AN ABSTRACT OF THE DISSERTATION OF

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Title: Calpain 2 Proteolysis Regulates Glioblastoma Cell Invasion

Abstract approved:

Jeffrey A. Greenwood

Glioblastoma is the most malignant primary brain tumor with the average patients surviving only one year after diagnosis, even with aggressive therapy. The formation of numerous micro-tumors dispersed into the brain due to rapid invasion of tumor cells, presents the primary challenge to the surgical removal of tumors and limits the effectiveness of current treatments. This dissertation presents studies aimed at understanding the molecular mechanisms regulating invasion of human glioblastoma cells. Transplantation of human glioblastoma cells in the zebrafish brain showed that the knockdown of calpain 2, a calcium-activated protease, resulted in a three fold decrease in the tumor cell invasion. The result was further verified in the

organotypic mouse brain slices where the knockdown cells demonstrated 2-fold decrease in the area of dispersal compared to control cells. Our data show that calpain 2 plays a role in the process of tumor cell angiogenesis. Glioblastoma cells were transplanted into the brain of zebrafish expressing GFP in the blood vessels and we observed that 23% of animals injected with control tumor cells demonstrated angiogenesis. In contrast, only 9% of fish that received calpain 2 knockdown cells showed the formation of new vessels. Consistent to the reports from human glioblastoma patients and rodent models, we did not observe metastasis of transplanted cells outside of the brain in the zebrafish, supporting for the use of zebrafish as an important model for glioblastoma cell invasion studies. These results provide evidence that calpain 2 protease activity is required for the dispersal of glioblastoma cells in the brain microenvironment. To determine the mechanism of calpain 2 regulation of tumor cell invasion, proteolysis of filamin by calpain 2 was studied.

Filamin is an important actin cross-linking protein which develops orthogonal actin networks in the periphery of the cell. In this study, we show that the expression of filamin inhibits glioblastoma cell invasion. Hence, knocking down filamin expression by 80% resulted in 220% increase in the invasion of glioblastoma cells through Matrigel extracellular matrix. The regulated proteolysis of filamin is a potential mechanism to facilitate the cyclic turnover of actin orthogonal networks which is required for glioblastoma cell invasion. In this study, we identified a novel

mechanism that the PI3 kinase activity regulates the cleavage of filamin by calpain 2 in glioblastoma cells. Binding of a membrane phospholipid phosphatidylinositol (3,4,5) triphosphate [PtdIns (3,4,5)-P_3] to filamin induces its proteolysis by calpain 2 after the amino acid lysine 268, removing the actin binding domain which in-turn abolishes the actin binding ability of filamin.

Calpain 2 Proteolysis Regulates Glioblastoma Cell Invasion

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Sangeet Kumar Lal, Author

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Dedicated to

My parents

Nagina Lal and Radha Raman Lal

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Chapter 1

Introduction

Origin of glioblastoma

Cancer is defined as the uncontrolled growth of abnormal cells. The oldest description of cancer dates to 1600 B.C. in Egypt, though the term "carcinoma" was first used by Hyppocrates, the father of medicine, 460-370 B.C (American Cancer Society). In spite of such a long history of diagnosis and constant development of therapeutic strategies, cancer still ranks as the second leading cause of death in America; accounting for 23% death in 2007, it was exceeded only by heart diseases at 25%. Approximately 2% of all cancer deaths are attributed to primary brain tumors (American Cancer Society). Although the exact cellular origin of brain tumors is still under debate, primary tumors are assumed to develop from neural stem cells or glial cells which retain the ability to divide throughout life, unlike nerve cells which stop dividing after differentiation (Kleihues et al. 1995; Schiffer et al. 2010).

Astrocytes, oligodendrocytes and microglia are the major types of glial cells which together perform a broad spectrum of roles essential for the survival and proper functioning of nerve cells. Astrocytes provide energy and nutrition to nerve cells by establishing close association with the blood vessels (Allen and Barres 2009). These cells are also involved in the formation of synapses (Ullian et al. 2001) and neurotransmission at synaptic junctions by controlling levels of K^+ ions and neurotransmitters such as glutamate (Barres 2008; Sontheimer 2008). Oligodendrocytes are responsible for the synthesis of myelin sheath around axons of nerve cells, which is crucial for the rapid conduction of nerve impulses, and microglia

are the resident immune cells of the nervous system (Barres 2008). The brain tumors derived from glial cells are collectively known as glioma (Kleihues et al. 1995).

Glioblastoma originate from astrocytes and are the most common and malignant tumors, accounting for 51.2% of glioma (CBTRUS 2008). The prognostic accuracy for glioblastoma tumors is poor and even the most aggressive therapeutic practices have failed to increase the median survival of patients more than 12 to 15 months (Gladson et al. 2010). This devastating diagnosis of glioblastoma is attributed primarily to the remarkable ability of tumor cells to invade over long distances into regions of the normal brain as single cells or small groups of cells. This unusual invasiveness of tumor cells makes the complete surgical resection of the tumor impossible and other therapeutic approaches, such as radiotherapy, ineffective. Post surgery invasion of remnant cells into surrounding tissues results in the recurrence of secondary and tertiary tumor foci, which contribute to the increased frequency of mortality. Therefore, the primary challenge for the therapeutic success against glioblastoma is to prevent rapid invasion of tumor cells into the normal brain. In order to do so, it is necessary to understand the molecular mechanisms regulating cell invasion.

Mutations in glioblastoma cells

The genetic screening of invasive glioblastoma cells has identified mutations in many genes involved in cell migration. Overexpression and/or mutation in the epidermal growth factor receptor (EGFR) gene have been reported in 36% to 60% of tumors (Rao et al. 2003; Soni et al. 2005; Wong et al. 1987). The most frequent mutation occurs due to an in-frame deletion of the exons 2 to 7 which results in the expression of a truncated form of the receptors that remains constitutively active despite being unable to bind the ligand. This activated receptor stimulates downstream signaling cascades such as the phosphatidylinositol 3-kinase (PI3 kinase) pathway to promote cell proliferation and migration (Ekstrand et al. 1992; Sugawa et al. 1990). The upregulation of the PI3 kinase pathway has been frequently reported in glioblastoma tumors (Guertin and Sabatini 2007; Li et al. 1997; Louis 2006). Gain-of-function mutations resulting in the activation of the p110α isoform of PI3 kinase have been reported in 27% of glioblastomas (Samuels et al. 2004). In a normal cell, the action of PI3 kinase is directly antagonized by PTEN (Phosphatase and TENsin homolog), a phosphatidylinositol (3,4,5)-triphosphate (PtdIns (3,4,5)-P_3) specific phosphatase. Genetic deletion of the PTEN gene has been reported in up to 70% of primary glioblastoma tumors due to loss of heterozygosity (LOH) of chromosome 10q and the mutations leading to the inactivation of the PTEN gene is found in 14% to 47% of glioblastoma tumors (Fujisawa et al. 2000; Gladson et al. 2010; Li et al. 1997; Ohgaki et al. 2004; Wang et al. 1997). Altogether, the amplification of EGFR

signaling, loss of PTEN activity and the over-activation of PI3 kinase itself, results in the net enhancement of PI3 kinase activity. PI3 kinase activity has been directly associated with increased invasion of glioblastoma cells (Kubiatowski et al. 2001). Additionally, the re-expression of the PTEN gene was reported to inhibit the growth and invasion of glioblastoma cells, (Furukawa et al. 2006; Koul et al. 2001; Li and Sun 1998), which further suggests that the PI3 kinase pathway plays a crucial role in the regulation of glioblastoma cell invasion. Hence, it is important to determine how PI3 kinase signaling is interpreted downstream to aid glioblastoma cell invasion.

Unique features of glioblastoma cells

Glioblastoma cells invade the surrounding tissues as single cells or small groups of cells but, with rare exceptions, do not metastasize outside of the brain (Kleihues and Sobin 2000). Further, these cells have been reported to migrate along the white matter tracts and blood vessel surfaces. However, they do not invade into the blood vessel wall, which is considered to be the reason for their inability to metastasize (Bernstein and Woodard 1995; Scherer 1940). Though the exact explanation for this observation is still not understood, the distinct pattern of glioblastoma invasion is assumed to be due to the fact that the extracellular matrix (ECM) of the brain is distinct from that of most organs. The ECM of the brain consists of tightly packed neuronal and glial processes leaving extracellular spaces of nanometer size (Thorne and Nicholson 2006).

The primary components of the brain matrix are hyaluronic acid, tenascin, thrombospondin and proteoglycans (Bellail et al. 2004). Hyaluronic acid, being highly anionic in nature, attracts Na^+ and other cations which increase an osmotic influx of water, making the brain a less rigid, water-rich microenvironment (Alberts et al. 2002). Further, hyaluronic acid interacts non-covalently with other brain-specific proteoglycans of the lectican family (aggrecan, versican, brevican and neurocan) (Bignami et al. 1993) which are all non-fibrous proteins and gives the brain a softer consistency. In contrast, the ECM of most other systemic organs is comprised of collagen, laminin and fibronectin which provide strong fibrous texture to the matrix. Inside the brain, this type of fibrous matrix is limited only to the perivascular areas (Gladson 1999). Thus, the brain microenvironment is heterogeneous with rigid fibrous matrix around the blood vessels and soft non-fibrous composition around the neuronal surfaces. However, it is important to note that glioma cells show migration along both the surfaces; blood vessels with defined fibrous basal lamina as well as axons in white matter-with softer and ill-defined ECM (Louis 2006). This finding suggests the involvement of more intricate mechanisms to glioblastoma invasion than just crawling over a rigid ECM, and has stimulated interest in determining unique characteristics of glioblastoma cells that help them adapt to the brain microenvironment.

A unique factor associated with glioblastoma cell invasion is the autocrine/paracrine signaling by glutamate. Glutamate is an endogenous neurotransmitter secreted at synaptic junctions that binds to receptors on the second

neuron at the synapse thus mediating fast signaling by inducing Ca^{2+} ion influx in post-synaptic neurons. The prolonged activation of glutamate receptors causes neuronal cell death by sustained calcium signaling known as excitotoxicity (Choi 1988). Hence, the normal concentration of glutamate in extracellular space is maintained at ~1 μM by the uptake of excessive glutamate by astrocytes through expression of specialized transporters which include excitatory amino acid transporters (EAAT) (Sontheimer 2008). Ye and colleagues first showed that malignant glial tumor cells release excessive amounts of glutamate instead of removing it from the extracellular space, and found a correlation with the failure of these cells to express glutamate transporters on their surface (Ye et al. 1999; Ye and Sontheimer 1999). Glutamate secretion was demonstrated to promote glioblastoma cell invasion *in vitro* and in mouse glioma tumor model (Lyons et al. 2007). These results are important because they point to a unique mechanism of dual advantage of glutamate secretion: (a) clearing space for tumor expansion by killing normal nerve cells by excitotoxicity, and (b) promoting tumor cell invasion.

Mechanisms of glutamate-induced invasion were provided by a study that showed that glutamate binding to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA) on the cell membrane stimulates calcium ion influx into the cell, which in turn induces cellular migration (Ishiuchi et al. 2002) (Fig 1.3). Calcium oscillations are required for focal adhesion disassembly in glioblastoma cells (Giannone et al. 2002) and there is more evidences supporting increase in glioma cell

motility by calcium influx (Maghazachi 2000; Manning et al. 2000). The exact targets of calcium signaling involved in regulation of glioblastoma invasion are not known. We proposed calpain 2, a calcium-activated protease, to be the potential effector molecule in invading cells.

Calpain in cell invasion

Calpain was first identified as a unique proteolytic enzyme in the cytosolic fraction of rat brain extract (Guroff 1964). It acquired its name from the absolute requirement of calcium ions for its activation and structural homology with the catalytic domain of the papain family of cysteine proteases (Carragher and Frame 2002). At present, the calpain family of cytoplasmic calcium-activated cystein proteases consists of 15 isoforms demonstrating a highly conserved evolutionary distribution with homologues present in invertebrates, plants, fungi, and mammals (Goll et al. 2003). Calpain 1 and calpain 2 are ubiquitously expressed isoforms and have been implicated in cell migration. Calpain 1 and 2 are heterodimeric proteins comprised of an 80 kDa large catalytic subunit and a 30 kDa small regulatory subunit (Franco and Huttenlocher 2005).

