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Calpain 2 Is Required for the Invasion of Glioblastoma Cells in the Zebrafish Brain Microenvironment

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Abstract

Glioblastoma is an aggressive primary brain tumor with a 5-year survival rate of less than 5%. The ability of glioblastoma cells to invade surrounding brain tissue presents the primary challenge for the success of focal therapeutic approaches. We previously reported that the calcium-activated protease calpain 2 is critical for glioblastoma cell invasion in vitro. Here, we show that expression of calpain 2 is required for the dispersal of glioblastoma cells in a living brain microenvironment. Knockdown of calpain 2 resulted in a 2.9-fold decrease in the invasion of human glioblastoma cells in zebrafish brain. Control cells diffusely migrated up to 450 μ m from the site of injection, whereas knockdown cells remained confined in clusters. The invasion study was repeated in organotypic mouse brain tissues, and calpain 2 knockdown cells demonstrated a 2.3-fold lower area of dispersal compared with control cells. In zebrafish brain, glioblastoma cells appeared to migrate in part along the blood vessels of the host. Furthermore, angiogenesis was detected in 27% of zebrafish injected with control cells, whereas only 12.5% of fish receiving knockdown cells showed the formation of new vessels, suggesting a role for calpain 2 in tumor cell angiogenesis. Consistent with the progression of glioblastoma in humans, transplanted tumor cells were not observed to metastasize outside the brain of zebrafish. This study demonstrates that calpain 2 expression is required for the dispersal of glioblastoma cells within the dynamic microenvironment of the brain, identifying zebrafish as a valuable orthotopic system for studying glioblastoma cell invasion.

Keywords

glioma; invasion; zebrafish; brain; calpain

Glioblastoma accounts for 53.7% of all primary glioma tumors, with only 4.75% of patients surviving 5 years after diagnosis (CBTRUS, 2011). However, a recent study with 573 patients reported that a concomitant treatment of temozolomide with radiotherapy after surgical resection of the primary tumor improved the 5-year survival rate to 9.8% whereas only 1.9% of patients receiving radiotherapy alone survived for 5 years postdiagnosis (Stupp et al., 2009). It is not clear which intracellular activities facilitate the rapid invasion of glioblastoma cells or the components of the brain used for traction by migrating cells. Incomplete knowledge of the molecular mechanisms regulating tumor cell invasion in the

brain has limited the potential for targeting invasion as a therapeutic intervention for malignant brain cancers.

Calcium influxes induced by glutamate activation of AMPA receptors have been identified as a unique characteristic increasing the invasiveness of glioblastoma cells (Lyons et al., 2007; Sontheimer, 2008); however, the roles of intracellular calcium targets are just beginning to be defined. We hypothesized that calpain 2 is a key downstream effector of calcium ions, facilitating glioblastoma cell invasion (Jang et al., 2010). Calpain is a family of calcium-activated cysteine proteases, and the ubiquitously expressed isoform calpain 2 has been implicated in various aspects of cell migration and invasion in many cancers (Goll et al., 2003; Mamoune et al., 2003; Lakshmikuttyamma et al., 2004; Franco and Huttenlocher, 2005; Cortesio et al., 2008). In addition, the knockdown of calpain 2 was shown to inhibit the VEGF-induced angiogenesis of pulmonary microvascular endothelial cells in vivo (Su et al., 2006). In our previous study, knockdown of calpain 2 expression decreased the invasion of human glioblastoma cells through an artificial matrix of Matrigel using in vitro transwell assays (Jang et al., 2010).

Although this initial study identified a potential role for calpain 2 expression in glioblastoma cell invasion, the in vitro assays were incapable of simulating the complex interplay between tumor cells and the local microenvironment of the brain. Furthermore, Matrigel was used at a concentration of 2 mg/ml, which corresponds to an average matrix pore size of ~6 μ m (Zaman et al., 2006). The brain, in contrast, is packed with glial and neuronal processes, vascular networks, and matrix components, leaving extracellular spaces in the submicrometer range (Thorne and Nicholson, 2006). Regulation of invasion-specific processes could be more completely understood by monitoring tumor cell invasion in a living brain; however, the lack of suitable animal models has been a limitation.

