kandel JJ. Vascular remodeling marks tumors that recur during chronic suppression of angiogenesis. *Mol Cancer Res*. 2004; 2(1):36–42. [PubMed: 14757844]

38. Weidner N. Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol.* 1995; 147(1):9–19. [PubMed: 7541613]
39. Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, Breier G. Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest.* 2002; 109(6):777–785. [PubMed: 11901186]
40. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene.* 1999; 18(38):5356–5362. [PubMed: 10498889]
41. Broom KA, Anthony DC, Blamire AM, Waters S, Styles P, Perry VH, Sibson NR. MRI reveals that early changes in cerebral blood volume precede blood-brain barrier breakdown and overt pathology in MS-like lesions in rat brain. *J Cereb Blood Flow Metab.* 2005; 25(2):204–216. [PubMed: 15678123]
42. Carbonell WS, Ansorge O, Sibson N, Muschel R. The vascular basement membrane as “soil” in brain metastasis. *PLoS One.* 2009; 4(6):e5857. [PubMed: 19516901]
43. Sutherland RM. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science.* 1988; 240(4849):177–184. [PubMed: 2451290]
44. Fidler IJ, Balasubramanian K, Lin Q, Kim SW, Kim SJ. The brain microenvironment and cancer metastasis. *Molecules and cells.* 30(2):93–98. [PubMed: 20799011]
45. Ribatti D, Vacca A, Dammacco F. New non-angiogenesis dependent pathways for tumour growth. *Eur J Cancer.* 2003; 39(13):1835–1841. [PubMed: 12932660]
46. Heddleston JM, Li Z, McLendon RE, Hjelmeland AB, Rich JN. The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. *Cell Cycle.* 2009; 8(20)
47. Fujiwara S, Nakagawa K, Harada H, Nagato S, Furukawa K, Teraoka M, Seno T, Oka K, Iwata S, Ohnishi T. Silencing hypoxia-inducible factor-1alpha inhibits cell migration and invasion under hypoxic environment in malignant gliomas. *Int J Oncol.* 2007; 30(4):793–802. [PubMed: 17332917]
48. Fukumura D, Xu L, Chen Y, Gohongi T, Seed B, Jain RK. Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. *Cancer Res.* 2001; 61(16):6020–6024. [PubMed: 11507045]
49. Glunde K, Guggino SE, Solaiyappan M, Pathak AP, Ichikawa Y, Bhujwalla ZM. Extracellular acidification alters lysosomal trafficking in human breast cancer cells. *Neoplasia.* 2003; 5(6):533–545. [PubMed: 14965446]
50. Sehgal V, Delproposto Z, Haddar D, Haacke EM, Sloan AE, Zamorano LJ, Barger G, Hu J, Xu Y, Prabhakaran KP, Elangovan IR, Neelavalli J, Reichenbach JR. Susceptibility-weighted imaging to visualize blood products and improve tumor contrast in the study of brain masses. *J Magn Reson Imaging.* 2006; 24(1):41–51. [PubMed: 16755540]
51. Dunn JF, O'Hara JA, Zaim-Wadghiri Y, Lei H, Meyerand ME, Grinberg OY, Hou H, Hoopes PJ, Demidenko E, Swartz HM. Changes in oxygenation of intracranial tumors with carbogen: a BOLD MRI and EPR oximetry study. *J Magn Reson Imaging.* 2002; 16(5):511–521. [PubMed: 12412027]
52. Baudalet C, Gallez B. How does blood oxygen level-dependent (BOLD) contrast correlate with oxygen partial pressure (pO₂) inside tumors? *Magn Reson Med.* 2002; 48(6):980–986. [PubMed: 12465107]
53. Yablonskiy DA, Haacke EM. Theory of NMR signal behavior in magnetically inhomogeneous tissues: the static dephasing regime. *Magn Reson Med.* 1994; 32(6):749–763. [PubMed: 7869897]
54. Robinson SP, Collingridge DR, Howe FA, Rodrigues LM, Chaplin DJ, Griffiths JR. Tumour response to hypercapnia and hyperoxia monitored by FLOOD magnetic resonance imaging. *NMR in biomedicine.* 1999; 12(2):98–106. [PubMed: 10392806]
55. Howe FA, Robinson SP, Rodrigues LM, Griffiths JR. Flow and oxygenation dependent (FLOOD) contrast MR imaging to monitor the response of rat tumors to carbogen breathing. *Magn Reson Imaging.* 1999; 17(9):1307–1318. [PubMed: 10576716]