Knockout mice were developed in order to understand the physiological functions of the ubiquitously expressed isoforms calpain 1 and 2. Knocking out the calpain 1 catalytic subunit gene [Capn1(-/-)] resulted in platelet dysfunction (Azam et al. 2001), whereas knocking out the gene for the large subunit of calpain 2 [Capn2(-/-

)] or the 30 kDa small regulatory subunit [Capn4(-/-)] present in both calpain 1 and 2 resulted in embryonic lethal conditions (Arthur et al. 2000). This showed that calpain 2 is critical for embryogenesis, whereas calpain 1 is not. Since cell migration is an active process during embryogenesis, it suggests a possible requirement of calpain 2 for cellular migration. The catalytic subunit of calpain has four domains, of which domain II possesses the active site, domain IV contains five calcium binding motifs (EF-hand motifs) and domain III apparently induces structural reorganization to form the active site when bound to calcium ions and/or phospholipids (Croall and Ersfeld 2007). The active site of calpain 2 contains the typical catalytic triad of the cysteine protease family, which includes cysteine (Cys 105), histidine (His 262), and asparagine (Asn 286). The enzymatic mechanism involves nucleophilic attack of Cys 105 on the partially positive carbonyl carbon of the susceptible peptide bond, forming a tetrahedral intermediate that quickly collapses to release the amine product. The enzyme is regenerated following hydrolysis of the acyl-enzyme complex releasing the carboxyl product (Storer and Menard 1994). A distinguishing feature of calpain proteolysis is that substrates are cleaved in a limited fashion to generate stable fragments rather than complete proteolytic digestion. Hence, calpain-mediated proteolysis is considered to be a post-translational modification that influences various aspects of cell physiology, including cell migration (Goll et al. 2003).

Calpain 2 has been frequently implicated in many physiological processes including focal adhesion dynamics, membrane protrusions and cell migration

(Carragher and Frame 2002; Franco et al. 2004; Franco and Huttenlocher 2005). Calpain was first shown to play a role in cellular adhesion dynamics by targeting the actin binding protein talin (Beckerle et al. 1987). It was reported to regulate the detachment of the rear end of the cell during migration (Huttenlocher et al. 1997; Palecek et al. 1998). The role of calpain in the regulation of cell migration was established when embryonic fibroblasts from the 28 kDa small subunit knockout mice were observed to have decreased rates of migration (Dourdin et al. 2001).

In recent years, an increasing amount of evidence has been presented showing that calpain 2 also plays an important role in tumor cell invasion. Selective downregulation of calpain 2 decreased invasion of prostate cancer cells by 50% in a rodent xenograft model (Mamoune et al. 2003). Calpain 2 knockdown resulted in the inhibition of breast cancer cell invasion by regulating the formation and stability of invadopodial projections (Cortesio et al. 2008). Our lab presented evidence that calpain 2 is required for the invasion of glioblastoma cells through the artificial Matrigel extracellular matrix (Jang et al. 2010). Together, these results are important because the data demonstrate that targeting calpain 2 during tumor invasion is a potential treatment for specific cancers. It is known that calpain 2 targets many cytoskeletal proteins including talin, vinculin, α-actinin, spectrin, ezrin, FAK and integrins to regulate the processes of cell spreading, membrane protrusions, focal adhesion dynamics and tail retraction to facilitatate cell migration. However, the substrates of calpain 2 in tumor cell invasion through a 3D matrix are not precisely

known. We propose that the actin cross-linking protein filamin is a likely substrate of calpain 2 in invading cells.

Filamin in cell invasion

Filamin is a V-shaped homodimeric protein composed of ~280 kDa subunits. Each monomer is comprised of an actin-binding domain (ABD) at the amino-terminus and the dimerization domain located at the C-terminus (Stossel et al. 2001). The two ends are separated by 23 homologous repeats (~96 residues each), which are divided into rod domain 1 (repeats 1 to 15) and rod domain 2 (repeats16 to 23). A relatively less ordered stretch of approximately 30 amino acids between repeats 15-16, designated as 'hinge 1' separates the two rod domains. Another hinge region between repeats 23-24, designated as 'hinge 2' separates rod domain 2 from the dimerization domain (Fig 1.1). The hinge regions provide overall flexibility to the protein dimer which facilitates cross-linking of branched actin filaments positioned at higher angles (>70%) and this unique cross-linking generates orthogonal networks of actin filaments (Nakamura et al. 2007; Stossel et al. 2001). These networks primarily provide the mechanical and elastic strength required for membrane protrusions and deformation resistance against the sheer forces generated during migration (Tseng et al. 2004).

Filamin is ubiquitously expressed in prokaryotes and eukaryotes (van der Flier and Sonnenberg 2001). The mammalian filamin family is comprised of three members: filamin A (FLNA), filamin B (FLNB) and filamin C (FLNC) which have

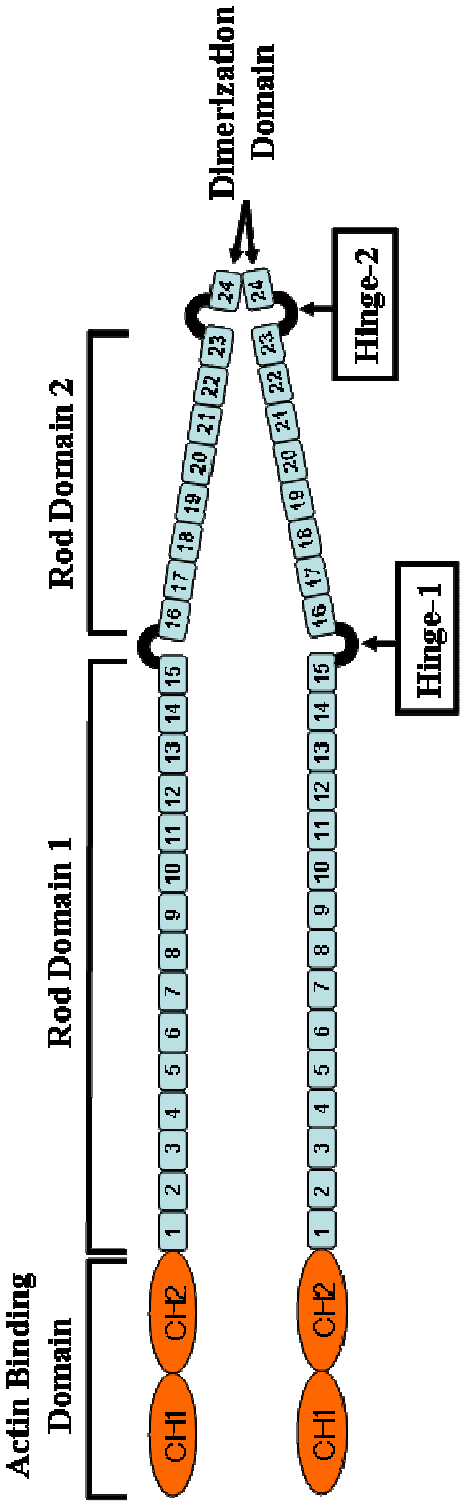
Actin Binding Domain
CH1
CH2
Rod Domain 1
Rod Domain 2
Hinge-1
Hinge-2
Dimerization Domain

Fig 1.1: The structure of human filamin (FLNA).

Filamin is a homodimeric protein with the actin binding domain (comprised of CH1 and CH2 subdomains) of each subunit located at the amino-terminus. The rest of the protein is made of 24 repeat units of ~96 amino acids each. Rod domain 1 contains repeats 1-15 and repeats 16-23 makes rod domain 2. The two monomers dimerizes non-covalently at the C-terminal 24^{th} repeat. The repeat units are interspersed by relatively less structured stretches of approximately 35 amino acids between repeats 15 and 16 designated as hinge 1 and between repeats 23 and 24 designated 'hinge 2'.

highly conserved genetic sequences and the proteins encoded by these genes show about 70% amino acid identity (van der Flier and Sonnenberg 2001). Out of three isoforms in vertebrates, filamin A and B are more ubiquitously expressed while filamin C expression is mainly restricted to skeletal and cardiac muscle cells (Popowicz et al. 2006; Stossel et al. 2001). This dissertation investigates the function and regulation of only the filamin A isoform, which henceforth will be referred to as filamin.

Filamin is an efficient actin filament cross-linker and a single filamin dimer per actin filament is sufficient to induce gelation. Also, the type of filamin cross-linking of actin filaments depends on the molar ratio of filamin to actin. At a higher ratio such as 1:10, parallel bundles of actin filaments are promoted, whereas a ratio of 1:150 to 1:740 leads to the formation of orthogonal actin networks (Brotschi et al. 1978). Filamin is maintained at lower concentration at the leading edge of cells and facilitates orthogonal actin networks formation (Flanagan et al. 2001). The reorganization and turnover of the actin cytoskeleton provides the driving force for cell migration.

Filamin has been shown to be important for cellular migration. Melanoma cells, with a genetic deficiency of filamin expression, do not migrate and exhibit extensive blebbing of the plasma membrane (Cunningham et al. 1992). Neuronal cells expressing a dysfunctional mutant filamin fail to migrate to the cortical region of the brain, resulting in periventricular heterotopia (Fox et al. 1998). The deletion of filamin

from *Dictyostelium* amoebae results in impared locomotion and phagocytosis (Cox et al. 1992; Ponte et al. 2000). These results demonstrate the importance of filamin for the efficient migration of a cell. Besides cross-linking actin filaments, filamin also binds to transmembrane integrin receptors and to many other signaling molecules localized at the cell membrane. By doing so, filamin serves as a scaffold protein, assembling them in close proximity to couple integrin mediated signal transduction and regulation of cytoskeletal reorganization. Filamin has been shown to be regulated by processes like phosphorylation by protein kinase (Chen and Stracher 1989), binding with phosphoinositides (Furuhashi et al. 1992a) and proteolysis by calpain.

The first evidence of calpain proteolysis of filamin was reported by Dr. Pastan's group (Davies et al. 1978), who suggested that cleavage affects cross-linking but not the actin binding ability of filamin. Human platelet filamin was further shown to be cleaved by calpain (Fox et al. 1985). The calpain cleavage site on human filamin was first mapped between residues 1761 and 1762 in the hinge I region, producing ~190 kDa and ~100 kDa fragments (Gorlin et al. 1990). Though of importance, all the work for calpain cleavage of filamin has been performed on purified proteins and the physiological relevance of this proteolysis is not yet understood. During migration in a three dimensional (3D) space, the cell body needs to modify its shape and stiffness to move through the pores of extracellular matrix. An overly stabilized actin network and cell-ECM attachments, as facilitated by filamin, would lead to the reduced flexibility of cell membrane and difficult translocation of the cell body. This highlights the

necessity for the cyclic turn over of actin networks in cell migration which can be facilitated by the regulated proteolysis of filamin. However, the mechanisms regulating calpain proteolysis of filamin in cells are not understood.

Phosphoinositides

Phosphoinositides are membrane lipids which have been shown to bind to and modulate the structure and function of many cytoskeletal proteins including α-actinin, talin, vinculin and filamin (Niggli 2005; Yin and Janmey 2003). Studies from our lab have shown that binding of phosphoinositides, PtdIns (3,4,5)-P_3 in particular, to the actin binding protein α-actinin modulates its three dimensional structure and regulate its function (Corgan et al. 2004; Fraley et al. 2005; Fraley et al. 2003; Full et al. 2007). Importantly, PtdIns (3,4,5)-P_3 binding to α-actinin enhances susceptibility to proteolysis by calpain 2 (Sprague et al. 2008). Filamin has been reported to bind phosphoinositides in the literature (Furuhashi et al. 1992b). Hence, we propose PtdIns (3,4,5)-P_3 binding as the mechanism to regulate filamin proteolysis in cells.

PtdIns (3,4,5)-P_3 belongs to a family of membrane glycerophospholipids, which are composed of a hydrophobic diacylglycerol backbone esterified to a polar inositol headgroup (Fig 1.2). The acyl chains integrate into the membrane whereas hydroxyl groups in the inositol ring are targeted for phosphorylation by kinases and dephosphorylation by phosphatases (Skwarek and Boulianne 2009). PI3 kinase catalyzes the phosphorylation of the phospholipid, phosphatidylinositol (4,5)-

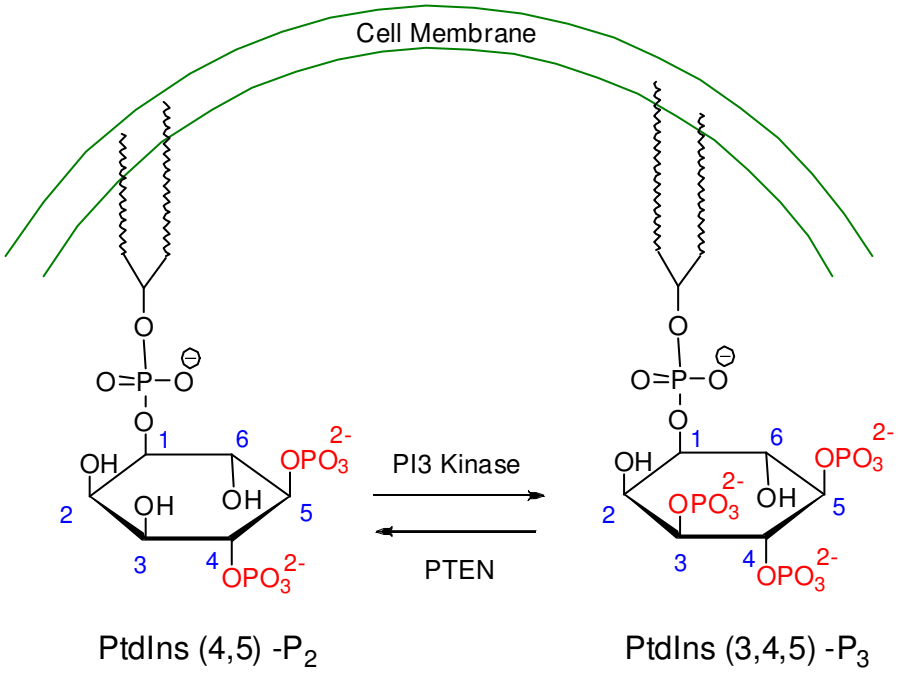

Fig 1.2: Diagram showing regulation of PtdIns (3,4,5)-P_3 in the cell membrane.

