Supplemental Materials and Methods

Materials

Dulbecco's Modification of Eagles Medium (DMEM), sterile Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} or Mg^{2+}, penicillin/streptomycin, L-glutamine, and trypsin were purchased from Cellgro; Mediatech Inc. (Manassas, VA). HyClone HyQTase was purchased from Thermo Scientific. Fetal bovine serum (FBS) and G418 selection antibiotic were purchased from Omega Scientific Inc. (Tarzana, CA). Secondary antibodies used throughout these studies were: Alexa Fluor 647-conjugated goat anti-rat IgG (H+L), Cat#: A21247; Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L), Cat#: A11035; Alexa Fluor 594-conjugated goat anti-mouse IgG (H+L), Cat#: A11032; Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L), Cat#: A11029; and Alexa Fluor 546-conjugated goat anti-human IgG (H+L). Cat#: A21089, all were purchased from Molecular Probes; Invitrogen Inc. (Eugene, OR). 4',6-diamidino-2-phenylindole (DAPI), Cat#: D21490; and ProLong Gold Antifade Reagent, Cat#: P36930, were purchased from Molecular Probes; Invitrogen Inc. (Eugene, OR). Molday ION Rhodamine B ultrasmall particles of iron oxide (USPIOs), Cat#: CL-50Q02-6A-50, were purchased from BioPal (Worcester, MA). Formvar/Carbon coated nickel 2x1mm slot grids for electron microscopy, Cat#: FCF2010-Ni, were purchased from Electron Microscopy Sciences (Hatfield, PA). DPX Mountant for Histology, Cat#: 44581, was purchased from Sigma (St. Louis, MO). Tris-buffered saline (TBS), phosphate buffered saline (PBS), Nissl stain, citrate solutions and their surfactant derivatives prepared in-house based on standard protocols.

Cell-culture Conditions

Gl26-Cit, CNS-1-Cit, U251, and HF2303 primary human GBM cancer stem-cell neurospheres were cultured *in-vitro* under humidified conditions in 95% air/5% CO_2 at 37°C. Culture medium for adherent glioma cell-lines (i.e. GL26-Cit, CNS-1-Cit, and U251) consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.3mg/ml L-glutamine, 50U/ml penicillin, 50µg/ml streptomycin, 6µg/ml G418 selection antibiotic (for selection of the mCitrine expression vector) and were passaged every 2-4 days. Culture medium for HF2303 primary human GBM cancer stem-cells neurospheres consisted of DMEM/F12 supplemented with N2 supplement (1x final concentration), 250mg/ml of 0.22µm filter sterilized bovine serum albumin (BSA), 10mg/ml gentimicin, and antibiotic-antimycotic reagent (0.5x final concentration). Fresh rhEGF and rhbFGF cytokines were added to neurosphere medium at each passage at a final concentration of 20ng/ml. Fresh cytokines were added every 3 days and neurospheres were deaggregated with HyClone HyQTase cell dissociation solution and split 1:3 every 5-6 days.

Engineering the GL26-Cit and CNS-1-Cit Glioma Cell-lines

The plasmid containing the mCitrine transgene (pRSET-B-Citrine) was subcloned into the pCI-neo expression vector backbone to afford a 6,199 base-pair plasmid to constitutively express mCitrine fluorescent protein (pCI-neo-mCitrine). To do so, the mCitrine transgene was released from pRSET-B-Citrine with BamH I and EcoR I restriction endonucleases followed by modification with T4 DNA polymerase to afford the blunt-ended mCitrine cDNA insert. The resulting excision product was then blunt ligated into the SmaI cloning site of the pCI-neo plasmid to yield pCI-neo-mCitrine, which was then transfected into the wild-type GL26 and

CNS-1 cell-lines. Resultant cells were sorted for high mCitrine expression by FACS (85th percentile) and subsequently cultured under G418 section antibiotic to maintain transgene expression.

