Automated Tracking of Nanoparticle-labeled Melanoma Cells Improves the Predictive Power of a Brain Metastasis Model

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Abstract

Biologic and therapeutic advances in melanoma brain metastasis are hampered by the paucity of reproducible and predictive animal models. In this work, we developed a robust model of brain metastasis that empowers quantitative tracking of cellular dissemination and tumor progression. Human melanoma cells labeled with superparamagnetic iron oxide nanoparticles (SPION) were injected into the left cardiac ventricle of mice and visualized by MRI. We showed that SPION exposure did not affect viability, growth, or migration in multiple cell lines across several in vitro assays. Moreover, labeling did not impose changes in cell-cycle distribution or apoptosis. In vivo, several SPION-positive cell lines displayed similar cerebral imaging and histologic features. MRI-based automated quantification of labeled cells in the brain showed a sigmoid association between metastasis frequency and doses of inoculated cells. Validation of this fully automated quantification showed a strong correlation with manual signal registration ($r^2 = 0.921$, $P < 0.001$) and incidence of brain metastases ($r^2 = 0.708$, $P < 0.001$). Metastasis formation resembled the pattern seen in humans and was unaffected by SPION labeling (histology; tumor count, $P = 0.547$; survival, $P = 0.686$). In summary, we present here a highly reproducible animal model that can improve the predictive value of mechanistic and therapeutic studies of melanoma brain metastasis. Cancer Res; 73(8); 2445–56. ©2013 AACR.

Introduction

Melanoma has the highest propensity, of all cancers, to metastasize to the brain (1). Patients often develop multiple brain metastases and have a dismal prognosis (2). Recent work with drugs targeting molecular subgroups of metastatic melanoma has shown encouraging results (3–5). However, these drugs have mostly transient and incomplete responses, and significant efforts are needed to develop more effective treatment strategies, especially for patients with brain metastases (6–8).

Reproducible and predictive animal models of brain metastasis are a prerequisite for therapeutic progress (9–12). Such models are required to uncover the enigmatic biology of melanoma brain metastasis (7, 8, 13, 14), to bridge the gap between preclinical and clinical efficacy studies (10, 15–18), and to functionally validate a vast array of new potential metastasis genes (19–22).

In brain metastasis research, one of the most used models includes freehand injection of cancer cells into the left cardiac ventricle of mice (23–25). When using this technique, it is necessary to verify that the right tumor cell load has been delivered to the brain to avoid making flawed conclusions from later observations (11, 24). Moreover, many cancers are heterogeneous with numerous cells of differing metastatic potential (26, 27), and the multistep metastatic process is generally highly ineffective (28–30). For these reasons, and to improve the predictive value of experimental brain metastasis models, it has been a long-standing goal to quantify the metastatic process (11, 28, 31).

MRI is the best modality for imaging brain pathology due to its high spatial resolution and excellent tissue contrast. Nonetheless, MRI alone cannot identify micrometastases or track single tumor cells in the brain. Nanoparticles have, therefore, seen an increasing use as MRI contrast agents (31–33). Superparamagnetic iron oxide nanoparticles (SPION) dominate the field of MRI-based cell tracking and have many advantages over conventional contrast agents, one of the most important...
being high magnetic signal strength (31–34). The coupling of MRI and nanoparticles can allow exceptional real-time visualization and quantification of cancer cells and tumors in the brain (31). It has even been reported that SPION labeling empowers in vivo tracking of single cancer cells in the brain (35). However, a quick, sensitive, and reproducible quantification of SPION-positive cells by MRI has proven to be a challenge (32, 36–38).

We here report on the development and validation of a novel and reproducible brain metastasis model with fully automated MRI-based quantification of SPION-labeled melanoma cells in the mouse brain after intracardial injection. This highly predictive model can provide valuable insight into the biology and therapy of melanoma brain metastasis, and finally help to increase the success rates of anticancer agents in clinical trials.

Materials and Methods

Superparamagnetic iron oxide nanoparticles

The synthesis and characterization of poly-L-Lysine–coated maghemite (PLL–γ-Fe₂O₃) nanoparticles has been reported previously (39). The toxicity of these particles as compared with other SPIONs has recently been evaluated (40).

Cell lines and cell culture

The H1, H2, and H3 cell lines were developed in our laboratory from resected tumors of 3 patients with melanoma brain metastases as previously described (ref. 41; Supplementary Fig. S1). We obtained written consent before tumor material was collected. The Regional Ethical Committee (no.1309) and the Norwegian Directorate of Health (9634) approved the tissue collection and storage. The SK-MEL-28 and A375 cell lines, established from primary cutaneous melanomas, were purchased from the American Type Culture Collection and used within 6 months. The H1, H2, and H3 cell lines were last authenticated in September 2012 using the AmpF/STR Profiler Plus PCR Amplification Kit (Applied Biosystems) and short tandem repeat profiles were matched to the parent tumor and cross-checked with cell line profiles at www.dsmz.de.