PI3 kinase catalyzes the transfer of γ-phosphate from ATP to the 3'-hydroxyl group of PtdIns (4,5)-P_2 to produce PtdIns (3,4,5)-P_3 in the plasma membrane. This activity of PI3 kinase is negatively regulated by the phosphatase PTEN which converts it back to PtdIns (4,5)-P_2 after dephosphorylation. Hence, loss of PTEN gene and constitutive activation of the PI3 kinase results in the constitutively enhanced levels of PtdIns (3,4,5)-P_3 in glioblastoma cells.

bisphosphate (PtdIns (4,5)-P_2) at the 3'-hydroxyl group of the inositol ring to produce PtdIns (3,4,5)-P_3 at the plasma membrane (Hawkins et al. 2006) (Fig 1.2). Therefore, the enhancement of the PI3 kinase activity in glioblastoma cells directly correlates to the increased PtdIns (3,4,5)-P_3 levels in the cell membrane (Fig 1.3). Once produced, PtdIns (3,4,5)-P_3 acts as a second messenger to initiate multiple signaling events. A major downstream effector of the PI3 kinase activity is protein kinase B (Akt) which is activated in 70% of gliomas (Mure et al. 2010). Binding of Akt to PtdIns (3,4,5)-P_3 is necessary for its activation by phosphorylation at amino acid Thr308 followed by a second phosphorylation at residue Ser473 which ensures maximum activity (Bayascas and Alessi 2005). Assessment of the phosphorylation status at these residues is a frequently used method to monitor PI3 kinase activity, and in particular, the levels of PtdIns (3,4,5)-P_3 in the cell membrane.

Zebrafish as a model for tumor cell invasion

Before the clinical trial on humans, a drug under discovery undergoes a series of biochemical and cellular assays with final validation in animal models. Though medical science has made significant advances aimed at treatment of malignant gliomas, the majority of targeted molecular drugs that have been evaluated to date have been disappointing, with response rates of 10% to 15% or less (Van Meir et al. 2010). Therefore, the search for novel targets and screening of a range of drugs are currently underway. A major limitation to understanding cancer cell biology is the

inability of current animal models to facilitate imaging of the dynamic process of tumor cell invasion and their interaction with the physiological microenvironments. Zebrafish has emerged as a useful experimental organism to study cancer cell invasion and metastasis (Beckman 2007).

Zebrafish, *Danio rerio*, is a tropical freshwater fish which originated from the Ganges river in Eastern India (Spence et al. 2008) and was originally used to study developmental biology and embryogenesis (Detrich et al. 1999). With a vertebrate body plan containing the full repertoire of genes and a counterpart to most mammalian organs, zebrafish exhibit a high degree of similarity to mammals with respect to molecular mechanisms of development and cellular physiology (Lieschke and Currie 2007). Zebrafish embryos are optically transparent, a characteristic that facilitates imaging of the internal organs and their interaction with the implanted cancer cells (Kari et al. 2007). The development of transgenic lines which express fluorescent markers in the vasculature makes it important for the tumor cell invasion and metastasis studies. The interaction of tumor cells with the fluorescent vascular system can be monitored with high-resolution microscopy in a living animal for several days to weeks (Stoletov et al. 2007). A specific advantage of zebrafish is that they can absorb small molecules and drugs directly from the water, through gills and skin, which makes administration of drugs and screening very easy. Although immature T-cells arise in the thymus by 3-4 dpf but the immune system is not functional until 28 dpf (Taylor and Zon 2009). This removes the complexity of immune responses which

is another advantage for the xenotransplantation of human cells. Zebrafish proteins display overall ~70% identity to their human orthologues, which approaches close to 100% in regions of conserved functional domains, such as a substrate binding site (Langheinrich 2003). A great attraction towards using zebrafish is the low cost involved in the handling and raising of these animals compared to mammalian models.

Thesis Outline

Glioblastoma cancer is difficult to treat because of the enhanced invasiveness of tumor cells in the brain, but the factors involved and the mechanisms regulating invasion of glioblastoma cells is not well understood. Based on the information from the literature, I proposed to test the central hypothesis that PI3 kinase mediated calpain 2 proteolysis of filamin regulates invasion of glioblastoma cells. This dissertation includes four chapters:

Chapter 1 presents the background information from the literature about the main players of the current studies which include; origin and specific features of glioblastoma, unique features of calpain 2 and role of calpain proteolysis in cell migration, structure and function of filamin and its value in cell migration, phosphoinositides and their role in regulating cytoskeletal proteins in cells, and zebrafish as the orthopic model to study tumor cell invasion.

Chapter 2 describes the study which shows that calpain 2 activity is required for the invasion of glioblastoma cells using ex-vivo assays and in the zebrafish brain.

We observed that tumor cells invaded the brain primarily in association with the blood vessels and inhibition of calpain 2 expression decreased the localization of tumor cells with the blood vessels indicating to a possible mechanism regulating dispersal of glioblastoma cells. Results from the zebrafish studies were further validated by repeating glioblastoma invasion in mouse brain slices in culture.

Chapter 3 discusses another novel finding that expression of filamin inhibits invasion of glioblastoma cells through dense extracellular matrix. Calpain 2 cleavage of filamin was considered as a mechanism controlling turn-over of orthogonal actin networks and hence glioblastoma cell invasion. Here, we show that PtdIns (3,4,5)-P_3 binding to filamin induces cleavage by calpain 2, removing the actin binding domain *in vitro* and filamin proteolysis is enhanced by PI3 kinase activity in glioblastoma cells.

Chapter 4 presents a summary of conclusions from the original research studies and discusses the contributions of this work to advance the field and future studies that can incorporate our findings to develop therapeutics for glioblastoma tumors.

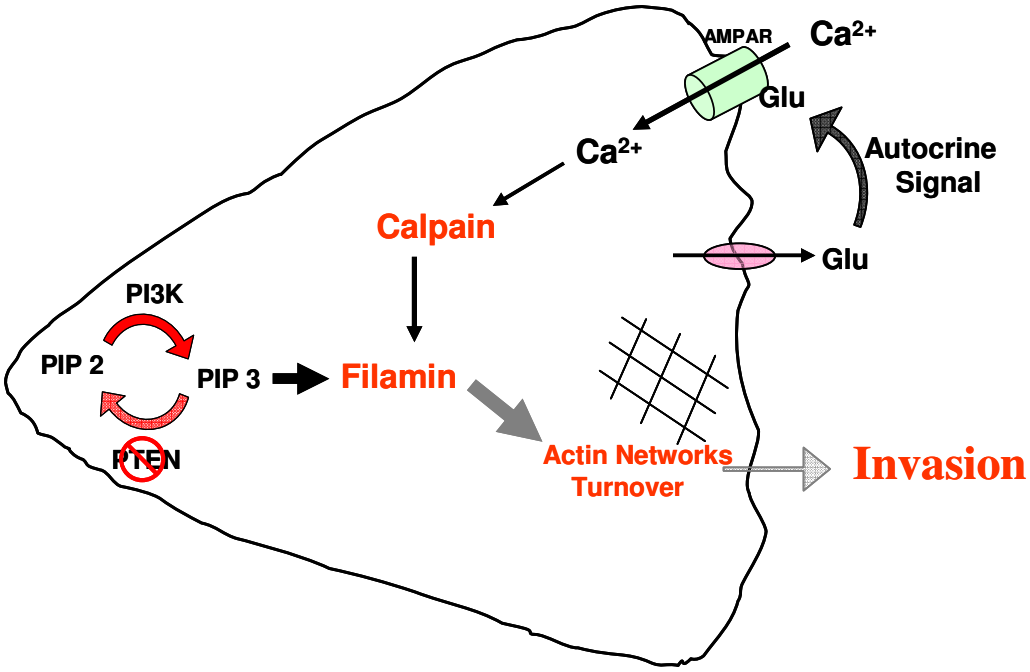

Fig 1.3: Schematic of the proposed model for the regulation of glioblastoma cell invasion by calpain.

The increased calcium signaling and increased PI3 kinase activity in glioblastoma cells synergizes to promote cell invasion. Secreted glutamate binds to the AMPA receptors and stimulates calcium influx in the cell. Loss of PTEN gene (block symbol) and activation mutations in PI3 kinase (highlighted arrow) results in the constitutively increased levels of PtdIns (3,4,5)-P_3 in the cell membrane.We propose that PtdIns (3,4,5)-P_3 binding to filamin induces its proteolysis by calcium ion activated calpain 2 which leads to the reorganization and turnover of actin networks at the leading edge providing pliability to the cell body. This increased flexibilty of the cell membrane enables the tumor cells to move through the pores of extracellular matrix.

Calpain 2 is required for the invasion of glioblastoma cells in the zebrafish brain microenvironment

Chapter 2

Abstract

Glioblastoma is the most aggressive type of primary brain tumor with an average survival time of only 14 months following diagnosis (Schiffer et al. 2010). The poor prognosis for patients diagnosed with glioblastoma is attributed to the rapid post-surgery recurrence of secondary and tertiary tumors resulting from the invasion of glioblastoma cells into the surrounding brain tissue. Although glioblastoma cells have been shown to migrate along blood vessels and the myelin sheath of axons, the molecular mechanisms regulating this dispersal pattern are not clear (Farin et al. 2006). A unique pathway implicated in the invasion of glioblastoma cells involves autocrine glutamate activation of AMPA receptors leading to calcium influxes in these cells (Lyons et al. 2007). Identification of the downstream effector molecules of calcium involved in the process of invasion is critical for understanding the rapid progression of glioblastoma, and we proposed that the calcium-activated protease calpain 2 is an essential target. In this study, we show that calpain 2 expression is required for the dispersal of glioblastoma cells in the living brain environment of the zebrafish. Knockdown of calpain 2 expression decreased cell invasion by 3-fold, and cells remained confined in clusters, in contrast to the control cells which dispersed far distances up to 450μm from the site of injection. The transplantation of control and calpain 2 knockdown glioblastoma cells in the transgenic zebrafish, *Tg(fli1:egfp),* expressing GFP in blood vessels, showed that angiogenesis induced by tumor cells appeared to be less prevalent in zebrafish injected with calpain 2 knockdown cells

compared to controls. Consistent results were observed in a common mammalian model showing reduced dispersal of calpain 2 knockdown glioblastoma cells compared to control cells in organotypic mouse brain slices.

Introduction

Malignant brain tumors have a poor prognosis resulting in parts from the ability of tumors cells to invade the tissue, limiting the effectiveness of primary tumor removal (Adamson et al. 2009). 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. Tumor cells accomplish this mechanical task by using matrix metalloproteinases 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. It is not clear which intracellular activities facilitate the rapid invasion of brain tumor cells, or the 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

Glioblastoma is the most invasive form of brain cancer with a 5-year survival rate of less than 4% (CBTRUS 2005). Despite therapeutic advances, treatments have been ineffective to increase the median survival time of patients more than 12 to 15 months (Stupp et al. 2005). Preventing glioblastoma cells from invading is the primary requirement for effective treatment with focal therapies such as surgical tumor resection and radiations (Lim et al. 2007). The mechanisms for the carcinogenesis and migration of glioblastoma cells are not clear, although upregulation of invasion-

specific processes such as enhanced matrix degradation by metalloproteinases and tumor cell angiogenesis, and mutations in several genes have been identified in many cases (Adamson et al. 2009; Kanu et al. 2009a; Kanu et al. 2009b). The most common genetic aberrations related to enhanced cell migration includes mutation in the epidermal growth factor receptor (EGFR), PTEN and PI3 kinase genes. Deletion of the PtdIns (3,4,5)-P_3 phosphatase PTEN is detected in 31% of glioblastoma cell lines (Li et al. 1997), 27% of tumors revealed mutations leading to activation of the p110α isoform of PI3 kinase (Samuels et al. 2004) and overexpression and/or mutation in EGFR gene have been reported in 36%-60% of tumors (Gladson et al. 2010; Lee et al. 2006). All of these mutations ultimately result in an increase in the level of PtdIns (3,4,5)-P_3 in glioblastoma cell membranes. Further, Kubiatowski et al. correlated PI3 kinase signaling with increased glioma cell invasion in rat brain implants (Kubiatowski et al. 2001). Together, these data indicate that increased levels of PtdIns (3,4,5)-P_3 in cell membranes play a role in the migration and invasion of glioblastoma cells. During migration, cells demonstrate asymmetric polarization with a distinct leading edge in the direction of migration and a tail at the opposite end (Lauffenburger and Horwitz 1996). The leading edge of the migrating cells displays dynamic remodeling of the actin cytoskeleton, which involves active proteolysis of several actin binding proteins by calpain a calcium-activated cytoplasmic cysteine protease (Franco and Huttenlocher 2005). Our lab showed that PtdIns (3,4,5)-P_3 binding to the actin bundling protein α-actinin regulates its proteolysis by calpain 2 (Sprague et al.