Stereotactic Tumor Implantation and Transcardial Perfusion

Six to seven week-old female mice or 200-240g male Lewis rats were anesthetized with a single i.p. injection of 75mg/kg ketamine and 0.5mg/kg medetomidine in sterile 0.9% NaCl for injection. Once deeply anesthetized, animals were placed into a stereotactic frame and a single midline incision was made from the frontal bone, caudally, to expose the cranium. A single hole was drilled into the cranium above the left cerebral hemisphere using a precision power drill equipped with a fine bit at the following coordinates from bregma: +0.5mm AP, +2.5mm ML, and -3.0mm DV for mice (or +1.0mm AP, +3.2mm ML, and -5.0mm DV for rats). Upon exposing the underlying dura, an injection of $3x10^4$ cells in 1μL of serum-free DMEM (or 2μL for CNS-1-Cit cells) was made using a 5μl Hamilton syringe equipped with a 33-gauge needle. Cells were allowed to settle at the injection site for 5min followed by slow needle withdrawal. Skin was then sutured and animals were given doses of atipamezole (1mg/kg i.p.) and buprenorphine (0.1mg/kg s.c.) for anesthesia reversal and post-operative pain relief, respectively. Animals were then allowed to recover in their cages under a heating lamp with wet chow and access to water.

Animals underwent transcardial perfusion at predetermined time-points post-tumor implantation or once reaching a moribund state. Prior to perfusion, animals were administered 50mg/kg i.p. injections of both ketamine and xylazine to induce deep anesthesia. Once all reflexes had been abolished, animals were transcardially perfused with oxygenated and

heparinized (100U/L) Tyrode's solution using a peristaltic pump followed by 4% paraformaldehyde (PFA) pH 7.4 in PBS. Brains were harvested and stored in 4% PFA at 4°C overnight, in the dark, prior to sectioning 50μm thick coronal tissue sections with a vibratome for immunolabeling and/or microscopic analysis.

Imaging Modalities

Epifluorescence and brightfield micrographs were taken with a Zeiss Axioplan-2 microscope equipped with a digital camera (Carl Zeiss MicroImaging, Inc.) and Axiovision Release 4.6 analysis software. Fluorescence scanning confocal micrographs were taken with a Leica DMIRE2 confocal microscope equipped with Leica Confocal Software version 2.61 (Leica Microsystems). Fluorescence channels were scanned sequentially to reduce inter-channel bleed. 488nm argon, and 542- and 590nm helium neon laser lines were used to excite Alexa dyes 488, 546, and 594, respectively. Glioma-bearing mouse brain tissue prepared for ultrastructural analysis was imaged with a transmission electron microscope at the Anatomical Pathology Laboratory at Cedars-Sinai Medical Center. Intravital imaging of malignant glioma through a cranial window at depths of up to 250μm within the somatosensory cortex of living mice was performed using a Prairie Optima multiphoton microscope with a Chameleon Ti- sapphire laser (Coherent) tuned sequentially to 1,030nm (peak excitation for mCitrine fluorescent protein) and 830nm (peak excitation for rhodamine B isothiocyanate conjugated-dextrans (mw=70kDa)). Images were captured every 15min for up to 12hrs while being attenuated with a Pockels cell to reduce the power to 50–70 mW at the brain surface.

Fractal Dimension Analysis

Tumor fractal dimension values (D-values) were calculated at predetermined time-points post-implantation using the box-counting algorithm of ImageJ (National Institutes of Health, Bethesda, MD). A series of calibration geometries with known D-values were used to validate the output given by the software. Among those geometries used were: the Koch snow flake [$D=1.260_{known}$ vs. 1.260_{calc}], the Sierpinski triangle [$D=1.585_{known}$ vs. 1.558_{calc}], the Sierpinski carpet [$D=1.893_{known}$ vs. 1.856_{calc}], the square [$D=2.000_{known}$ vs. 1.988_{calc}], and the line segment [$D=1.000_{known}$ vs. 1.019_{calc}]. Each calibration geometry was determined to be within 2% error of the respective reference value. Tumor D-values were derived from maximum projections of 10x fluorescence confocal z-stacks taken at the center of the tumor mass using an identical step-size and total z-thickness. The mCitrine channel was used alone to reveal tumor morphology, negligible background in this channel allowed for the definitive visualization of tumor morphology. Box sizes utilized for all analyses (including calibration geometries) were 1-16, 32, 64, 90, 91. The method used to determine tumor fractal dimension was as follows: tumor micrographs were opened in ImageJ software and converted to 8-bit. Thresholds were adjusted equivalently. Each micrograph was then converted from grayscale to binary. The edges of each tumor were then found and images were analyzed using the aforementioned box sizes.