Zebrafish are a cost-effective model containing the full repertoire of vertebrate genes (Lieschke and Currie, 2007) and demonstrate a molecular basis of development similar to that of humans (Granato and Nusslein-Volhard, 1996). Recent studies have shown that transplanted human melanoma cells proliferate, migrate, and induce angiogenesis in zebrafish (Lee et al., 2005; Haldi et al., 2006). An advantage of zebrafish is the optical transparency of the animal, which allows time-course visualization of invading cancer cells (Feitsma and Cuppen, 2008). In addition, transgenic zebrafish, *Tg(fli1:egfp)*, expressing green fluorescent protein (GFP) in the endothelial cells makes it possible to image the interaction of tumor cells with the vasculature containing active blood circulation (Langheinrich, 2003; Kari et al., 2007; Stoletov et al., 2007; Lee et al., 2009). Finally, rodent xeno-transplantation models of cancer require injection of millions of cells, whereas, in zebrafish, fewer than 100 cells can be transplanted and imaged using high-resolution microscopy, simulating earlier stages of tumor development.

In this study, real-time monitoring of cell invasion in vitro showed that the requirement of calpain 2 for glioblastoma cell invasion was a function of the density of extracellular matrix. By transplanting human glioblastoma cells in the zebrafish brain, we demonstrate that calpain 2 expression is required for the dispersal of tumor cells in the brain microenvironment. Transplanted glioblastoma cells were observed to migrate along the abluminal surface of blood vessels, but did not metastasize outside the brain of zebrafish, which is consistent to reports from human patients and rodent models (Bernstein and Woodard, 1995; Farin et al., 2006; Montana and Sontheimer, 2011). These results support the hypothesis that calpain 2 expression is required for glioblastoma cell invasion in the living brain microenvironment and demonstrate the potential of zebrafish as an orthotopic model for brain tumor cell invasion studies.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium (DMEM) and trypsin/EDTA were purchased from Mediatech (Manassas, VA). L-glutamine, Geneticin, Dulbecco's phosphate-buffered saline (D-PBS), and carboxymethylbenzamido derivative of dialkyl-carbocyanine (CMDiI) cell tracker dye were purchased from Invitrogen (Eugene, OR). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). Matrigel was from BD Biosciences (Bedford, MA), and transwell permeable support (6.5-mm diameter, 8.0- μ m pore size) was purchased from Corning (Corning, NY). FuGENE HD and CIM-16 plate for xCELLigence were ordered from Roche Applied Sciences (Indianapolis, IN).

Cell Culture and Labeling

Human U87MG glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 1% L-glutamine and 10% fetal bovine serum (FBS) at 37°C and the standard conditions of 100% humidity, 95% air, and 5% CO₂. The preparation of stable cell lines of control and calpain 2 knockdown glioblastoma cells has been described elsewhere (Jang et al., 2010). Briefly, glioblastoma cells were transfected with scrambled or calpain 2 sequence-specific shRNA (SA Biosciences, Frederick, MD) using FuGENE HD, and cells with stable incorporation of shRNA were selected using 400 μ g/ml Geneticin according to the manufacturer's protocol. For transplantation in the zebrafish brain and mouse brain slices, glioblastoma cells were labeled with CMDiI diluted at 2 μ M in D-PBS according to manufacturer's protocol and suspended at a final concentration of 5×10^6 cells/ml.

Real-Time Invasion Assay

Real-time monitoring of glioblastoma cell invasion was performed using the xCELLigence system from Roche Applied Sciences (Indianapolis, IN). This system uses a specialized transwell apparatus, Cell Invasion and Migration (CIM) plate 16, and the Real Time Cell Analyzer-Dual Plate (RTCA-DP) instrument for performing electrical measurements. The lower side of the transwell membrane is integrated with gold microelectrodes, which maintain electrical impedance when connected to electricity. The impedance changes when a cell invades through and reaches the lower side of the membrane. The relative change in the impedance between measurement at any time (t) and the background value (t₀) is expressed as cell index (CI), providing real-time quantitative information about the number of invading cells. The invasion assays were performed as per the manufacturer's instructions. Briefly, Matrigel diluted in cold serum-free media at 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml concentrations was coated onto the upper chamber of CIM plates. The lower and upper chamber plates were assembled, and, immediately before adding cells, the background impedance of the media was recorded. Fifty thousand cells suspended in serum-free media were added to the Matrigel, and invasion was stimulated by using 10% FBS in the lower chamber as chemoattractant. Cells were allowed to settle for 30 min, and the CIM plate assembly was placed in the RTCA-DP analyzer to record the electrical impedance of the membrane every 15 min for 23 hr. The invasion data was analyzed in the RTCA software.