2008). Although this suggests a possible mechanism for how PI3-kinase activity enhances cellular migration, the role of calpain in glioblastoma cell motility and the mechanisms of calpain activation are still not understood. Calpain is a family of cysteine proteases activated by the binding of calcium ions and is important for the regulation of processes important for cell migration (Franco and Huttenlocher 2005). Calpain 2 is a ubiquitously expressed isoform which requires millimolar concentration of calcium ions *in vitro* for half maximal activity, which is significantly higher than the nanomolar range in a normal cell (Goll et al. 2003).

Calcium appears to play an important role in promoting the invasion of glioblastoma cells, as recent studies have demonstrated that autocrine glutamate activation of AMPA receptors induce calcium influxes regulating glioblastoma cell adhesion and migration (Lyons et al. 2007; Sontheimer 2008). The relation between calcium ion influx and increased invasion is not clearly understood, but the activation of calpain 2 is a potential candidate. In the last decade, the evidence for association of calpain with cancer has significantly increased. Increased levels of calpain activity has been detected in breast cancer tissues (Shiba et al. 1996) and the overexpression and increased activity of calpain 2 was reported in colorectal cancer (Lakshmikuttyamma et al. 2004). Inhibition of calpain 2 activity by chemical inhibitors or siRNA mediated knockdown was reported to decrease the invasiveness of prostate cancer cells by ~50% in an *in vivo* xenograft model (Mamoune et al. 2003). The isoform-specific role of calpain 2 in breast cancer cell invasion was elucidated when the knockdown of

calpain 2 resulted in reduced invasion through regulation of the stability of invadopodial projections necessary for movement through the extracellular matrix (Cortesio et al. 2008). Furthermore, inhibition of calpain 2 activity was reported to attenuate the process of angiogenesis mediated by vascular endothelial growth factor (VEGF) in pulmonary endothelial cells (Su et al. 2006). These reports motivated us to test the hypothesis that calpain 2 is necessary for the invasion of glioblastoma cells.

Recently, using a shRNA mediated knockdown technique, we demonstrated that the knockdown of calpain 2 resulted in an 80% decrease in the invasion of glioblastoma cells through "Matrigel", an artificial extracellular matrix (Jang et al. 2010). We also observed 36% lower levels of active matrix metalloproteinase (MMP-2) in the extracellular media in calpain 2 knockdown cells which points to one possible mechanism for the reduced invasiveness of these cells (Jang et al. 2010). Though the knowledge gained from this study is valuable, much of the work reporting calpain involvement in cancer progression and tumor cell invasion has been performed on *in vitro* systems, which are incapable of simulating the complex interplay between tumor cells and the local microenvironment. Calpain 2 regulation of invasion-specific processes, such as formation and stability of invadopodia and the tumor cell angiogenesis, can be more completely examined with high-resolution imaging of the tumor cell-vascular interactions in a live microenvironment, but, a lack of suitable animal models has been a limitation. Searching for an *in vivo* system to facilitate high-resolution imaging of early stage cancer progression, we settled on zebrafish (*Danio*

rerio) as an orthotopic model for the time-lapse monitoring of invasion and angiogenesis of glioblastoma cells transplanted into a live brain microenvironment.

Primarily used as a developmental and embryological model in the 1930s, zebrafish have become an important and widely used animal model for many human diseases in recent years (Detrich et al. 1999; Feitsma and Cuppen 2008). The primary benefit of using zebrafish is that they have a vertebrate body plan essentially containing the full vertebrate repertoire of genes and possess a counterpart to most mammalian organs (Lieschke and Currie 2007). Previously, it has been reported that zebrafish have a similar molecular basis of patterning and development as humans (Granato and Nusslein-Volhard 1996). Furthermore, researchers have observed that zebrafish spontaneously develop almost any type of cancer with blood vessels, brain, gill, nasal epithelium, and the lymphomyeloid system being less common target tissues (Kent et al. 2002; Matthews 2004). Neoplasms, histologically similar to human tumors, have been induced in all zebrafish organs using carcinogens (Grabher and Look 2006). Significant similarities between the molecular expression profiles of orthologous genes in human and zebrafish liver tumors have been identified, which builds confidence in using zebrafish as a cancer model (Lam et al. 2006). Recent publications have established that melanoma cells survive, proliferate, migrate and induce angiogenesis in zebrafish (Haldi et al. 2006; Lee et al. 2005). In addition, zebrafish embryos are transparent, allowing continuous visualization of invading cancer cells, and the availability of the transgenic fish expressing GFP in the

vasculature makes it possible to investigate tumor cell-blood vessel interactions (Kari et al. 2007; Langheinrich 2003; Stoletov et al. 2007). Rodent models of cancer cell transplantation requires millions of cells to start with, whereas in zebrafish, fewer than 100 cells can be microinjected and imaged using high resolution microscopy, simulating the early stages of cancer progression. Together, this evidence provides support for the use of zebrafish as a cancer model.

Results and Discussion

Calpain 2 is required for glioblastoma cell invasion through dense extracellular matrix

To study the role of calpain 2 in invasion, we previously used shRNA to generate stable human U87MG glioblastoma cells with calpain 2 expression decreased by more than 80%. Using *in vitro* transwell assays, we reported that knockdown of calpain 2 resulted in a 90% decrease in the invasion of glioblastoma cells through an artificial extracellular matrix (Jang et al. 2010). However, conventional methods to measure cellular motility and invasion, including transwell assays, have limitations: single end point data measurement, required post- experimental processing such as labeling of cells, and manual data analysis. This approach also lacks any quantitative measurement of cell behavior or invasion patterns in real time. Live-cell microscopy is useful for the continuous monitoring of cells, but expression of an exogenous

florescent marker is necessary which risks adding unknown variability to the cellular system. In this study, we quantified the invasion of glioblastoma cells in real time using microelectronic impedance measurement technology (xCELLigence) as described in the Materials and Methods.

The biophysical properties such as density and rigidity of the extracellular matrix have been strongly correlated with the motility of tumor cells (Zaman et al. 2006). Therefore, we used different concentrations of Matrigel to assess the effect of varying extracellular matrix density on the rate of invasion of glioblastoma cells expressing control or calpain 2-specific shRNA. Cells were placed on top of Matrigel in the upper chamber of the transwell inserts and the impedance of the microelectrodes on the lower side of the membrane was recorded every 15 min for 19 hrs. The change in the impedance value correlates to the number of cells which invaded through the Matrigel. In the absence of matrix, simulating cell migration, an immediate sharp increase in the cell index value was observed for both control and knockdown cells, and the cell index curve plateaued within 4 hrs after the measurement was started. This result is consistent with our previous report that calpain 2 knockdown does not affect the migration of glioblastoma cells. In contrast, in the presence of matrix coating, a lag phase showing no change in the impedance of the electrode was observed for 9 hrs, 6 hrs and 4 hrs in case of 0.8, 0.4 and 0.2 mg/ml dilution of Matrigel respectively. Thereafter, a gradual increase in cell index was recorded for both control and knockdown cells; however, the increase was significantly higher in control cells

compared to the knockdown cell index (Fig 2.1). The rate of invasion was determined by calculating the slope of cell index curves over the period of 20hrs, which showed that knockdown of calpain 2 resulted in 59%, 22%, and 9% decrease in invasion compared to control cells in presence of 0.8, 0.4, and 0.2mg/ml of Matrigel density respectively when stimulated with FBS as a chemoattractant (data not shown).

The results are in agreement with the data from our previous study showing a 90% reduction in the invasion of knockdown cells when 2 mg/ml Matrigel was used in conventional transwell assay (Jang et al. 2010). However, continuous monitoring of invasion provided important information that calpain is not a temporal requirement such as initiation of migration; rather, it constitutively increases the invasiveness of glioblastoma cells through densely packed matrix. The brain microenvironment is packed with glial and neuronal processes, matrix proteins and proteoglycans leaving extracellular spaces of submicrometer range (Thorne and Nicholson 2006). Therefore, the rigidity of brain matrix presents a severe constraint on free cellular motility of cancer cells. The composition and the density of extracellular matrix vary widely between tissues and organs. Calpain has been linked with the invasion of prostate cancer cells but it is not known if this protease helps glioblastoma cells to infiltrate in the brain tissues. Based on our real time data showing requirement of calpain 2 for glioblastoma cell invasion through dense matrices, we proceeded to test this hypothesis in a live brain microenvironment with functional circulatory system.

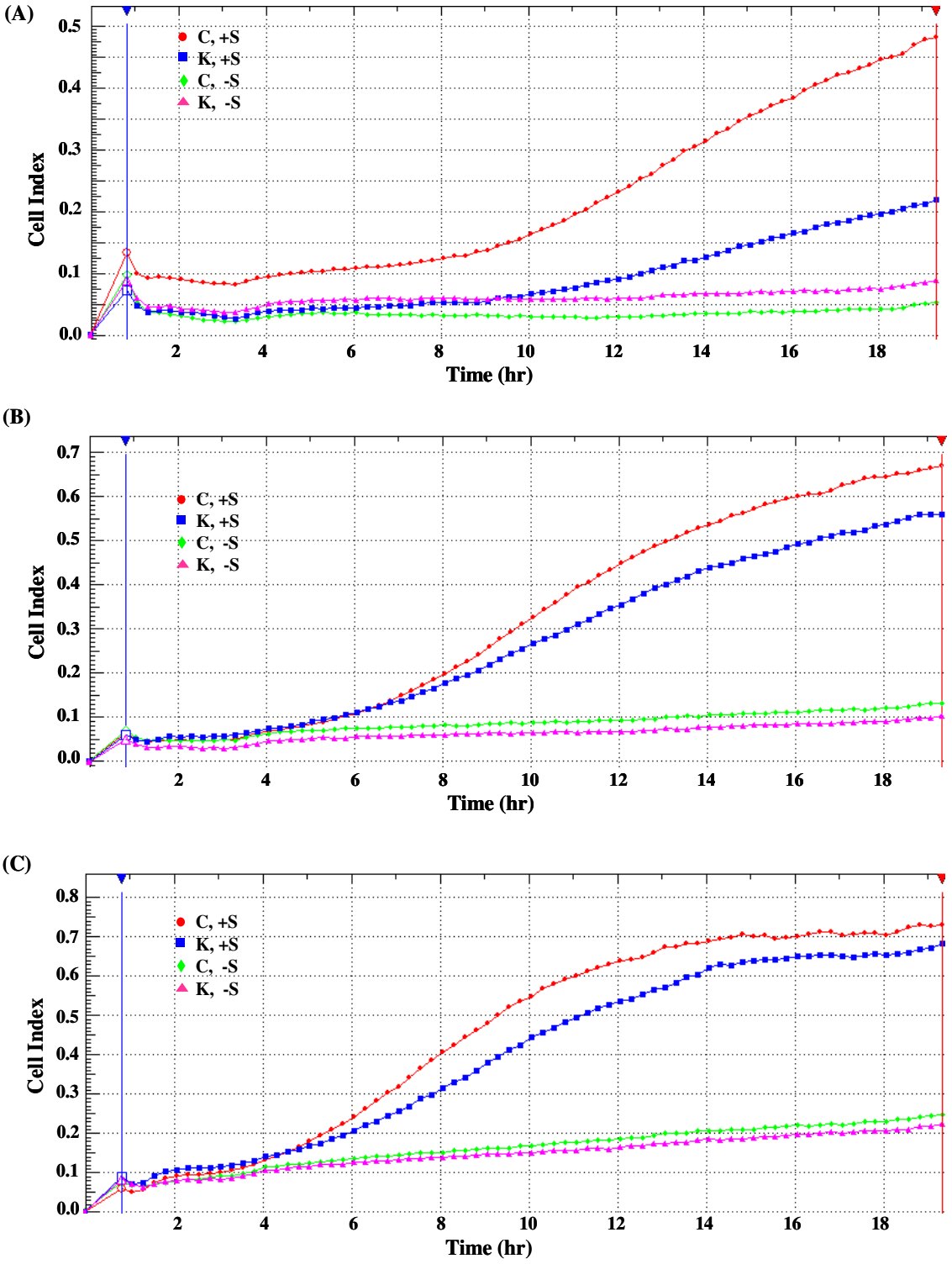
(A)
Cell Index
Time (hr)
C, +S
K, +S
C, -S
K, -S
(B)
Cell Index
Time (hr)
C, +S
K, +S
C, -S
K, -S
(C)
Cell Index
Time (hr)
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Fig 2.1: Real time analysis of invasion demonstrates requirement of calpain 2 for glioblastoma cell movement through dense extracellular matrix.