The H1, H2, SK-MEL-28, and A375 cell lines have the BRAFV600E mutation, whereas the H3 cell line is BRAFWT (see Supplementary Material, ref. 42).

The H1 cell line was transduced with the genes for GFP and luciferase as previously described (41). The H2, H3, and SK-MEL-28 cell lines were transduced with a lentiviral pGFP-CMV reporter vector that coexpresses copGFP and firefly luciferase linked by the self-cleaving peptide T2A (System Biosciences). Flow cytometric isolation of cells by GFP expression was conducted on a BD FACSAria (BD Biosciences). The transduced cell lines were used in all experiments.

All cells were grown on Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum, 4 times the prescribed concentration of nonessential amino acids, 2% L-glutamine, penicillin (100 IU/mL), and streptomycin (100 μg/mL; hereafter, called complete DMEM (BioWhittaker)). The cells were kept in a standard tissue culture incubator at 37°C (100% humidity, 5% CO₂). The growth medium was exchanged twice a week.

Cell proliferation and labeling assays

The H1, H2, H3, and SK-MEL-28 cells were seeded in 6-well plates (Nunc), each well with 10⁵ cells in 2 mL medium. Twenty-four hours later, SPIONs suspended in complete DMEM were added to the wells at 2.5, 5, 10, or 15 μg/mL. Wells with unlabeled cells were used as controls. Cells were counted 24, 48, and 72 hours after labeling (4 unlabeled and 3 labeled wells for each concentration and time point). Before counting, each well received 0.5 mL trypsin for 3 minutes followed by 0.5 mL complete DMEM. A 100 μL of the single-cell suspension was mixed with 100 μL Trypan blue and kept on ice. Live and dead cells were quantified using a hemacytometer. All proliferation assays were conducted in triplicate and paralleled by assessments of labeling efficacy with Perls’ Prussian blue (PPB) staining, which renders SPION-positive cells blue (see Supplementary Material).

Scanning electron microscopy and transmission electron microscopy

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) procedures are described in the Supplementary Material.

Flow cytometric DNA analysis

H1 cells were seeded in 25 cm² culture flasks (260,000 cells/flask) and grown for 24 hours before SPIONs were added to a concentration of 10 μg/mL in 3 of 6 flasks. Twenty-four hours later, the cells were trypsinized, centrifuged at 900 rpm for 4 minutes, and fixed in ice-cold 70% ethanol. They were stored at −20°C until the next day when they again were centrifuged (900 rpm for 15 minutes) before removing the ethanol. Each sample was stained with a 100 μL freshly made mixture of PBS, ribonuclease (1 mg/mL), and propidium iodide (PI; 1 mg/mL). The cells were then incubated in the dark for 30 minutes at room temperature. The cellular DNA content was measured using a BD Accuri C6 flow cytometer (BD Biosciences). DNA histograms were obtained by gating a 2-parameter forward- and side-scatter cytogram to a 1-parameter DNA histogram. Unlabeled H1 cells were used as negative controls. Cell-cycle distribution was determined using ModFit LT 3.3 (Verity Software House). Experiments were conducted in triplicate.

Flow cytometric analysis of apoptosis

H1 cells were prepared as described earlier for the DNA analysis. Annexin V staining for apoptosis detection was conducted using the Alexa Fluor 647 Conjugate kit (Invitrogen) according to the manufacturer’s instructions. The percentage of apoptotic cells was measured using 2-parameter histograms on a BD Accuri C6 flow cytometer (BD Biosciences). Experiments were conducted in triplicate.

In vitro wound healing and spheroid growth

The wound healing and spheroid growth assays are described in the Supplementary Material.

Mice

Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice that were 6 to 8 weeks old were bred...
and maintained in animal facilities certified by the Association for Assessment and Accreditation of Laboratory Animal Care International (see Supplementary Material).

**Cell line preparation and injection**

A total of 8 \( \times \) 10^5 tumor cells were incubated in 75 cm^2 culture flasks (Nunc) for 24 hours before adding SPIONs to a concentration of 10 μg/mL in complete DMEM for another 24 hours. Flasks with control cells did not contain nanoparticles. Cells were subsequently washed with PBS to remove unincorporated particles before trypsinization. After centrifugation at 900 rpm for 4 minutes, syringes were prepared with a total volume of 0.1 mL of PBS and 10^5, 5 \( \times \) 10^5, or 10^6 cells. The cell-containing syringes were kept on ice.