Zebrafish Handling and Cancer Cell Transplantation

Zebrafish (*Danio rerio*) were housed and reared at Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. Adult tropical 5D strain and Tg(fli1:egfp) strain of zebrafish were kept under standard laboratory conditions of 28°C on a 14 hr light/10 hr dark photoperiod in fish water consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean). All animal experimental

procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Zebrafish 5D were transplanted with glioblastoma cells at 4 days postfertilization (dpf) for the experiments represented in Figures 1 and 5, whereas the transgenic fish *Tg(fli1:egfp)* were transplanted with glioblastoma cells at 10 dpf for the experiments represented in Figures 2–4. The needles were prepared from a borosilicate glass pipette with internal diameter 0.5 mm and external diameter 1.4 mm, using a glass micropipette puller (Sutter Instrument, Novato, CA). The CMDiI-labeled glioblastoma cell suspension was mixed with phenol red (1:10 v/v) before loading the needle, and 50–100 cells were injected into zebrafish, which had been anesthetized with 0.004% Tricaine in fish water, at 30 psi using an ASI MPP1-2 air-driven pressure injector. Fish that survived injection and were active after 12 hr were transferred to individual wells of a six-well plate containing 10 ml fish water and maintained at 29°C. The immune system of zebrafish is not functional until 28 dpf, which removes the complexity of immune responses for the xenotransplantation of human cells (Taylor and Zon, 2009).

Organotypic Brain Slice Invasion Assay

Brain tissue slices were obtained from 9-week-old B6SJL female mice. Animals were anesthetized with isoflurane, and the brain was immediately obtained by decapitation and maintained in 4% low-melting-point agarose solution in PBS (pH 7.4). The 400- μ m-thick horizontal slices were prepared using a vibratome (Leica VT 1000S) and transferred onto a transwell cell culture insert (0.4- μ m pore size) placed in a six-well plate containing 1,500 μ l media in the lower well and 500 μ l media (10% FBS-DMEM supplemented with 600 μ g/ml geneticin and 2 $\times$ penicillin/streptomycin) in the upper chamber of the insert. The brain slice culture was incubated at 37°C under standard culture conditions of 100% humidity, 95% air, and 5% CO₂. Control and calpain 2 knock-down glioblastoma cells were stained with CMDiI dye and suspended to the final concentration of 10⁷ cells/ml. With a Hamilton syringe fixed on a micromanipulator, 10⁴ cells were gently injected onto the tissue in 1 μ l transfer volume over 1–2 min. Images were captured with a Zeiss LSM510 confocal microscope over 7 days to monitor dispersal of cells. Area of the tumor cell dispersal was measured using Zeiss LSM510 image analysis software.

Microscopy and Data Analysis

For images shown in Figures 1 and 5, zebrafish were anesthetized with 0.004% tricaine in fish water and imaged with a Zeiss Axiovert 100 fluorescence microscope with a $\times$ 10 objective lens. CMDiI dye is diluted with each cell division, so the exposure time was adjusted to capture all the labeled cells on different days of imaging. The area occupied by tumor cells in each fish was measured at 1 and 6 days postinjection (dpi) using the distance calibration tool of MetaMorph 6.2 image analysis software (Molecular Devices, Sunnyvale, CA). The difference in the area occupied by tumor cells between 1 dpi and 6 dpi in an individual fish was defined as the area of dispersal. Only the animals that had received localized cell injection were chosen for imaging to allow monitoring of cell dispersion over time. The images presented in Figures 2–4 and 6 were captured with a LSM510 confocal microscope with $\times$ 10 objective lens. The association of tumor cells with blood vessels was quantified using the colocalization measurement tool of the Zeiss LSM image analysis software. The colocalization coefficient was measured from approximately 15 consecutive slices of the z-stack (100 μ m) from different fish, which represented the ratio of colocalizing pixels of CMDiI fluorescence to the total number of pixels above threshold in that channel. The measurements from individual slices were averaged to determine the overall percentage of tumor cells colocalized with blood vessels for control and calpain 2 knockdown cells at 1 and 6 dpi. Identical threshold intensity was applied for all the measurements.