Control and calpain 2 knockdown (KD) cells were monitored in real time for invasion through Matrigel matrix of varying concentration using microsensor impedance measurements. The upper chamber was coated with 0.8, 0.4 and 0.2 mg/ml concentrations of Matrigel and the invasion was stimulated with ±10% FBS in the lower chamber of the transwells. Suspended in serum free media, 50,000 cells were placed over Matrigel and the measurements were obtained every 15 min for 19 hrs. The cell index represents the number of cells that reached the bottom surface of the transwells. The curve shows cell index value at each point of measurement for cells invading through (A) 0.8 mg/ml, (B) 0.4 mg/ml, and (C) 0.2 mg/ml Matrigel. Legends: C-control; K-knockdown; S-10% FBS. The results are representative of two independent experiments.

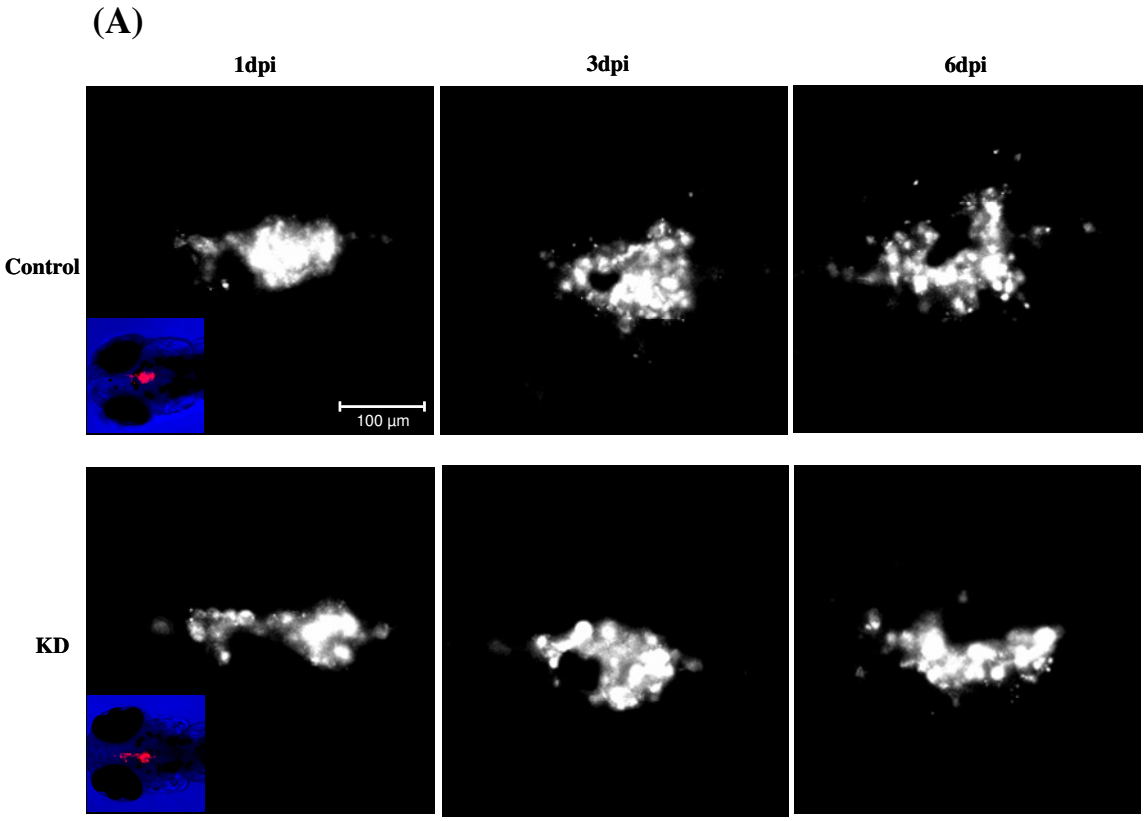
Knockdown of calpain 2 decreases dispersal of glioblastoma cells in zebrafish brain

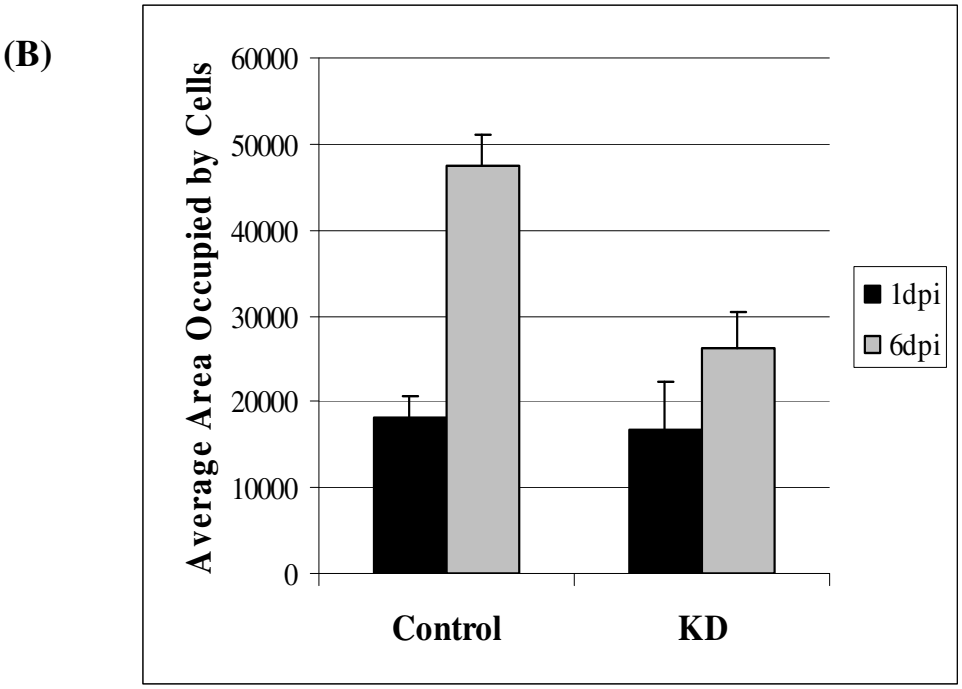
We used zebrafish as the orthotopic model to monitor glioblastoma cell invasion in a living brain. Human tumor cells when transplanted in the zebrafish peritoneal cavity and yolk sac have been shown to survive, proliferate, migrate and induce angiogenesis as observed in mammalian models and during cancer progression. In addition, the early structure and the developmental pattern of the zebrafish brain are very similar to that of human beings, in terms of overall organization of the major brain components, generation, differentiation and connectivity of neurons, and onset of specific signaling mechanisms-for example, Hedgehog pathway, which regulate cell fate specification in neural tube (Tropepe and Sive 2003). The homologous brain microenvironment and ability to image the dispersal of individual cells over time motivated us to use the zebrafish to study glioblastoma cell invasion.

To determine if calpain 2 regulates glioblastoma cell invasion in live brain, 50 to 150 control or calpain 2 knockdown glioblastoma cells were transplanted into the brain of four-day-old fish, the age at which development of brain ventricles and the vascular system is complete in zebrafish (Isogai et al. 2001). The cells were stained with CMDiI, a photostable probe that becomes highly fluorescent after incorporation into the membranes of living cells (Molecular Probes, Invitrogen). After injection, zebrafish were monitored overnight and the healthy and active fish selected to examine the morphology and distribution of injected cells in the brain. Fluorescence

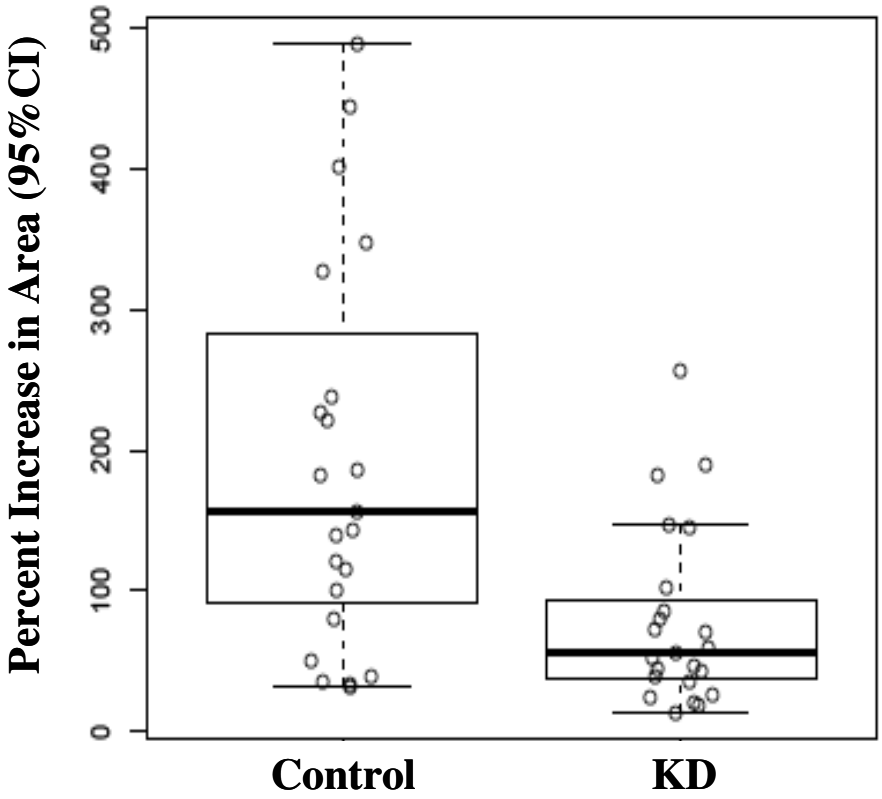
microscopy was used to image the animals for six days post injection (6 dpi), and careful attention was paid to similar alignment of fish on successive days of imaging to be able to correlate the dispersal pattern of cells in the brain tissue. Sixteen hours after injection, the cells appeared as a compact cluster in the midbrain of the zebrafish (Fig 2.2A). By 3 dpi, control cells had started to migrate away from the initial cell cluster into the surrounding tissues, whereas, Knockdown cells remained as a compact cluster and separation of any surface cells was rarely observed. By 6 dpi, dispersal of control cells was extensive with individual cells migrating as far as 450 μm within the brain. In contrast, calpain 2 knockdown cells maintained a loose cluster with little dispersal, mostly in close proximity to the site of injection (Fig 2.2A).