Tissue Preparation for Transmission Electron Microscopy (TEM)

To facilitate the discrimination of tumor cells under the electron microscope, medium from 50-70% confluent T75 culture flasks containing GL26-Cit glioma cells was exchanged for 10mls of serum-supplemented DMEM containing 50µl of Molday ION Rhodamine B USPIO nanoparticles [2mg/ml], achieving a final USPIO concentration of 10µg/ml. Cells were then

cultured overnight under standard conditions. Cells were trypsinized and resuspended at $3x10^4$ cells/μL in serum-free DMEM. Stereotactic microinjections where then performed in the same manner as described in the Stereotactic Tumor Implantation and Transcardial Perfusion section. At each time point, animals were anesthetized and transcardially perfused with oxygenated and heparinized (100U/L) Tyrode's solution followed by a fixative combination of 1% Glutaraldehyde (GA) and 2% PFA in 0.1M PBS (i.e. EM fixative). Brains were harvested and placed into 10mls of EM fixative at 4°C overnight in the dark. A vibratome was then used to cut a single 2mm-thick coronal section that contained the tumor lesion. Using a scalpel and dissecting microscope, the contralateral hemisphere was dissected away followed by a single medio-lateral cut through the center of the tumor lesion, bisecting the ipsilateral hemisphere. Visualization of the lesion was made possible by the red color afford by the iron-containing nanoparticles (USPIOs) under the brightfield dissecting microscope and the green fluorescence given by mCitrine when visualized under the epifluorescence microscope. Once the precise location of the lesion was identified, further cuts were made in order to fashion two equivalent $2mm^3$ blocks of tissue, each containing approximately half of the tumor lesion. Prepared specimens where then stored in 300μl of EM fixative in 0.5ml microcentrifuge tubes at 4°C in the dark prior to TEM imaging. The technical staff at the electron microscopy core at Cedars-Sinai Medical Center performed the tissue embedding and further cutting steps prior to TEM imaging.

Cranial Window Installation and Multiphoton Laser Scanning Microscopy

The craniotomy and window installation were performed based on a previously described protocol (5). Modifications in the orientation of the stereotactic frame were made to allow for

tumor injections at oblique angles relative to the top of the cranium in order to achieve lateral tumor implants within 1mm of the cortical surface. GL26-Cit tumor cells ($3x10^4$ cells in 1μL serum-free DMEM) were injected into the somatosensory cortex of C57BL/6J mice while under injectable anesthesia. A 4mm glass coverslip was then installed over the exposed brain and glued with cyanoacrylate-based adhesive followed by the application of dental cement in the shape of a well to fill with 0.9%NaCl and immerse the imaging objective. Animals were allowed to recover under a heating lamp. To achieve direct visualization of tumor cell growth and migration in real-time *in-vivo* we employed the use of time-lapse multiphoton laser scanning microscopy. At the time of imaging, animal anesthesia was induced with a controlled inhalation of 4% isoflurane followed by continuous inhalation of 1.5-2% isoflurane to maintain anesthesia throughout the imaging procedure. 200μL of a 5% solution of rhodamine B isothiocyanate conjugated-dextran (mw=70kDa) diluted in 0.9%NaCl was injected via the tail-vein into mice to visualize brain microvasculature. Animals were then placed on a heating pad to maintain physiologic body temperature and the head was secured in a stereotactic frame. Eyes were lubricated with petrolatum to prevent drying while under long-term anesthesia. Multiphoton Z-stacks upwards of 250μm within the somatosensory cortex were taken of the $mCitrine^+$ tumor (peak excitation: 1030nm) and dextrans (peak excitation: 830nm) every 15min for up to 12hrs. Once imaging was complete, animals were removed from the stereotactic frame and allowed to recover in a clean cage under a heating lamp.

Immunohistochemistry

Brains were coronally sectioned every 50μm in a serial fashion using a Vibratome and stored in 12-well plates containing PBS-0.1% sodium azide at 4°C in the dark. Brain sections

chosen for immunolabeling were placed into fresh 12-well plates containing TBS-0.1% Triton-X (TBS-Tx) for 20min while shaking. When antigen retrieval was required, TBS-Tx was replaced with boiling 10mM sodium citrate solution and allowed to shake at room temperature for an additional 15min. Non-specific antibody binding was blocked by incubating sections in 10% normal goat serum in TBS-Tx for 1hr at room temperature. Sections were incubated in primary antibody diluted in TBS-Tx containing 1% NGS and 0.1% sodium azide overnight at room temperature while shaking. Sections were washed in TBS-Tx and incubated in secondary antibody solution containing 1% NGS in TBS-Tx overnight at room temperature, in the dark. Sections were then washed with TBS-Tx followed by incubation with a [1:1,000] dilution of DAPI [5mg/ml] in PBS for 6min. Sections were washed twice in PBS, mounted on microscope slides, and coversliped using an anti-fade reagent.