Mice received a 0.02 mL subcutaneous injection of 0.3 mg/mL buprenorphine hydrochloride (Temgesic; Reckitt Benckiser Pharmaceuticals Inc.) for prolonged pain relief post injection. A 0.1 mL tumor cell suspension was then slowly injected into the left cardiac ventricle freehand using a 30G insulin syringe (Omnican50; B. Braun Melsungen AG) as previously described (43). Mice were given 10^4 (n = 6), 10^5 (n = 6), 5 \( \times \) 10^5 (n = 11), or 10^6 (n = 7) H1 cells labeled with SPIONs. Control mice received 10^6 (n = 9) unlabeled H1 cells. We also injected 5 \( \times \) 10^5 labeled cells from H2 (n = 6), H3 (n = 6), and SK-MEL-28 (n = 4).

**Bioluminescence imaging**

Bioluminescence imaging (BLI) was done at the day of injection to evaluate inoculation failure and after 2, 4, and 6 weeks to study systemic tumor development (see Supplementary Material).

**Magnetic resonance imaging**

MRI was carried out using a 7 Tesla small-animal scanner (PharmaScan; Bruker BioSpin MRI) equipped with a circular mouse head transmit/receive coil. FOV 2.0 cm, matrix size 256 \( \times \) 256, and slice thickness 1 mm were constant across the optimized sequences. We obtained coronal images with a T2w sequence (TR 1,500 ms; TE, 2.8, 7.2, 11.6, 16, 20.4, and 24.8 ms; FA 30 degrees; 1 average; and scan time, 6 minutes 21 seconds). A T1w image series of variable quality. The mean detection count.

One mouse injected with 5 \( \times \) 10^5 labeled H1 cells developed 10 brain metastases. Twenty-four hours after sacrifice at 8 weeks, we scanned the fixed brain (4% paraformaldehyde in PBS) with a high-resolution T2w RARE sequence (TR 4,200 ms; TE 35 ms; slice thickness 0.5 mm; FOV 2.0 \( \times \) 2.0 cm; matrix size 256 \( \times \) 256; and 16 averages). A three-dimensional (3D) brain model illustrating the total metastasis burden was then made by manual segmentation in Imaris 6.1.5 (Bitplane AG).

**Quantification of SPION-labeled cells in the brain**

Using FSL 4.1 (www.fmrib.ox.ac.uk/fsl), a 3D brain mask was drawn for one of the mice on the basis of a high-quality T2w series. To avoid time-consuming drawings for a large number of datasets, an affine registration was found between the first echo of the T2’w sequence from every mouse to the brain mask space using FSL’s FLIRT (FMRIB’s Linear Image Registration Tool). The second echo (TE 7.2 ms) was used for signal detection, as this represented the best trade-off between the hypointensive signals from the SPION-positive cells and the structural features of the brain.

Using MATLAB 7.14 (MathWorks), we made a signal detection algorithm, enabling fully automated quantification of SPION-positive cells (Supplementary Fig. S2). A binary image with probable detections was generated from the second echo by applying adaptive thresholding. This procedure generated many false-positive detections due to a high sensitivity and noise as well as inhomogeneities in the MR images.

We, therefore, trained a multilayer feedforward neural network based on expert labeling using MATLAB’s Neural Network Toolbox (MathWorks). Expert labeling of a selection of true-positive and false-positive signals was carried out on deidentified second echoes from 10 random mice. We extracted the signal intensities from a 5 \( \times \) 5 window around all expert labels, and rearranged these into 25 \( \times \) 1 vectors. These vectors were matched with the corresponding vectors from the first echoes and transformed into distinctive 50 \( \times \) 1 feature vectors that were assembled into a large training set. This training set describing the unique signal properties of all expert labels was used to generate a neural network database that could be integrated in the automated classification of true or false signal detections.

The binary signal detection image of the SPION-positive cells was transformed into the brain mask space using the obtained affine transformation, and the detected signals were intersected with the brain mask to remove candidates outside the brain. The remaining detections were counted. As a baseline, automated quantification was carried out on 3 control mice with T2’w image series of variable quality. The mean numbers of false-positive detections in these control mice (mean 7, values 0 – 8 – 14) were subtracted from the automated signal detection count.

As a validating measure, the deidentified second echo series of all T2’w sequences were examined in OsiriX 4.1 (Pixmeo), and all SPION-positive cells were manually identified and counted.
Histology and immunohistochemistry

Mouse brains from all labeled and unlabeled groups were stained with hematoxylin and eosin (H&E) to evaluate morphology, PPB to visualize iron, and HMB-45 to identify melanocytic cells (see Supplementary Material).