56. Burrell JS, Walker-Samuel S, Baker LC, Boulton JK, Ryan AJ, Waterton JC, Halliday J, Robinson SP. Investigating temporal fluctuations in tumor vasculature with combined carbogen and ultrasmall superparamagnetic iron oxide particle (CUSPIO) imaging. *Magnetic resonance in medicine: official journal of the Society of Magnetic Resonance in Medicine/Society of Magnetic Resonance in Medicine*. 2011; 66(1):227–234. [PubMed: 21305600]

Figure 1.

Phase contrast MRI of breast cancer metastases in the brain. Three adjacent slices from a single animal are shown at 4 weeks after intracardiac injection of unlabeled 10^6 MDA-MB-231BRL cells. Of the tumors that were visible on T_2 -weighted images (A), the competing effects of edema and susceptibility rendered most tumors nearly isointense or hyperintense on T_2^* -weighted magnitude images (B). These same tumors were highly conspicuous on phase images (C), with different metastases displaying different spatial patterns. Carbogen (95/5 % O_2/CO_2) was used as the inhalation gas to decrease the magnetic susceptibility effects of the vasculature. Only one of the four metastases shown had BBB permeability as evidenced by leakage of Gd-DTPA (D).

Figure 2. Phase contrast in metastatic tumors is not maintained ex vivo following perfusion fixation. T₂-weighted images depict an edematous metastatic lesion in vivo (A,B) and ex vivo (D,E). The phase contrast between the tumor and the surrounding brain is evident in vivo (C) but is absent ex vivo (F), whereas the contrast between white matter and gray matter is maintained. This suggests that the origin of the phase change is vascular and is not a result of the increased edema. The H & E stained sections (G & H) demonstrated both the invasive growth of 231BRL metastases and the edema seen as an enlargement of the extracellular space. Scale bars indicate 500 μ m.

Figure 3.

Serial MRI of metastatic tumor development. Rats underwent MRI before (week -1) and after intracardiac injection of FePro labeled MDA-MB-231-BRL cells. T₂-weighted spin-echo images (A) show a metastatic tumor visible at week 4 (arrowheads). At 1 day post-injection (week 0), labeled cells were visible on both T₂*-weighted magnitude (B) and phase (C) images, although phase images provided higher contrast to visualize the labeled cells. The iron label substantially decreased in successive weeks and disappeared completely by week 3. A developing tumor, although small, was visible at week 3 and became more pronounced at week 4. Despite the use of carbogen, baseline images were essential to differentiate preexisting large vessels (arrows) from developing tumors. The region of magnification is depicted in D. Ex vivo T₂-weighted MRI is shown in E and H&E staining is shown in F. Scale bars indicate 0.5 mm.

Figure 4.

Susceptibility changes preceded blood-brain barrier permeability. Demarcation of the tumor border was performed at week 5 on the T_2 -weighted images (arrowhead, top right). Spatial registration of images allowed the identical regions of interest to be applied to T_2^* maps, phase images, and enhancement maps from previous weeks. At 4 weeks post-injection, susceptibility changes within the metastasis were visible as a decreased T_2^* and an increased phase dispersion within the tumor margin. These changes progressed at week 5 and were accompanied by leakage of Gd-DTPA within the tumor indicative of a disrupted BBB. The mean and standard deviation of the pixel values within the tumor are displayed for each quantitative imaging measure.