Supplemental Text on the Agent-based Computational Model

We observe that the number of glioma cells in the simulation increases exponentially with time (Figure S3). Indeed, an exponential fitting of the number of tumor cells at time t denoted $N(t)$ shows that:

$$N(t) \approx 867 e^{0.0312 * t} \qquad eq.\ \ 4$$

providing an exact fit with exponential growth. We note that the growth coefficient 0.0312 is slightly less than the optimized growth rate parameter $\mu_b = 1/30\ (\approx 0.0333)$. This reflects the fact that glioma cells not in contact with a blood vessel do not divide and eventually die, thus negatively contributing to the total number of glioma cells at any time t (i.e. $N(t)$).

We have also written our model using a density distribution of tumor cells rather than defining each glioma cell separately in order to analyze the model at a larger scale. In this version we denote by the density distribution of glioma cells at the position $(\vec{x})$ with direction $(\vec{\omega})$ and at time (t) by $f(t, \vec{x}, \vec{\omega})$. The evolution of glioma cells results in the following partial differential equation for f :

$$\partial_t f + \nabla_{\vec{x}} \bullet (C(\vec{x}) f) + \nabla_{\vec{\omega}} \bullet (v P_{\vec{w}} T(\nabla g(\vec{x})) f) = \sigma \Delta_{\vec{w}} f + \mu(\vec{x}) f \qquad eq.\ \ 5$$

where $C(\vec{x})$ and $P\vec{\omega}T$ are given by eq. 2 and 3 respectively. The diffusion term $\sigma \Delta_{\vec{w}} f$ is the expression of the random perturbations exerted on glioma cells and the growth rate $\mu(\vec{x})$ is given by:

$$\mu(\vec{x}) = \begin{cases} -\mu_d & if\ g(\vec{x}) = 0 \quad (\vec{x}\ not\ on\ a\ BV) \\ \mu_b & if\ g(\vec{x}) > 0 \quad (\vec{x}\ on\ a\ BV) \end{cases} \qquad eq.\ \ 6$$

The speed and the growth rates are coupled with the environment, namely by the presence of blood vessels described by the density $g(\vec{x})$. As glioma cells migrate over time, their spatial distribution influences the number of total glioma cells at any time t during the simulation. To

measure this effect, we estimate the density distribution of tumor cells $\rho(\vec{x})$ every 60hrs using a Particle-in-Cell (PIC) method:

$$\rho(\vec{x}) = \left\{ density\ of\ \ glioma\ cells\ at\ \vec{x} \right\}$$

We plot the domain where the density ρ is greater than a given threshold (i.e. $h \geq 0.5\ cells/\mu m^2$) at different times (Figure S4*A* and S4*B*). More precisely, we represent this by the set:

$$C(t) = \left\{ \rho(t,\ \vec{x}) \geq 0.5 \right\} \qquad eq.\ \ 7$$

at time t =0, 60, 120, 180, and 240hrs. We observe a fast spreading during the first 60hrs followed by slow spreading thereafter.

A correlation between the density of blood vessels (g) and the density of tumor cells (ρ) emerges due to the increasing intensity of attraction of glioma cells as they come in proximity to nearby blood vessels. We quantify this correlation by estimating the proportion (p) of tumor cells on blood vessels:

$$(p) = \frac{\#\left\{ glioma\ cells\ on\ a\ BV \right\}}{\#\left\{ total\ glioma\ cells \right\}} \qquad eq.\ \ 8$$

and observe that the initial proportion $(p) = 0.20$ (or 20%) quickly converges to a constant value of $(p) = 0.96$ (or 96%) after the first 60hrs (Figure S4*C* and S4*D*). Consequently, the spread of glioma cells is significantly slowed due to their reduced migration speed on blood vessels and their tendency to remain in contact with blood vessels once in contact.