Statistical analysis

Statistical analyses were conducted with SPSS 19.0.0 for Mac (SPSS Inc.). The statistical tests used are specified in the Results section and in the Supplementary Material. Values are presented as means ± SD. A 2-tailed \( P < 0.05 \) was considered significant.

Results

SPIONs do not affect cellular viability across multiple cell lines

To investigate the optimal labeling concentration, we assessed cellular viability in proliferation assays. H1 cells did not display any changes in viability with increasing SPION concentrations up to 15 \( \mu \text{g/mL} \) (Figs. 1A and B). In a comparative proliferation assay, the viability of the H1, H2, H3, and SK-MEL-28 cell lines was not affected by 10 \( \mu \text{g/mL} \) of SPIONs for 1, 2, or 3 days (Fig. 1C).

Tumor cell labeling efficacy is dependent on SPION concentration and exposure time

To further determine the best labeling concentration and to define the ideal SPION exposure time, we evaluated labeling efficacy at different concentrations and at different lengths of time. The fraction of labeled cells increased up to 10 \( \mu \text{g/mL} \) of SPIONs and did not improve beyond 24 hours of exposure (Fig. 2A). We decided on a labeling protocol using 10 \( \mu \text{g/mL} \) of SPIONs for 24 hours, and tested this protocol in a comparative assay with the H1, H2, H3, and SK-MEL-28 cell lines (Fig. 2B). Throughout, we found a labeling fraction of more than 95%.

SPIONs are trapped at the cell surface and transported to the cytoplasm

We carried out SEM to study the cellular surface morphology of SPION-positive and -negative cells. SEM showed elongated, stellate H1 cells with several filopodia at the leading and trailing edges (Fig. 2C). Numerous pseudopodia could be seen at the cell surface, and SPIONs were trapped inside this dense meshwork. We used TEM to explore the intracellular distribution of SPIONs. TEM displayed SPIONs in cytoplasmatic vesicles, often localized close to the cell membrane.

Figure 1. Cellular viability and death is not affected by increasing concentrations of SPIONs. Increasing concentrations of SPIONs (0, 2.5, 5, 10, and 15 \( \mu \text{g/mL} \)) were added to H1 monolayer cultures, and the number of live (A) and dead (B) cells were determined at 1, 2, and 3 days (\( n = 3 \), mean ± SD) and tested with 1-way ANOVA analysis. A, live H1 cells \([\cdot, F(4,61) = 0.17, P = 0.95; \cdot, F(4,61) = 0.95; \cdot, F(4,61) = 0.65, P = 0.65; \cdot, F(4,61) = 0.74, P = 0.57]\). B, dead H1 cells \([\cdot, F(4,61) = 2.0, P = 0.10; \cdot, F(4,61) = 1.63, P = 0.18; \cdot, F(4,61) = 0.64, P = 0.29]\). C, a SPION concentration of 10 \( \mu \text{g/mL} \) did not affect the viability of the H1, H2, H3, and SK-MEL-28 cell lines over a 3-day period (\( n = 3 \)) as evaluated by the Student t test \( H1 (P = 0.72, 0.51, \text{ and } 0.91), H2 (P = 0.13, 0.49, \text{ and } 0.86), H3 (P = 0.99, 0.14, \text{ and } 0.80), \text{ and SK-MEL-28 (P = 0.39, 0.26, \text{ and } 0.57)} \). The low proliferation rate of H3 cells is also evident in Fig. 2B and was confirmed in vivo (data not shown).
Labeling does not affect in vitro wound healing or spheroid growth

To test whether SPION exposure affected the directional migration of tumor cells, we conducted a scratch wound assay. Unlabeled and labeled H1 cells showed similar wound closure ability (Supplementary Fig. S3). In addition, we studied unlabeled and labeled H1 tumor spheroids to assess cell proliferation in a 3D multicellular environment. SPION exposure did not influence spheroid growth over 28 days (Supplementary Fig. S3).

Cell-cycle distribution and apoptosis is not disturbed by SPION exposure

We wanted to find out whether cell-cycle distribution and apoptosis were affected by SPIONs. Flow cytometric analysis did not show any difference between unlabeled and labeled H1 cells (Fig. 3).

Melanoma cell lines labeled with SPIONs show similar MRI and histologic features in the mouse brain

We used in vivo MRI to examine the imaging qualities and reproducibility of our labeled cell lines. T2’w MRI (TE 7.2 ms) showed comparable signal intensity and frequency of SPION-positive H1, H2, and SK-MEL-28 cells 24 hours after intracardial injection of 5 × 10⁶ cells (Fig. 4A; Supplementary Movie S1). Similar results were also obtained with the H3 cell line, the H1_Br1, H1_Br2, and H2_Br1 cell lines derived from serial in vivo passages in mice brains, and the A375 cell line (data not shown).