Statistical Analysis

Statistical analysis of the data was performed via Student's *t*-test. The nonparametric Wilcoxon rank sum test was performed to compare the tumor dispersal and colocalization of tumor cells with blood vessels between control and knockdown groups.

RESULTS

Calpain 2 Is Required for Glioblastoma Cell Invasion in the Zebrafish Brain

Previously, we reported a 90% decrease in the invasion of human glioblastoma cells through Matrigel when calpain 2 expression was significantly knocked down (Jang et al., 2010). Interestingly, no difference between control and calpain 2 knockdown glioblastoma cells was observed when two-dimensional migration over matrix substrates was compared. To understand better the role of calpain 2 in glioblastoma invasion, we examined the ability of control and knockdown cells to navigate three-dimensional environments of varying density, including the zebrafish brain. Invasion through Matrigel of increasing density was quantified in real time by using microelectronic impedance measurement technology (xCELLigence). Control and calpain 2 knockdown cells were added on top of 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml concentrations of Matrigel in the upper chamber of transwells, and 10% FBS was added to the lower chamber to stimulate invasion. The impedance of the microelectrodes integrated on the lower side of the membrane was recorded every 15 min for 23 hr (Fig. 1A). The impedance reading changes when cells traverse the Matrigel matrix and reach the lower side of the membrane and is represented as the cell index. A lag phase showing no change in CI was observed for 9 hr, 6 hr, and 4 hr in case of 0.8, 0.4, and 0.2 mg/ml concentration of Matrigel, respectively. Thereafter, a gradual increase in CI was recorded for both control and knockdown cells; however, the increase was higher in control compared with knockdown cells (Fig. 1A). The rate of invasion was quantified by calculating the slope of the CI curves, and calpain 2 knockdown cells were observed to have a 59%, 22%, and 9% lower rate of invasion compared with control cells in the presence of 0.8, 0.4, and 0.2 mg/ml Matrigel, respectively, suggesting that calpain 2 is important for tumor cell movement through dense extracellular matrix.

The various components of the brain provide a densely packed microenvironment, with extracellular spaces in the submicrometer range (Thorne and Nicholson, 2006). In comparison, the Matrigel concentration of 0.8–0.2 mg/ml used for the transwell assays forms pore sizes ranging between 6 and 60 μ m (Zaman et al., 2006). To examine the role of calpain 2 in glioblastoma cell invasion in the brain, control or calpain 2 knockdown cells were transplanted into the brain of 4-day-old zebrafish, the age at which brain ventricles and the overall wiring of the head vascular system are fully formed (Isogai et al., 2001). Live fish were imaged for 6 dpi to examine the distribution of injected cells in the brain. Careful attention was paid to the alignment of fish during imaging on successive days to correlate precisely the dispersal pattern of cells in the brain tissues. At 1 dpi, cells appeared as a compact cluster in the midbrain of zebrafish (Fig. 1B). By 6 dpi, control cells had dispersed in the brain, with individual cells migrating up to 450 μ m from the site of injection. In contrast, calpain 2 knockdown cells remained confined in clusters, with little dispersal, mostly in close proximity to the site of injection. Long-distance migration was rarely observed for knockdown cells (maximum of 250 μ m). The area of the region occupied by the tumor cells at 1 and 6 dpi was quantified, and overall we observed a $158\% \pm 12.7\%$ ($P = 0.007$) increase for the control tumor cells after 6 days compared with a $54.8\% \pm 10.9\%$ ($P = 0.006$) increase for the knockdown cells (Fig. 1C). Therefore, knockdown of calpain 2 expression resulted in a 2.9-fold decrease in the dispersal of glioblastoma cells after 6 dpi in the zebrafish brain. The difference in the tumor cell dispersal between control and knockdown cells was statistically significant ($P < 0.001$, Wilcoxon rank sum test; Fig. 1D).

To verify that the CMDiI staining was stable during the time course, a fraction of cells prepared for transplantation was maintained in culture and imaged under conditions similar to those used for the zebrafish. The morphology of the cells appeared normal and healthy, with the CMDiI stain distributed throughout the cellular membrane systems, including plasma membrane projections (Fig. 1E). These results demonstrate that calpain 2 expression is important for the invasion of glioblastoma cells within the dense microenvironment of the brain.