The area occupied by the glioblastoma cells at 1, 3 and 6 dpi was measured as described in Materials and Methods. The dispersal area for control cells increased by 160% over 6 days compared to only 50% increase for the calpain 2 knockdown cells (Fig 2.2B). Regression analysis of the data shows that if the initial area occupied by both cell types is same, knockdown of calpain 2 expression was calculated to decrease the final dispersal area by 39% compared to control cells with the 95% confidence interval between 24 to 47% (two sided p value <0.0002) (Fig 2.2C). To verify that the CMDiI staining was stable during the time course, a fraction of the glioblastoma cells prepared for transplantation into zebrafish was plated in tissue culture dish, maintained under standard *ex-vivo* culture conditions and imaged through one week post labeling. The morphology and spreading of cells appeared healthy with the CMDiI stain

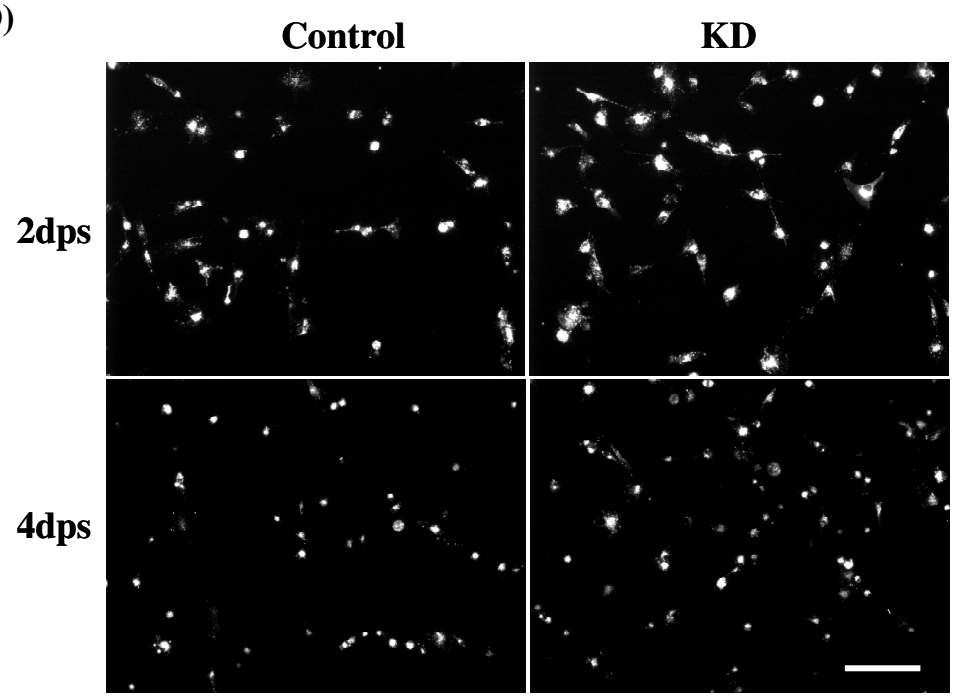

(A)
1dpi
3dpi
6dpi
Control
KD
100 µm


(B)
Average Area Occupied by Cells
60000
50000
40000
30000
20000
10000
0
Control
KD
1dpi
6dpi

(C)


Percent Increase in Area (95% CI)
0
100
200
300
400
500
Control
KD

(D)


Control
KD
2dps
4dps

2.2: Calpain 2 expression is required for dispersal of glioblastoma cells in zebrafish brain microenvironment.

(A) Control and calpain 2 knockdown (KD) glioblastoma cells were labeled with CMDiI dye and microinjected in the brain of zebrafish 4 days post fertilization. Animals were imaged at 1, 3 and 6 days post injection (dpi) with Zeiss axiovert 100 fluorescence microscope to assess the distribution of transplanted cells in the brain. The inset shows the initial position of the cells in the brain at 0 dpi in the overlay of brightfield (colored blue) and fluorescence (red) images. The image is representative of >100 injected fish for each cell line from three separate experiments.

(B) The bar graph represents the average area occupied by control and KD cells at 1 day and 6 dpi (μm^2). The graph is representative of three independent experiments and error bars show SEM. Area of tumor cell dispersal was quantified using distance calibration tool in Metamorph 6.2 as described in materials and methods.

(C) The box plot compares the percent increase in the area occupied by cells from day 1 to day 6. When Initial area is fixed, calpain 2 knockdown decreases the final dispersal area by 39% and the 95% confidence interval (CI) ranges between 24 to 47%. Two sided p value <0.0002.

(D) Comparison of the stability of CMDiI fluorescence of control (2pC) and calpain 2 knockdown (2pR) cells over time. Images were captured at 2 and 4 days post staining (dps). High fluorescence intensity was maintained without any obvious adverse effects on the morphology and spreading of cells. The rounding appearance of cells at 6 dpi is merely due to the more restricted distribution of the stain on the cell membranes. Scale bar= 100µm.

distributed throughout the cellular membrane systems including plasma membrane projections (Fig 2.2D). This result demonstrates that calpain 2 expression is essential for the invasion of glioblastoma cells in a live brain microenvironment.

Human glioblastoma cells disperse primarily along blood vessels in zebrafish brain

In 1940 Scherer suggested that glioma cells do not migrate randomly, but follow certain preferred paths, such as blood vessels or axon fiber tracts in the brain (Scherer 1940). Since then, many reports have improved our understanding of the pattern of glioma invasion based on results inferred from fixed tissues or organotypic tissue culture studies (Bernstein and Woodard 1995; Farin et al. 2006; Guillamo et al. 2001). Nevertheless, until now the biggest limitation has been to visualize the migration of cancer cells in a living brain with functional blood circulation. The easy visualization of the zebrafish vasculature makes it possible to monitor the interaction of cancer cells with the blood vessels during migration. To investigate preferred patterns of dispersal, we transplanted glioblastoma cells, stained with CMDiI dye, into the brain of *Tg(fli1:egfp)* line of zebrafish expressing GFP in the vascular system, and the animals were examined for two weeks post injection using a LSM510 confocal microscope.

By 1 dpi, cells were localized in clusters in the midbrain and appeared to have begun interacting with the blood vessels such as primordial midbrain channel (PMBC)

and anterior cerebral vein (ACeV). By 6 dpi, most of the cells had aligned in small groups along the abluminal surface of blood vessels in proximity and invaded into the surrounding regions in the brain (Fig 2.3). A distinct pattern of preference was noticed where glioblastoma cells demonstrated increased association with the larger blood vessels such as PMBC or ACeV even though the original cell mass at 1 dpi was observed to be surrounded with a meshwork of smaller vessels such as central arteries (Fig 2.3). This may simply have been due to the increased area for attachment on the vessel wall or due to the volume of blood flowing through the channel which provides a rich source of nutrients, oxygen and signaling factors to help stimulate cellular motility, although, the exact reason requires further investigations. After 12 dpi, cells were extensively dispersed and were found at far distances in the brain from the original site of injection. However, we noticed significantly decreased intensity of CMDiI staining of cells, which can be accounted based on the dilution of the dye due to cell proliferation, as well as the dispersal of cells which made weak fluorescence from individual cells difficult to detect (data not shown).

To further dissect the pattern of glioblastoma cell organization along blood vessels, the orthogonal view (Ortho) analysis was performed on the digital sections of the identical region on a blood vessel from the images of the same fish captured on 1 and 6dpi (Fig 2.3). At 1dpi, cells displayed much localized distribution with limited interaction with the surface of blood vessels. By 6 dpi, extensive reorganization of the cell mass around the vessels was observed. The spatial distribution of cells in the z-

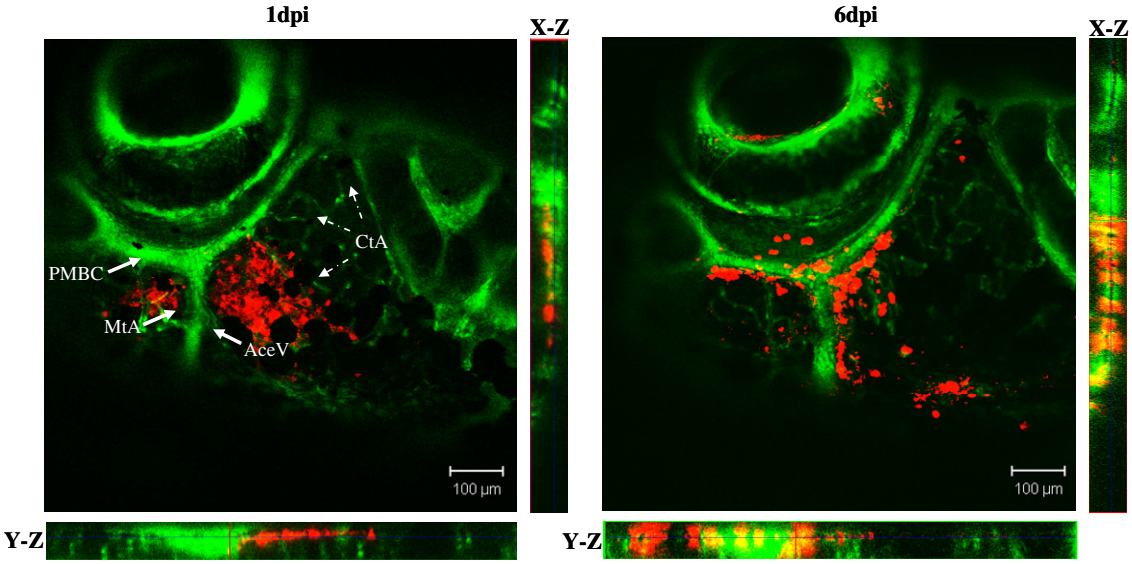


Fig 2.3: Human glioblastoma cells migrate along blood vessels in zebrafish brain.

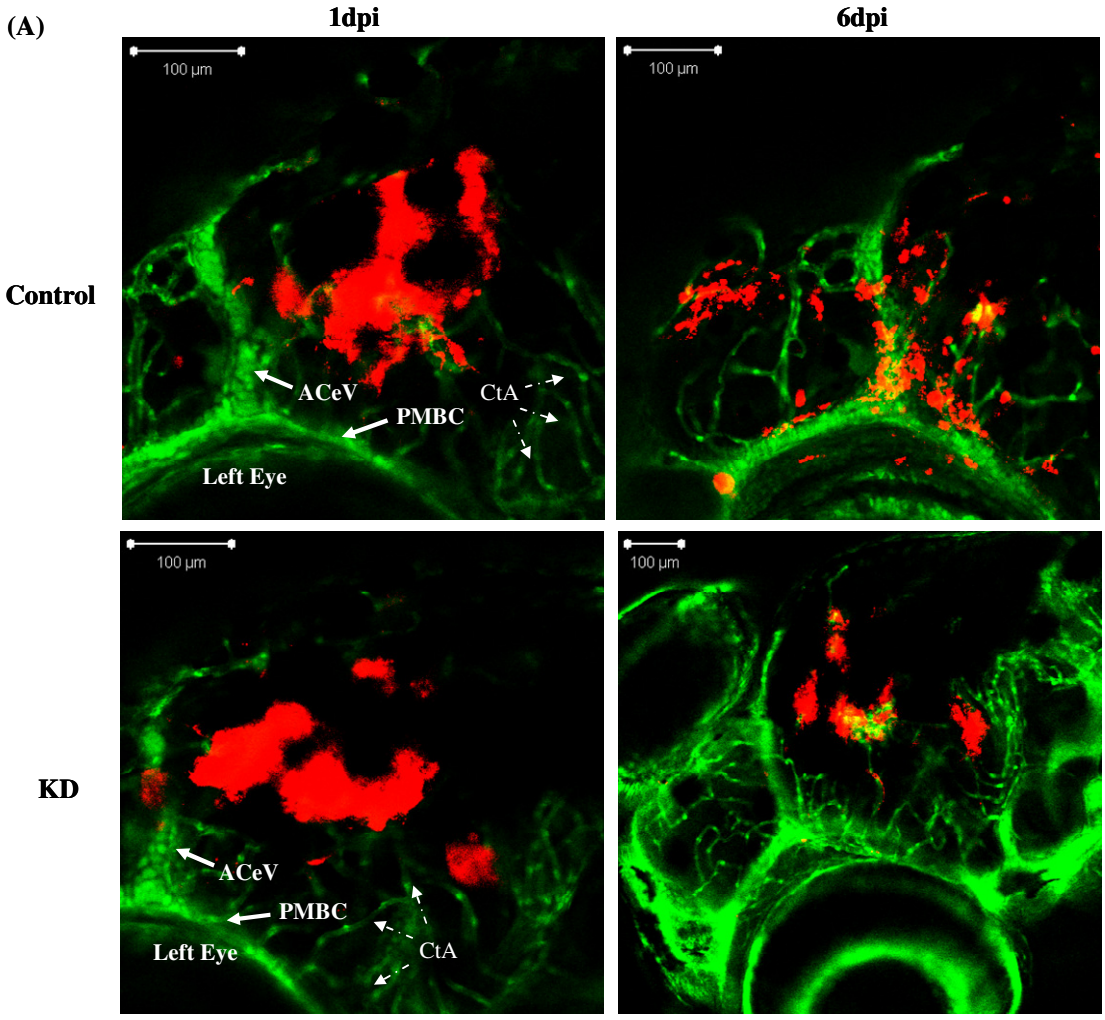
Four days post fertilization, Tg (*fli*1:egfp) zebrafish, expressing GFP in endothelial cells, were microinjected with CMDiI labeled human glioblastoma cells (red). Animals were imaged through 6 days using Zeiss LSM510 confocal microscope. Cells were observed to migrate along the abluminal surface of host's blood vessels (green) as single cells or in small groups. The image represents 60 injected animals. Invasion was assessed in three dimensions by capturing z-stacks at the thickness of 5.6 µm. Ortho analysis of the z-planes was performed to monitor the distribution of migrating cells along the surface of blood vessels. Image in the Y-Z plane shows relative distribution of CMDiI labeled cells along the primordial midbrain channel whereas that in the X-Z plane shows the cells spread on the anterior cerebral vein. The yellow regions refer to close association of cells with the blood vessels. Blood vessel nomenclature; PMBC: primordial midbrain channel, ACeV: Anterior cerebral vein, MtA: metencephalic artery, CtA: central artery.

stack along the primordial midbrain channel over the period of 6 days has been shown in the X-Z plane, whereas the distribution of cells along the anterior cerebral vein has been shown in the Y-Z plane. The pattern of cellular distribution showed that during migration, cells associate closely with the outer surface of the blood vessels as shown by the intense yellow regions along the circumference of vessels at 6dpi.