To study the initial distribution and fate of SPION-positive cells in the mouse brain, we acquired serial T2’w images directly after injection of 5 × 10⁵ labeled H1 cells. At 1 hour, we could clearly observe an increase in the number of hypointensive signals (Fig. 4B, top left), and larger and more confluent signals than at 24 hours (Fig. 4A, top left). The corresponding 1-hour T2w image showed normal cerebral anatomy, and a number of small, barely discernable hypointensive signals (Fig. 4B, bottom left). We did not observe any consistent change in MRI signal frequency from 1–6 hours post injection (data not shown). H&E staining showed normal mouse brain anatomy (fixed 6 hours after injection of 5 × 10⁵ labeled H1 cells; Fig. 4B, top middle). HMB-45 staining confirmed the melanocytic properties of the intravascularly entrapped tumor cells (Fig. 4B, bottom middle and right), whereas PPB staining showed that these cells were labeled with iron particles (Fig. 4B, top right).

Automated quantification of labeled cells reveals a sigmoid relationship to the injected cell quantity, a strong correlation to manual cell counts, and a decrease in labeled cells over time

To test the performance of and validate our automated quantification algorithm, we injected increasing cell concentrations. We observed an increase in the number of hypointensive signals from the lower to the higher concentrations; however, there was a relative reduction with the larger and less discrete signals in the animals that were given 10⁶ cells (Fig. 5A; Supplementary Movie S1). This resulted in a nonlinear relationship between the number of injected SPION-positive cells and the mean number of signals at 24 hours as detected by automated analysis (Fig. 5B). The mean values from the manual counts of SPION-positive cells were 15.5 ± 1.8 for 10⁵ cells, 92.8 ± 1.98 for 10⁶ cells, 345.2 ± 65.8 for 5 × 10⁶ cells, and 334 ± 226.8 for 10⁷ cells. We found significant differences between the 10⁵ and 10⁶ groups and the 10⁶ and 5 × 10⁷ groups (Student t test; both P < 0.001). There was no difference between the 5 × 10⁵ and 10⁶ groups (Student t test; P = 0.903).

There was a strong linear relationship between the manual and automated quantification results with an r² = 0.921 (Pearson linear correlation, P < 0.001; Fig. 5C). We observed
a decrease in detected SPION-positive cells over a 6-week period, as shown for the $5 \times 10^5$ group in Fig. 5D.

The pattern of dissemination seen in human melanoma brain metastasis is recapitulated in the mouse model

To consider the clinical relevancy of the animal model, we studied the morphologic features of the brain metastases. Figure 6A shows MR images of the maximum tumor burdens in the mice as evaluated by their final T2w images and T1w images with contrast before euthanization. Only 1 mouse in the $10^4$ group developed a noncontrast-enhancing cerebral tumor after 22 weeks (data not shown).

The melanoma brain metastases were melanocytic and widespread in the mouse brains (Fig. 6B, 2 left images; Supplementary Movie S2). H&E and HMB-45 stainings showed small, circumscribed tumors in the brain parenchyma (Fig. 6B, 2 images on the right). Histopathologic examination of tumor brains did not show any difference between mice that received labeled versus unlabeled cells (data not shown).

Tumor burden and survival relies on injected cell load and is unaltered by SPION labeling

To further characterize and validate the animal model, we evaluated the relationship between the number of cells injected and the number of cells detected versus the number of brain metastases. The number of contrast-enhancing tumors per animal increased significantly from the $10^4$ group to the $5 \times 10^5$ group (Fig. 6C and D). Thereafter, there was no difference between the $5 \times 10^5$ group and the $10^6$ group, or between the labeled and unlabeled $10^6$ groups (Student t test; $P > 0.05$; for details see Supplementary Table S1).

The $10^5$ and $5 \times 10^5$ groups developed larger tumors than both $10^6$ groups (Fig. 6A), had longer survival (Figs. 6C, D, and F; Supplementary Table S1), and were more often sacrificed due to brain tumor-related symptoms. Mice injected with $10^5$ cells more often succumbed to extracranial than intracranial disease as evaluated by cerebral MRI, whole-body optical imaging, and histologic investigations of visceral organs, femora, and spine (data not shown). Mice that did not develop tumors had
Figure 4. Labeling yields equivalent MRI and histologic characteristics for multiple cell lines. See text for details on MRI images in A and B. A, 24 hours after intracardial injection of $5 \times 10^5$ labeled H1, H2, and SK-MEL-28 cells. B, 1 hour after injection of $5 \times 10^2$ labeled H1 cells. Scale bar, MRIs 0.25 cm. H&E staining of the brain revealed no gross, morphologic alterations (top middle; scale bar, 2 mm). PPB staining (top right, scale bar, 10 μm) and HMB-45 staining (bottom middle, scale bar, 50 μm; bottom right, scale bar, 20 μm) showed intraluminally located tumor cells in longitudinal and transverse sections of capillaries throughout the brain.

consistently lower brain tumor cell loads at 24 hours and longer survival times (Fig. 6E and F).