Human Glioblastoma Cells Disperse Along Blood Vessels in Zebrafish Brain

Many researchers have reported that glioma cells follow certain preferred paths for migration such as along the blood vessels or axon fiber tracts in the brain (Scherer, 1940; Nagano et al., 1993; Bernstein and Woodard, 1995; Guillamo et al., 2001; Lamszus et al., 2003; Farin et al., 2006). However, success in imaging of individual glioblastoma cells in a living brain has been limited. To determine the route of dispersal, glioblastoma cells were transplanted in the midbrain of the *Tg(fli1:egfp)* fish and invading cells were examined for 6 dpi. At 1 dpi, cells were localized in clusters, with little interaction with blood vessels observed. By 6 dpi, the cells were observed closely aligned along the abluminal surface of blood vessels (Fig. 2). The distribution of tumor cells along blood vessels was further examined by “ortho” analysis on identical regions of a blood vessel from the same fish imaged at 1 and 6 dpi (Fig. 2). The spatial distribution of cells in the entire z-stack along the primordial midbrain channel is shown in the X-Z plane, whereas the distribution of cells along the anterior cerebral vein is represented in the Y-Z plane. The pattern of cell dispersion suggests that, during migration, cells associate closely with the outer surface of the blood vessels as shown by the yellow regions spread along the circumference of vessels at 6 dpi.

The association of control and calpain 2 knockdown glioblastoma cells with blood vessels was also compared by transplanting in the midbrain of *Tg(fli1:egfp)* zebrafish brain. By 1 dpi, both control and knockdown cells were observed in clusters surrounded by a network of smaller central arteries and equidistant from the larger vessels such as anterior cerebral vein and primordial midbrain channel. By 6 dpi, control cells exhibited a diffuse pattern of infiltration in the brain, and, in 90% of zebrafish, association with the anterior cerebral vein, primordial midbrain channel, and connected vessels was observed. In contrast, calpain 2 knockdown cells remained confined in clusters at 6 dpi in 75% of the zebrafish (Fig. 3A). Localization of the tumor cells to the blood vessels was quantified using the Zeiss LSM colocalization image analysis software as described in Materials and Methods. At 6 dpi, $28.7\% \pm 13.1\%$ of the CMDiI fluorescence signal for the control tumor cells directly overlapped with the EGFP fluorescence signal from the blood vessels compared with $9.5\% \pm 7.4\%$ at 1 dpi ($P = 0.003$). In contrast, colocalization of the calpain 2 knockdown cells with blood vessels increased only 6.4%, from $11\% \pm 3.6\%$ to $18\% \pm 5.7\%$, over the period of 6 days ($P = 0.007$; Fig. 3B). The difference in the colocalization was compared for the set of fish injected with control and knockdown cells. Calpain 2 knockdown cells demonstrated threefold less colocalization with blood vessels compared with control cells ($19\% \pm 8.8\%$ vs. $6.4\% \pm 2.8\%$; Wilcoxon rank sum test, $P = 0.022$). These results support the conclusion that calpain 2 expression is required for glioblastoma cell invasion in the living brain and identify blood vessels as an important path of distribution. Because axonal nerve tracts of white matter have also been observed as a path for the glioma tumor cell migration (Pedersen et al., 1993; Giese and Westphal, 1996; Guillamo et al., 2001), further studies are underway investigating the dispersal of transplanted tumor cells along the axonal fibers in the zebrafish brain. Consistent with previous reports that human glioma cells do not metastasize, we did not observe transplanted cells either in the lumen of blood vessels or outside the brain of zebrafish.