Holash and colleagues introduced the term "vessel cooption" to describe the process in which tumor cells use the preexisting host vessels to migrate along and fulfill their need of nutrients and oxygen (Holash et al. 1999). To determine if calpain 2 plays a role in vessel cooption, approximately 150 control and calpain 2 knockdown cells were transplanted in the same location in *Tg(fli1:egfp)* zebrafish brain, to expose them to the similar surrounding of blood vessels. By 1 dpi, cells were observed in clusters surrounded by a network of smaller central arteries, and equidistant from the larger blood vessels such as anterior cerebral vein and primordial midbrain channel (Fig 2.4A). By 6 dpi, control cells exhibited a diffuse pattern of infiltration in the brain, and in ~90% of zebrafish, extensive encasing of the anterior cerebral vein and associated vessels with invading cells was observed. In contrast, the transplanted knockdown cells remained confined in clusters at 6 dpi in 75% of the zebrafish. The localization of tumor cells to the blood vessels was quantified by using the histo analysis tool of the LSM image analysis software. At 6 dpi, 29% of control tumor cells were found co-localized with the blood vessels compared to 9.5% at 1 dpi, (paired t-test, $p=0.003$). In contrast, the association of knockdown cells with blood vessels

increased only 6%, from 11% to 17%, over the period of 6 days (paired t-test, p=0.007) (Fig 2.4B). Further, the difference in the co-localization was compared for the set of fish injected with control and knockdown cells. Co-localization with blood vessels was three fold less for the calpain 2 knockdown cells compared to control cells (paired t-test, p=0.02), supporting the conclusion that calpain 2 expression is required for glioblastoma cell invasion in the brain microenvironment of zebrafish.

Glioblastoma cells appeared to migrate predominantly along the blood vessels suggesting that was the preferred path of invasion in the brain, which is consistent with the observation from the rodent orthotopic model of glioma invasion (Guillamo et al. 2001). Glioblastoma cells were not observed outside the brain consistent with previous reports that human glioma cells do not metastasize and have rarely been found in tissues outside the brain, strengthening the validity of using zebrafish as a model for glioblastoma cell invasion. In summary, we report that human glioblastoma cells injected into the zebrafish brain microenvironment survive and preferentially disperse along blood vessels during invasion of the surrounding brain tissue.


(A)
1dpi
6dpi
Control
KD
100 µm
ACeV
PMBC
CtA
Left Eye

(B)

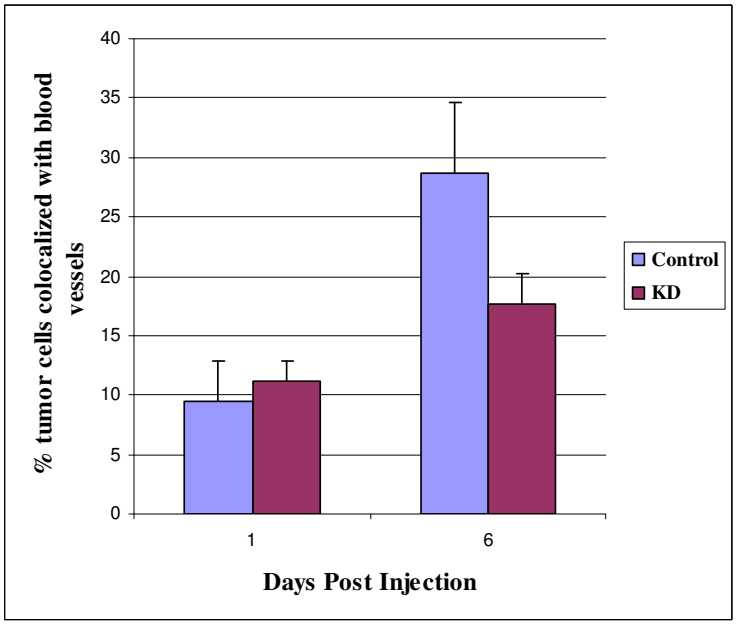


Fig 2.4: Decreased association with blood vessels shows that knockdown of calpain 2 inhibits dispersal of glioblastoma cells.

Control and calpain 2 knockdown (KD) human glioblastoma cells were transplanted in the brain of 4 dpf Tg(*fli*1:egfp) zebrafish. Fish were imaged for 6 dpi using Zeiss LSM510 confocal microscope. Animals were aligned identically while imaging on successive days to visualize same blood vessels.

(A) Control cells extensively infiltrated the surrounding brain by migrating long distances as single cells or in small groups primarily along blood vessels. In contrast, calpain 2 knockdown cells remained confined in clusters. The yellow regions refer to close association of cells (red) with blood vessels (green). Images are representative of 30 animals injected with each cell line. Blood vessel nomenclature; PMBC: primordial midbrain channel, ACeV: Anterior cerebral vein, CtA: central artery.

(B) Colocalization of glioblastoma cells with blood vessels in the zebrafish brain was calculated using image analysis tool on LSM510 confocal microscope. When compared with the starting localization of tumor cells with blood vessels at 1 dpi, control tumor cells exhibited 3-fold increased association with blood vessels at 6 dpi ($p=0.003$) in contrast to only 1.5-fold increase in the colocalization of calpain 2 knockdown cells ($p=0.007$). The error bar shows standard error of mean.

Knockdown of calpain 2 attenuates tumor cell angiogenesis in zebrafish brain

Angiogenesis is defined as the elongation of new capillaries or the branching of existing blood vessels (Holash et al. 1999; Vajkoczy et al. 2002). Angiogenesis and the degradation of extracellular matrix by MMPs are two well-studied phenomena associated with the invasion and metastasis of cancer cells (Fischer et al. 2005; Gagner et al. 2005; Nakada et al. 2003). Our lab reported 36% lower levels of active MMP2 in the surrounding media of calpain 2 knockdown cells than in control glioblastoma cells which correlated with 90% reduction in invasion of knockdown cells through Matrigel matrix (Jang et al. 2010). Further, knockdown of calpain 2 was shown to inhibit the VEGF induced angiogenesis of pulmonary microvascular endothelial cells *in vivo* (Su et al. 2006). However, the result was inferred indirectly from the amount of hemoglobin content observed in the Matrigel plug grafted subcutaneously in the mouse skin. The ability to clearly visualize the blood vessels in transparent vessels in *Tg(fli1:egfp)* zebrafish provided us with the advantage to better determine the reorganization of existing vessels or formation of new blood vessels, before and after the transplantation of cancer cells. We asked if calpain 2 regulates tumor cell angiogenesis to enhance the invasion of glioblastoma cells.

The organization of blood vessels, in z-stacks and the 3-D reconstructed images, was compared for each zebrafish on 1 and 6 dpi. Twenty-three percent of the fish injected with control glioblastoma cells demonstrated clear evidence of

angiogenesis and the newly formed vessels were densely surrounded with tumor cells (Fig 2.5). Another 20% of control cells injected fish displayed vessel reorganization, marked by the spatially altered alignment of preexisting vessels presumably caused by the invading cells. In contrast, only 9% of fish injected with calpain 2 knockdown cells showed low levels of angiogenesis where short new vessels appeared to form close to many preexisting vessels (Fig 2.5). Furthermore, the number of animals showing spatial reorganization of existing vessels around tumor cells was also less (approximately 10%) than control cells.

According to previous studies, an increase in the number of invading cells along the perivascular surfaces was observed when anti-angiogenic treatments were applied (Lamszus et al. 2003). We also observed that tumor cells invaded long distance (up to 450 μm) in the brain along blood vessels without showing significant remodeling of associated vessels or formation of new vessels. Increased angiogenesis was demonstrated by cells that either remained in clusters or migrated short distances in local vicinity. Together, our data suggests that calpain 2 plays a role in the process of angiogenesis, however, consistent with previous reports, angiogenesis was not observed as the essential requirement for glioblastoma cell invasion in the brain.