Figure 6E shows that there was good concordance between the automated signal detections at 24 hours and the tumor count of the mice at the last MRI with an $r^2 = 0.708$ (Pearson linear correlation, $P < 0.001$). The $r^2$ was slightly lower for manual signal registrations ($r^2 = 0.691$; data not shown).

The mean survival time for the $10^4$ group was 156.7 ± 37.8 days, for the $10^5$ group 68.0 ± 12.5 days, for the $5 \times 10^5$ group 53.1 ± 5.1 days, for the $10^6$ group 39.4 ± 10.3 days, and for the control group 46.0 ± 2.5 days (Fig. 6F). The Mantel–Cox log-rank test showed significant differences between all group pairs ($P < 0.05$), except the $10^6$ labeled and unlabeled groups ($P = 0.547$).

Discussion

The main objective of the present study was to increase the predictive power of a melanoma brain metastasis model by controlling methodologic variation due to inherent technical limitations (23–25) and the heterogeneous nature of metastatic melanoma (8, 26, 27). This is essential to draw valid conclusions of biologic differences or therapeutic efficacy from experimental brain metastasis models (Fig. 7). To the best of our knowledge, this is the first brain metastasis model that routinely incorporates a fully automated, baseline quantifying of tumor cell deployment in the brain. This model should be a valuable complement to other recent models exploring the different aspects of melanoma progression (44–47).

De Vries and colleagues have illustrated to what extent variation in cell delivery may affect experimental reproducibility (48). They quantified SPION-positive dendritic cells injected into the lymph nodes of patients with melanoma and observed that cells were erroneously injected in 50% of patients despite ultrasound guidance by an experienced radiologist. Consequently, they proposed that inadequate delivery could explain why only a limited fraction of patients responded in clinical trials of dendritic cell vaccines.

Unfortunately, only 5% of anticancer agents that show preclinical effect are eventually licensed (18). Recent studies also indicate a drop in success rates in phase II trials, and further discouraging, a 58% failure rate of new anticancer drugs in phase III trials (15, 16). Adding to this, only a fraction of new approvals are for agents directed at novel targets, which, in part, has been attributed to the lack of proper in vivo validation (17). Evidently, more reproducible animal models are needed to elucidate the biology of brain metastasis (7, 8, 13, 14), and to functionally validate large-scale genomic and pharmacologic data obtained from human tumor material (19–22).

It has been shown that one subcutaneously implanted melanoma cell can develop into a tumor, and real-time imaging with multiphoton laser scanning microscopy has unraveled the unique steps of melanoma brain metastasis formation from the single-cell level (45, 47). However, as shown by Fidler and others, the metastatic cascade is generally highly inefficient (28–30), and many cancers are heterogeneous, harboring cells of variable metastatic potential (26, 27). We also found brain metastasis formation to be very inefficient; the majority of signal voids disappeared during the first 24 hours and, to a lesser extent, during the first 4 weeks (Figs. 4 and 5). Supposing a one-to-one relationship between cells and signals, approximately 0.05% of the injected cells were arrested in the brain at 24 hours, and approximately 3.5% of these cells later gave rise to contrast-enhancing tumors (Figs. 5 and 6). Taken together, it seems apparent that it is critical to deliver a sufficient and comparable number of cells to the brain to gather reliable data.
Intracardial injections in mice have usually been carried out freehand, sometimes guided by low-resolution ultrasound imaging (23–25). Injection successes have typically been inferred from an absent BLI “chest” signal, and tumorigenicity has been evaluated by brain metastasis burden with endpoint histology or in recent years by longitudinal MRI. Anything in between failure and success has seemingly been disregarded. This is essential because most existing data from mouse studies investigating drug efficacy against brain metastases are generated from models where it is impossible to know with certainty whether or not the mice have had an adequate cancer cell exposure to the brain (11, 24). This study shows that it is necessary to consider the actual cancer cell load in the brain at baseline, as this is related to the formation of contrast-enhancing brain metastases in a dose–response fashion (Figs. 5B, 6E, and 7). Moreover, MRI was superior to BLI in discriminating all degrees of injection success; several animals interpreted as successfully injected by BLI were classified as partial failures by