Knockdown of Calpain 2 Attenuates Tumor Cell Angiogenesis in the Zebrafish Brain

Tumor angiogenesis is an important process for the progression of glioblastoma (Holash et al., 1999; Nakada et al., 2003; Fischer et al., 2005; Gagner et al., 2005). Although the zebrafish xenograft system was developed with the intent to study the mechanisms involved in the invasion and dispersal of glioblastoma cells in the microenvironment of the brain, images were also examined for evidence of tumor angiogenesis. Organization of blood vessels in z-stacks and the 3-D reconstructed images were compared for each zebrafish on 1 and 6 dpi. Twenty-seven percent of the fish injected with control glioblastoma cells demonstrated clear evidence of tumor angiogenesis, with newly formed vessels densely surrounded by tumor cells. An additional 18% of the fish injected with control cells displayed vessel reorganization, characterized by spatial realignment of preexisting vessels. In contrast, evidence of tumor angiogenesis was observed in only 12.5% of the fish injected with calpain 2 knockdown cells with a less extensive network of vessels (Fig. 4). In addition, spatial reorganization of existing vessels was observed in only 12.5% of the animals injected with knockdown cells. Consistent with previous studies (Holash et al., 1999; Lamszus et al., 2003), we also observed many tumor cells that traversed long distances in the brain along blood vessels, known as “vessel co-option,” without showing any remodeling of the associated vessels or formation of new vessels. Tumor angiogenesis was observed around cells that remained in clusters or migrated short distances in the local environment. Together, the data suggest that calpain 2 expression in glioblastoma cells may play a role in the induction of tumor angiogenesis. Further studies are necessary to test the role of calpain 2 in the tumor angiogenesis induced by glioblastoma cells.

Glioblastoma Cells Do Not Invade the Yolk Sac of Zebrafish

Recently, human melanoma cells microinjected in the yolk sac of zebrafish were shown to proliferate and invade into the surrounding organs, including intestine, pancreas, and liver (Haldi et al., 2006). To examine the ability of glioblastoma cells to disperse in tissues other than the brain, control and calpain 2 knockdown cells were microinjected into the yolk sac of 4 dpf zebrafish. We did not observe dispersal of control or knockdown cells in the yolk sac, with no trace of invasion detected in the surrounding visceral organs (Fig. 5A). The increase in the average area occupied by the transplanted cells after 5 dpi was minimal for both control and calpain 2 knockdown glioblastoma cells (Fig. 5B). The lack of glioblastoma cell dispersal might, in part, be explained by the lack of vascular networks in the yolk sac. The vascular system in the yolk contains only right and left supraintestinal veins (SIV) after 4 dpf, which degenerate by 7–8 dpf (Isogai et al., 2001). In addition, the yolk sac lacks the myelinated tracts of axonal surface, which is another potential route of the glioma tumor cell dispersion in the brain (Nagano et al., 1993; Pedersen et al., 1993; Giese and Westphal, 1996).

Calpain 2 Is Important for Glioblastoma Cell Invasion in Organotypic Mouse Brain Slices

Although zebrafish possess a vertebrate body plan with a cellular composition in the brain and blood vessels homologous to mammals (Isogai et al., 2001; Lowery and Sive, 2005), experiments were conducted to test the role of calpain 2 in glioblastoma invasion using a mammalian system. The ex vivo culture of mouse brain slices has been used to examine the invasiveness of glioma cells (Valster et al., 2005), and studies have shown that brain slices preserve tissue architecture and composition for several weeks in culture (Gahwiler et al., 1997). In this study, 10,000 CMDiI-labeled glioblastoma cells were injected onto 400- μ m-thick sections of brain tissue, and tumor cell invasion was examined for up to 7 dpi by confocal microscopy. Both control and calpain 2 knockdown cells invaded the surrounding brain tissue; however, by 7 dpi, a decreased area of dispersal was observed for the knockdown cells compared with control (Fig. 6A). The average area occupied by control cells increased from $3.1 \pm 1.6 \text{ mm}^2$ to $12 \pm 7.2 \text{ mm}^2$ ($P = 0.06$) over the period of 7 days. In

contrast, the average area occupied by calpain 2 knockdown cells increased from $2.5 \pm 1.2 \text{ mm}^2$ to only $6.2 \pm 3.0 \text{ mm}^2$ ($P = 0.06$) after 7 days. Hence, calpain 2 knockdown cells demonstrated a 2.3-fold decrease in the area of dispersal compared with control cells ($8.6 \pm 6.1 \text{ mm}^2$ vs. $3.7 \pm 2.4 \text{ mm}^2$; Fig. 6B). Although the results with the organotypic culture system demonstrated reduced invasion for the calpain 2 knockdown cells, the decrease was not of the same extent as observed in zebrafish. The difference might be explained by the absence of a live microenvironment in the organotypic culture containing a dynamic extracellular matrix and blood vessels with active circulation, highlighting one of the advantages of the zebrafish system.