Figure 5. Region of interest analysis of developing metastases. * - $p < 0.05$, ** - $p < 0.001$ compared to baseline. Graphs indicate mean $\pm$ inter-animal s.d. (n=52 tumors in 8 animals)

Figure 6.

Vascular changes in MDA-MB-231-BRL brain metastases. 231BRL brain metastases, identified both as CD44-positive cells and DAPI-stained hypercellular clusters, (A) grow by coopting the existing vasculature as evidenced by the location of tumor cells situated in the perivascular space surrounding a central endothelial cell. Compared to vessels in the normal rat brain cortex (B), vessels co-opted by 231BRL cells were less dense (C) and enlarged (D). Endothelial cells lining the co-opted vessels (E,F) were tortuous and convoluted (arrow), indicative of vascular remodeling. Scale bars indicate 100 μ m. Graphs indicate mean $\pm$ s.e.m (n=16 tumors in 4 animals).

Figure 7.

Hypoxia and pro-invasive markers in 231BRL brain metastases. 231BRL cells express the hypoxia marker HIF1 α (A, B) and pro-invasive markers β 1-integrin (C) and MMP9 (D). Co-opted vessels lack VEGF expression (E, arrowheads), whereas metastases that progress to solid masses include vessels with increased VEGF expression (E; arrows). Increased astrocyte activity surrounding the metastasis is shown by GFAP immunoreactivity (E), and the tumor hypercellularity is demonstrated by DAPI staining. Scale bars indicate 100 μ m.

Table 1

Region of interest serial analysis.

Parameter	Region of Interest	Week						
		-1	0	1	2	3	4	5
Phase Mean (Hz)	Tumor	0.09 ± 0.15	0.14 ± 0.22	0.07 ± 0.12	0.06 ± 0.09	0.03 ± 0.09	-0.01 ± 0.13	-0.06 ± 0.03
	Contralateral	0.10 ± 0.13	0.19 ± 0.15	0.05 ± 0.10	0.06 ± 0.11	0.06 ± 0.10	0.07 ± 0.12	-0.06 ± 0.24
	p-value	ns	ns	ns	ns	ns	ns	ns
Phase s.d. (Hz)	Tumor	0.41 ± 0.11	0.71 ± 0.16	0.43 ± 0.16	0.42 ± 0.15	0.49 ± 0.21	0.59 ± 0.11*	0.77 ± 0.13**
	Contralateral	0.38 ± 0.07	0.63 ± 0.12	0.39 ± 0.09	0.39 ± 0.10	0.39 ± 0.08	0.36 ± 0.07	0.41 ± 0.14
	p-value	ns	ns	ns	ns	ns	0.0002	0.0101
T ₂ [*] (ms)	Tumor	34.83 ± 3.55	32.50 ± 8.12	37.48 ± 3.00	37.52 ± 5.36	35.45 ± 4.82	36.89 ± 3.59	34.90 ± 4.18
	Contralateral	35.34 ± 4.47	33.63 ± 7.05	38.64 ± 3.34	37.38 ± 5.82	37.11 ± 4.84	38.72 ± 4.95	36.41 ± 4.59
	p-value	ns	ns	ns	ns	ns	ns	ns
Enhancement (%)	Tumor	-	-	-	3.18 ± 0.84	4.92 ± 1.51	3.78 ± 1.70	14.26 ± 10.67 [†]
	Contralateral	-	-	-	3.69 ± 1.87	3.18 ± 2.02	1.51 ± 1.07	0.73 ± 1.19
	p-value	-	-	-	ns	ns	0.0114	0.0453
Normalized T ₂ w Signal		1.02 ± 0.07	1.02 ± 0.06	1.00 ± 0.04	0.99 ± 0.03	0.99 ± 0.07	1.05 ± 0.05	1.12 ± 0.02**

* - p < 0.05 compared to week -1;

** - p < 0.001 compared to week -1;

[†] - p < 0.05 compared to week 2; ns – not significant