Figure 5. Automated quantification of labeled cells shows a sigmoid association with inoculated cell load, great concordance with manual registration, and a continuous decrease over time. A, T2-weighted MR images at 24 hours after intracardial injection of increasing concentrations of labeled H1 cells (top row, see also H2 in Supplementary Movie S1). The middle row displays the segmented signals inside the 3D brain mask that were registered for quantitative analysis (all blue signals were counted as one regardless of size and shape). The bottom row shows an overlay of the detected signals. Scale bar, 0.25 cm. B, the number of H1 cells injected plotted against the number of automated signal detections at 24 hours (Student t test). Mice injected with $10^4$ labeled cells had a mean number of 20.0 ± 4.6 SPION-positive cells at 24 hours ($n = 6$). The corresponding numbers were 58.3 ± 16.7 for $10^5$ cells ($n = 6$), 231.5 ± 39.2 for $5 \times 10^5$ cells ($n = 11$), and 159.0 ± 131.0 for $10^6$ cells ($n = 7, 1$ data point missing). The 2 animals with less than 100 detected signals in the $10^6$ group were injection failures not identified by BLI. C, there was a high degree of covariation between automated and manual quantification data. D, automated signal detection displayed a significant decrease over time (paired samples t test).

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Figure 6. Brain metastasis is dependent on the brain cell load, recapitulates the widespread nature seen in humans, and is unaffected by SPION labeling. A, MRI T2w and postcontrast T1w images show typical tumor burden before sacrifice for the $10^5$ group at 8 weeks, the $5 \times 10^5$ group at 6 weeks, and the labeled and unlabeled $10^6$ groups at 5 weeks. Scale bar, 0.25 cm. B, a macroscopic image of a mouse brain with melanocytic metastases (far left). A 3D model of a mouse brain with multiple metastases (middle left; see also Supplementary Movie S2). An H&E staining from the cerebellum shows a circular metastasis with typical noninvasive characteristics (middle right; scale bar, 200 μm). A cerebral tumor stained with HMB-45 shows similar features growing at the interface between the gray and white matter (far right; scale bar, 200 μm). C, an increasing number of contrast-enhancing tumors developed from the $10^4$ group to the $5 \times 10^5$ group. However, there was no significant difference between the $5 \times 10^5$ group and the $10^6$ group or between the labeled and unlabeled $10^6$ groups as evaluated by the Student t test ($P > 0.05$; see also Supplementary Table S1). D and E display the number of brain tumors at the last MRI before sacrifice; all animals were included, thus providing information about tumor burdens to expect within the different lifespans (F). D, the maximum number of contrast-enhancing tumors that developed in each mouse (Student t test; see also Supplementary Table S1). E, there was a good correlation between the automated signal detection and the maximum number of brain metastases. F, there was no difference in animal survival between the labeled and unlabeled $10^6$ groups (Mantel–Cox log-rank test; $P = 0.547$). Increasing cell loads were, otherwise, associated with decreasing survival times (Mantel–Cox log-rank test; $P < 0.05$). The $10^6$ group is not featured because the mice did not develop any contrast-enhancing tumors and the animals died of natural causes.
MRI (e.g., low brain tumor cell load as the two outliers in the 10^6 group in Fig. 5B which reappear in Fig. 6D with no tumors).

It is important to tailor each study to the tumorigenicity of the cell line used by injecting an appropriate number of cells. Thus, a foreseeable tumor burden can be expected in the brain and elsewhere in the body, as well as a probable survival time, and thereby, a practicable time frame for comparative analysis of biologic disparities or therapeutic interventions (Figs. 6A, C, D, and F; e.g., a tumor cell load of 10^6 H1 cells is apparently too high with a relative reduction in contrast-enhancing brain tumors because of extensive extracranial tumor burdens and shorter lifespans). As such, our model can be tuned to increase the precision level of studies on metastasis biology or treatment. This is important because the predictive value of most current preclinical data is limited by the fact that the drugs are usually tested in a preventive scenario with microscopic or low-volume metastatic disease, whereas in most clinical trials, the patients have advanced, high-volume metastatic disease (14, 49).

MRI coupled with SPION labeling is an excellent method for tracking cells in the brain (32, 33). T2*w sequences are commonly used to image SPION-labeled cells which appear as local hypointensive spots (50). SPIONs are generally considered to be safe with good biocompatibility and low toxicity (34, 50). Nonetheless, there are many different SPIONs available, and possible cytotoxic effects need to be addressed for each SPION and cell type used (31). The SPIONs used in the present study were recently tested in combination with human bone marrow mesenchymal stromal cells (40). To ensure the safe use of these SPIONs, the authors suggested keeping the labeling concentration as low as possible. Thus, we used two-thirds of the SPION concentration for one-third of the time and achieved almost 100% SPION-positive cells (Fig. 2). Extensive in vitro and in vivo testing on our melanoma cell lines did not reveal any significant effects of SPION labeling (Figs. 1, 3, 4, and 6; Supplementary Fig. S3).