DISCUSSION

Glioblastoma is the most common primary brain cancer, with a median survival of 12–18 months after diagnosis (Stupp et al., 2005; Gladson et al., 2010; CBTRUS, 2011). Malignant brain tumors have a poor prognosis, resulting in part from the ability of tumor cells to invade the tissue, limiting the effectiveness of primary tumor removal (Furnari et al., 2007; Adamson et al., 2009; Van Meir et al., 2010). In addition, secondary tumors formed by the invasive cells are often resistant to radiation and chemotherapy (Drappatz et al., 2009). Invasion requires tumor cells to navigate submicrometer pores within the microenvironment of the brain, which act as the substrate for cell membrane attachments necessary to generate traction as well as a barrier to the advancing cell body. Tumor cells accomplish this mechanical task by using matrix metalloproteinases (MMPs) to loosen the extracellular matrix structure of the confining tissue and an actin cytoskeleton-based machinery to adhere and crawl through the extracellular space within the brain (Rao, 2003; Sabeh et al., 2009). It is not clearly understood which intracellular activities facilitate the rapid invasion of brain tumor cells or the specific components of the brain used for traction by migrating cells. Incomplete knowledge of the molecular mechanisms involved in tumor cell invasion in the brain has limited the potential for targeting invasion as a therapeutic intervention for malignant brain cancers.

Calpain 2 has been implicated in different invasion-related processes such as the regulation of MMPs, formation of invadopodial projections, and angiogenesis in different types of cancer cells (Su et al., 2006; Cortesio et al., 2008; Jang et al., 2010). Specifically, in our previous report, we demonstrated that knockdown of calpain 2 in glioblastoma cells resulted in a 39% decrease in extracellular MMP2 (Jang et al., 2010). However, in addition to modulating the extracellular matrix, efficient invasion of tumor cells requires flexibility of the membrane and deformability of the cell in order to migrate through the narrow pores of the microenvironment (Wolf et al., 2003). Reorganization of the actin cytoskeleton at the cortical cell surfaces is the primary requirement for the pliability of cell membranes, and the remodeling of actin cytoskeletal structures depends on the structural and functional regulation of actin binding proteins, which are responsible for the polymerization, branching, bundling, and cross-linking of actin filaments (Ayscough, 1998; Wolf and Friedl, 2006). In addition, lamellar actin networks provide stability to the membrane and control the productive migration of the cell (Ponti et al., 2004). Cell migration within three-dimensional environments involves regulation of orthogonal networks of actin filaments at the cell periphery, and filamin is a key actin-associated protein that cross-links branched actin filaments into orthogonal networks. Previously, calpain proteolysis of filamin was implicated in increased breast tumor cell invasion (Xu et al., 2010), and we demonstrated that filamin is a major substrate for calpain 2 in glioblastoma cells (Jang et al., 2010). Hence, we propose that the proteolysis of filamin by calpain 2 is a mechanism important for glioblastoma cell invasion downstream of the calcium fluxes induced by autocrine glutamate activation of AMPA receptors. Future studies will be aimed at testing this hypothesis.

By using the zebrafish xenograft system, allowing the imaging of individual tumor cells moving through the dynamic microenvironment of a living brain, we observed that the transplanted human glioblastoma cells migrated primarily along the abluminal surface of blood vessels (Figs. 2, 3). Consistent with reports examining human brain tissues and mammalian models, we did not observe tumor cell intravasation into the lumen of blood vessels or metastasis to other tissues. In addition, knockdown of calpain 2 decreased the invasion of glioblastoma cells within the zebrafish brain and organotypic mouse brain slices (Figs. 1, 6). The findings from this study will be strengthened by future studies examining the invasion of different glioblastoma cell lines and primary tumor cells in the zebrafish in comparison with rodent systems. Also, transgenic lines of fish possessing fluorescent nerve fibers as well as blood vessels will provide an important tool with which to examine comparatively the routes and mechanisms of glioblastoma tumor cell invasion in the brain. Together the results presented here identify zebrafish as an important new approach for imaging individual tumor cells to determine specific mechanisms of invasion and calpain 2 as a potential therapeutic target to inhibit glioblastoma cell invasion. Furthermore, the zebrafish tumor cell dispersal assay provides a discovery platform to dissect chemically and genetically the pathways that promote or block invasion in the brain.

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Fig. 1.

Calpain 2 expression is required for invasion of human glioblastoma cells in the zebrafish brain. **A:** Real-time analysis of human glioblastoma cell invasion in vitro. Control and calpain 2 knockdown glioblastoma cells were added to the Matrigel matrix at 0.8 (top), 0.4 (middle), and 0.2 (bottom) mg/ml concentrations in the upper chamber of transwells and invasion stimulated with $\pm 10\%$ FBS in the lower chamber. The impedance of microelectrodes on the lower surface of membrane was measured every 15 min for 23 hr and represented as cell index. C, control; K, knockdown; S, 10% FBS. The curves are representative of two independent experiments. **B:** Control and calpain 2 knockdown (KD) glioblastoma cells labeled with CMDiI were microinjected in the brain of 4-day-old zebrafish. Monochrome images of cells in the brain of zebrafish captured 1 and 6 days postinjection (dpi). The **insets** show the initial position of the transplanted cells in the brain

at 1 dpi. **C:** The bar graph represents the average area occupied by control and KD cells in the brain quantified in Metamorph 6.2; $n = 25$; mean $\pm$ SEM. **D:** Box plot showing percentage increase in the area occupied by cells after 6 dpi between control and KD groups ($*P < 0.001$). **E:** Monochrome images of CMDiI stained cells maintained under standard cell culture conditions; dps, days poststaining.

Fig. 2.

Human glioblastoma cells disperse along blood vessels in the zebrafish brain. Ten days postfertilization, *Tg(fli1:egfp)* zebrafish, expressing GFP in endothelial cells, were microinjected with CMDiI-labeled human glioblastoma cells (red) and imaged by confocal microscopy. Invasion was assessed in three dimensions by capturing z-stacks at a thickness of 5.6 μm . Ortho analysis of z-stacks was performed to monitor the distribution of migrating cells along blood vessels (green). The image in the X-Z plane (below) shows relative distribution of tumor cells along the primordial midbrain channel, whereas that in the Y-Z plane (right) shows cells spreading along the anterior cerebral vein. The yellow regions identify close association of cells with the blood vessels. Images are representative of 30 injected fish. Blood vessel nomenclature: PMBC, primordial midbrain channel; ACeV, anterior cerebral vein; MtA, metencephalic artery; CtA, central artery.

Fig. 3. Calpain 2 knockdown (KD) glioblastoma cells remain confined in clusters and demonstrate decreased localization with blood vessels. **A:** Human glioblastoma cells transplanted in the brain of 10-day-old *Tg(fli1:egfp)* zebrafish were imaged for 6 dpi by confocal microscopy. The yellow regions represent close association of cells (red) with blood vessels (green). Blood vessel nomenclature: PMBC, primordial midbrain channel; ACeV, anterior cerebral vein; CtA, central artery. **B:** Colocalization of tumor cells with blood vessels was quantified using Zeiss Physiological Software v3.2; n = 6 (control) and 5 (knockdown); mean $\pm$ SEM.

Fig. 4.

Knockdown of calpain 2 attenuates tumor cell angiogenesis induced by injected glioblastoma cells. *Tg(fli1:egfp)* zebrafish, transplanted with control and calpain 2 knockdown glioblastoma cells, were imaged for 6 dpi by confocal microscopy. Three-dimensional reconstruction of tumor cells invading the brain of zebrafish at 1 and 6 dpi shows blood vessels in green and tumor cells in red. The newly formed blood vessels at 6 dpi are shown with arrows; n = 11 (control) and 8 (knockdown).

Fig. 5. Human glioblastoma cells do not invade in the yolk sac of zebrafish. **A:** CMDiI-labeled control and calpain 2 knockdown cells (KD) were microinjected into the yolk sac of 4-day-old zebrafish. Animals were imaged for 5 days using a Zeiss axiovert fluorescence microscope. Cells did not show significant dispersal and remained confined in clusters in the yolk sac. **B:** Area occupied by cells at 2 and 5 dpi was quantified using Metamorph 6.2; $n = 5$; mean $\pm$ SEM.

Fig. 6. Knockdown of calpain 2 results in decreased invasion of glioblastoma cells in organotypic mouse brain slices. **A:** Human glioblastoma cells (10,000), stained with CMDiI, were added to the brain tissue obtained from 62-day-old mice and imaged for 7 days by confocal microscopy. **B:** Area of dispersal was quantified using Zeiss Physiology Software v3.2; $n = 3$; mean $\pm$ SEM.