In the present work, we have developed a robust and practical method for quantification of tumor cell load to the brain based on commonly used and readily available MRI sequences, image processing software, and machine learning tools. After a valid training set is procured for the combination of cell line and SPION used, later acquired T2*w images (scan time only 6 minutes 24 seconds) can be analyzed in a fully automated fashion using the described signal detection algorithm (Supplementary Fig. S2). This fully automated image analysis and the short MRI sequences considerably strengthen the applicability and throughput of the brain metastasis model, although with an inevitable trade-off between sensitivity and specificity (Fig. 5A and B; e.g., the relative reduction in detected signals for the 10^6 group).

The automated quantification system is not without limitations. As with most other available MRI protocols, there is a lack of a one-to-one relationship between cells and signals, making it difficult to differentiate individual signal voids as single or multiple cells (Fig. 5A, ref. 37). Individual signal voids should rather be interpreted as local concentrations of SPIONs. This is also illustrated by our histologic observations of a mixture of intravascular single cells and clusters of cells in 4-μm tissue sections (Fig. 4B) versus the MRI slices of 1 mm. Furthermore, the signal registration is only conducted within the predefined brain mask to avoid complicating artifacts and, consequently, does not count signals detected in the periphery of the brain (Fig. 7; Supplementary Fig. S2). Conclusively, MRI-based quantification of SPION-positive cells is challenging, and significant hardware and software improvements are still needed to facilitate a fast and reproducible protocol for single tumor cell detection throughout the brain (32, 36–38). Nonetheless, for practical purposes, most metastasis studies do not require absolute numbers, as long as the relative numbers are proportional to the injected quantities and predictive of the ensuing tumor development (Figs. 5B, 6E, and 7).

In summary, we have presented a novel and reproducible method for fully automated quantification of cancer cells in the brain within a robust animal model of metastatic melanoma. This model represents a powerful tool for both spatial and temporal monitoring of cancer cell dissemination and tumor progression, and it can improve the predictive value of biologic and therapeutic data gained from current experimental brain metastasis models, eventually reducing the number of clinical trials that fail to show patient benefit.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T. Sundstrøm, I. Daphu, M. Lund-Johansen, R. Bjerkvig, F. Thorsen, M. Babic

Figure 7. Quantitative tracking of nanoparticle-labeled melanoma cells improves the predictive power of an experimental brain metastasis model. Mice in groups A and B were injected intracardially at time 0 (left). T2*w MRI is analyzed at 24 hours post injection to determine the brain cell load (middle). Only animals that have comparable tumor cell exposure are routed to further follow up, for example, testing a standard treatment versus a novel treatment (right).
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.)
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis)

Writing, review, and/or revision of the manuscript

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases)
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Acknowledgments
The authors thank Ole Johnny Steffensen and Torild Halsebakke Bjerke, Department of Pathology, Ålesund Hospital (Ålesund, Norway), for assistance with histologic and immunohistochemical procedures; Kyaw Moe, Department of Biomedicine, University of Bergen (Bergen, Norway) for help with developing the 3D brain model; Anja Torevik, Department of Biomedicine, University of Bergen for support with cell authentication; Jian Wang, Department of Biomedicine, University of Bergen, for assistance with cell authentication; Christian Tønnessen, Department of Biomedicine, University of Bergen for support with cell authentication; Jian Wang, Department of Biomedicine, University of Bergen Norway and Brett Fite, Department of Biomedical Engineering, University of California Davis (Davis, CA) for manuscript discussions and comments.

Grant Support
This study was funded by the Western Norway Regional Health Authority (grants 911645 and 911558), the University of Bergen, the Norwegian Cancer Society, the Norwegian Research Council, the Grant Agency of the Czech Republic (grant P304/12/1370), and the Centre de Recherche Public de la Santé Luxembourg.

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Received September 5, 2012; revised December 17, 2012; accepted January 22, 2013; published OnlineFirst February 19, 2013.

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www.aacajournals.org Cancer Res; 73(8) April 15, 2013
2455

Published OnlineFirst February 19, 2013; DOI: 10.1158/0008-5472.CAN-12-3514

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Automated Tracking of Nanoparticle-labeled Melanoma Cells Improves the Predictive Power of a Brain Metastasis Model

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