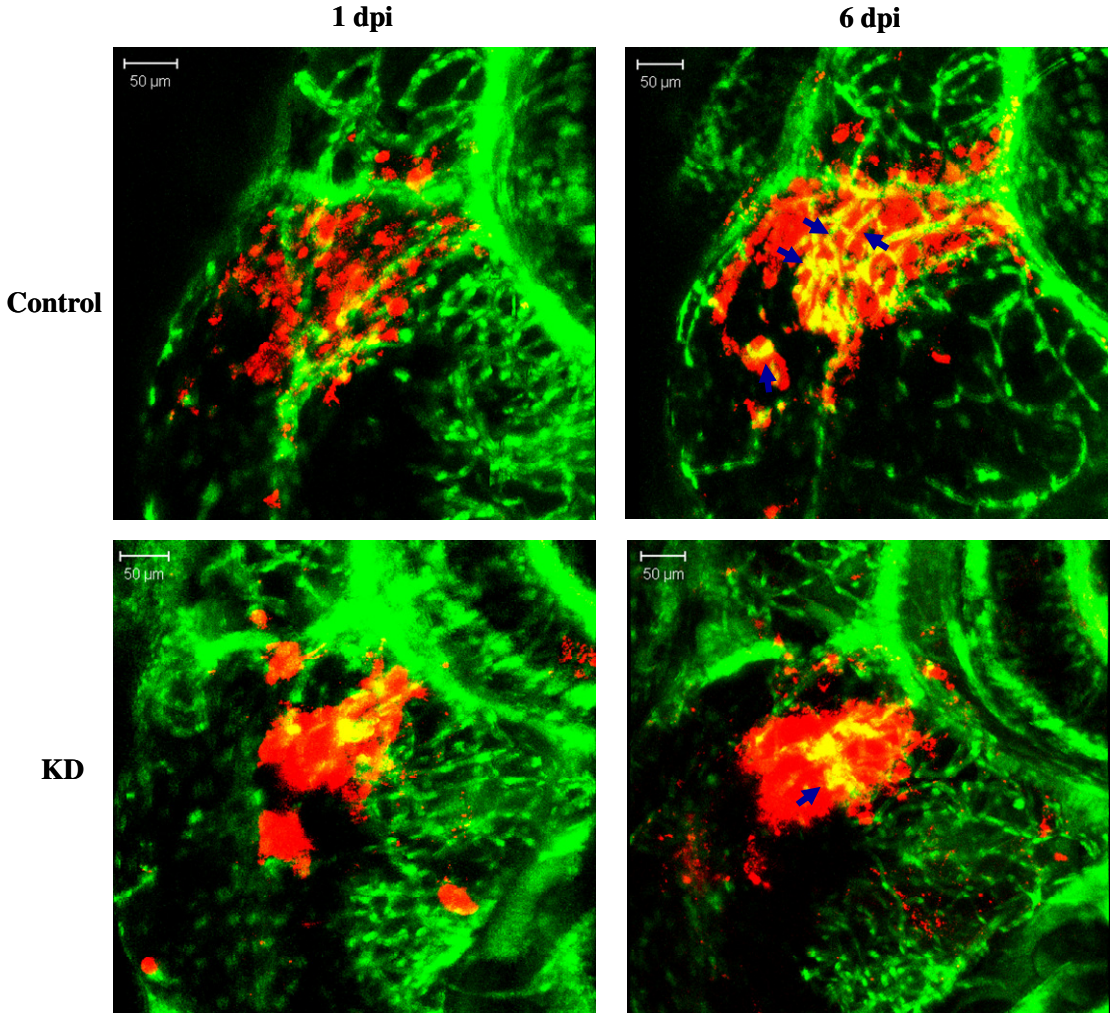

1 dpi
6 dpi
Control
KD
50 µm
50 µm
50 µm
50 µm

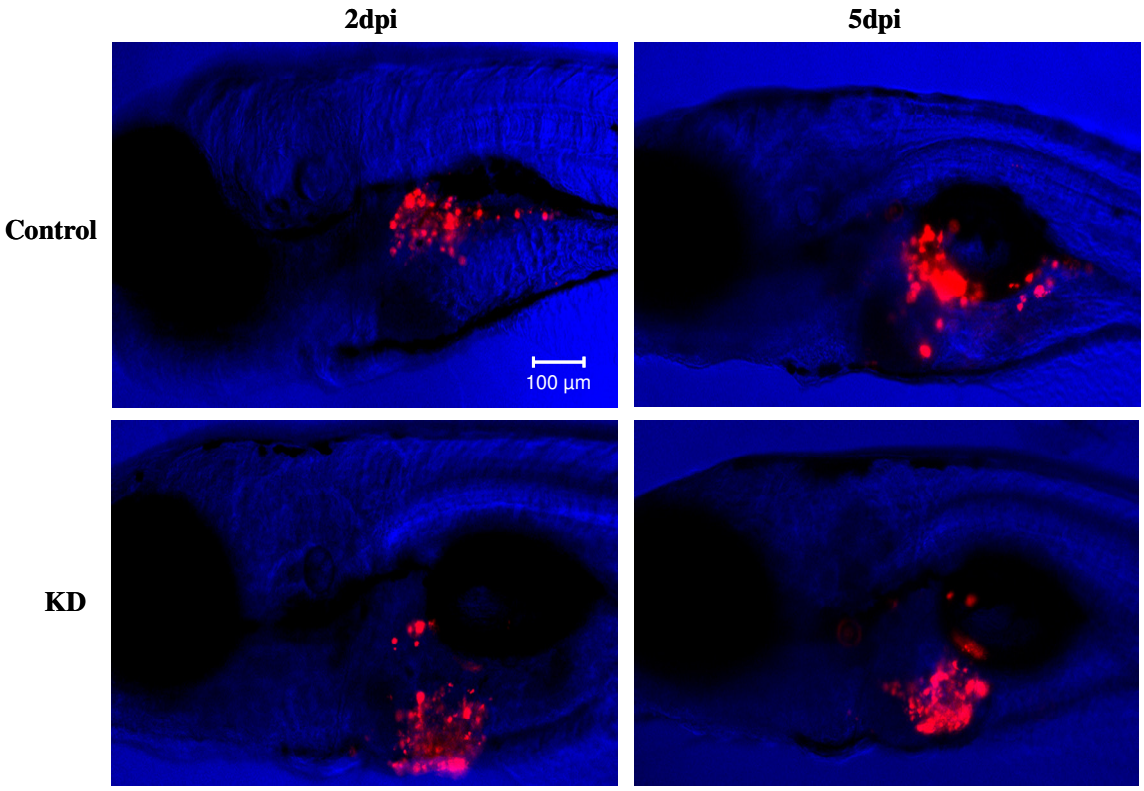
Fig 2.5: Knockdown of calpain 2 attenuates tumor cell angiogenesis in glioblastoma.

Tg(*fli*1:egfp) zebrafish, 14 dpf, transplanted with control (top panel) and calpain 2 knockdown (bottom panel) human glioblastoma cells were imaged for 6 days post injection using a LSM510 confocal microscope. Three-dimensional reconstruction of tumor cells invading the brain of zebrafish at 1 dpi and 6 dpi shows blood vessels in green and tumor cells in red. The newly formed blood vessels at 6 dpi are shown with blue arrows. Images were obtained from the same animal on consecutive days. Images demonstrate the frequency of angiogenesis in control and calpain 2 knockdown (KD) cells.

Glioblastoma cells do not invade in the yolk sac of zebrafish

The interaction of cells with the extracellular matrix is an important regulator of cell motility (Ulrich et al. 2009). The density and composition of extracellular matrix components is characteristically defined for different tissues and organs. Recently, the human melanoma cells microinjected in the yolk sac of zebrafish were shown to proliferate and migrated into the surrounding organs including intestine, pancreas and liver (Haldi et al. 2006). To determine the effect of extracellular matrix density on glioblastoma cell invasion in live animals, ~100 control and calpain 2 knockdown cells were microinjected in the yolk sac of 4dpf zebrafish. Both control and knockdown cells failed to demonstrate any significant extent of migration or invasion in spite of the nutrient rich environment of the yolk sac (Fig 2.6A). Although cells showed localized spread within the yolk sac boundary, no trace of their invasion in the surrounding visceral organs outside the yolk sac was noticed. The increase in the average area occupied by transplanted cells after 5 dpi was minimal for both control and calpain 2 knockdown glioblastoma cells (Fig 2.6B). This indicates that calpain 2 expression does not drive glioblastoma cell invasion in a non-brain microenvironment. This could be partly explained on the basis of the lack of rich vascular networks in the yolk sac. The complexity of the yolk vascular system is negligible compared to the brain, with only right and left supraintestinal vein (SIV) remaining after 4 days post fertilization (dpf). These also degenerate by 7 to 8 dpf

(A)



(B)

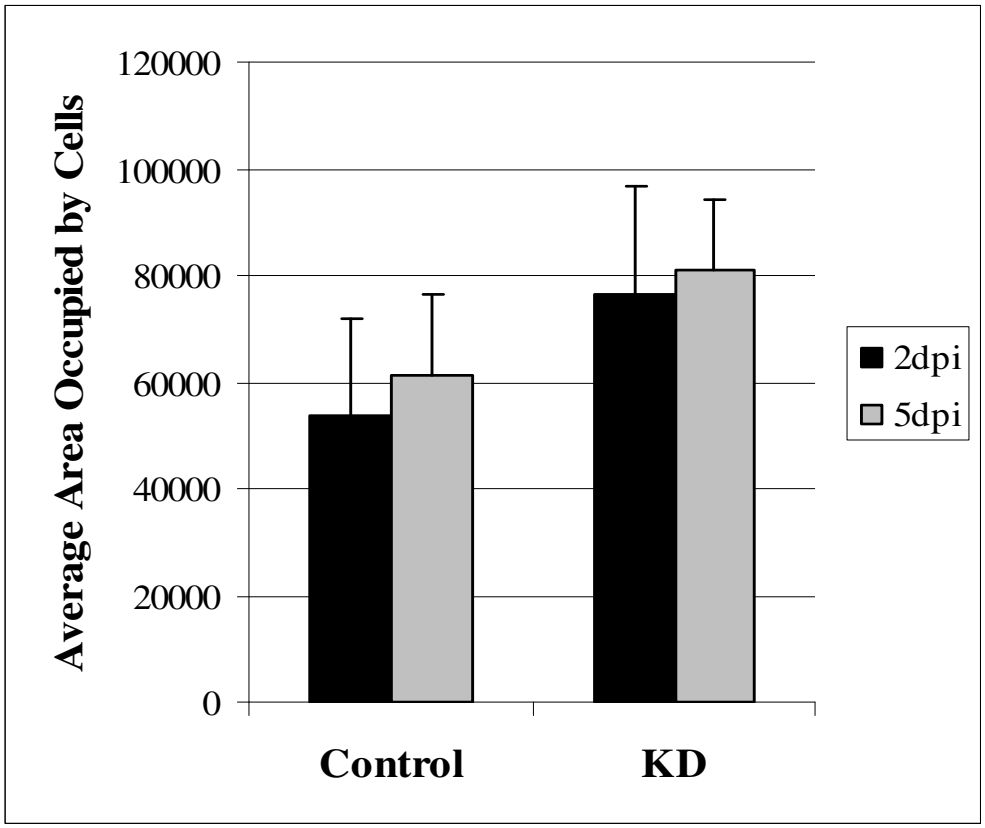


Fig 2.6: Human glioblastoma cells do not invade in the yolk sac of zebrafish.

(A) CMDiI labeled control and calpain 2 knockdown cells (KD) were microinjected in the yolksac of zebrafish at 4 dpf. Animals were imaged for 5 consecutive days using Zeiss axiovert fluorescence microscope as described in materials and methods. Images were captured with brightfield (colored blue) and with rhodamine fluorescence filter in place (red). Cells demonstrated negligible tendency of invasion and remained confined into the yolk sac. The images are representative of 40 injected fish.

(B) The area occupied by cells (μm^2) at day 2 and 5 post injection were quantified as described in materials and
methods. n=3; error bars represent standard error of mean.

when yolk is completely consumed. Since, our results have indicated that calpain 2 regulates the invasion of glioblastoma cells primarily along blood vessels, the lack of vessels and hence the basement membrane of the vascular wall presumably presents a tough challenge for the migration of glioblastoma cells.

Calpain 2 is required for glioblastoma cell invasion in organotypic mouse brain slices

Considering the differences between the complex microenvironment between mammalian and zebrafish brain such as lack of cerebral hemispheres in zebrafish, it is important to verify the requirement of calpain 2 for glioblastoma cell invasion in a mammalian brain environment. 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 control and calpain 2 knockdown glioblastoma cells were transplanted in 400µm thick sections of brain tissue in culture and tumor cell invasion was examined for 5 dpi with a LSM510 confocal microscope. Both control and knockdown cells demonstrated rapid invasion in the tissue, however, by 5 dpi, knockdown cells showed decreased area of dispersal compared to control cells (Fig 2.7). The area occupied by control cells progressively increased from 1.22mm^2 (0 dpi) to 5.68mm^2 (5 dpi) in the z-slice showing maximum diffusion of cells. In contrast, the area occupied by calpain 2 knockdown cells

increased from 1.29mm^2 at 0 dpi to 2.97mm^2 at 5 dpi. In essence, compared to 365.5% increase in the area of dispersal for control cells, knockdown cells demonstrated only 130% increase in the area of dispersal after 5 days of injection. This is comparable to the difference of the area of dispersal between control and calpain 2 knockdown cells observed in the zebrafish brain.

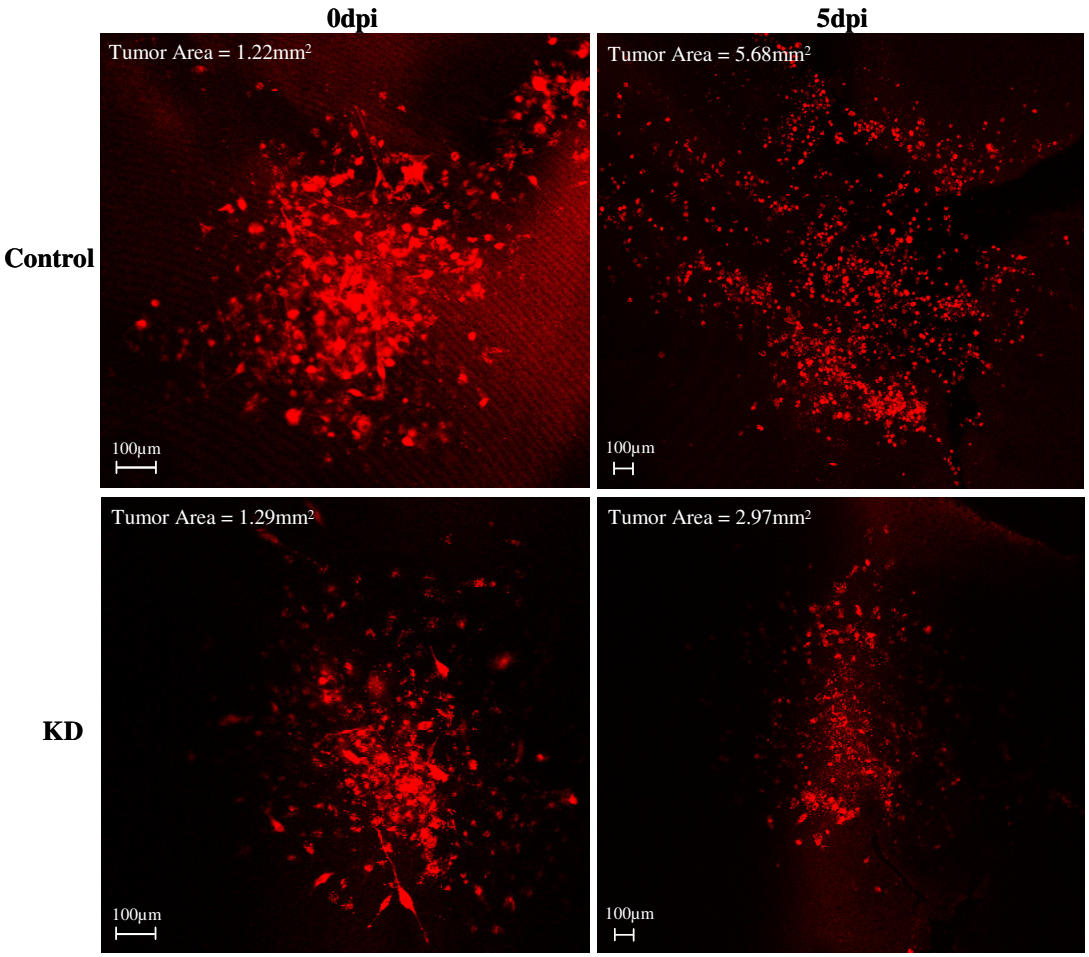


Fig 2.7: Knockdown of calpain 2 inhibits glioblastoma cell invasion in organotypic mouse brain slices.

Control and calpain 2 knockdown (KD) glioblastoma cells were stained with CMDiI and 10,000 cells were placed in the top layer of brain tissue slices obtained from 62 days old mice and maintained in culture as described in the Materials and Methods. The slices were imaged for 5 successive days using LSM510 confocal microscope to monitor the invading tumor cells. The area of dispersal (mm^2) was measured using the LSM image analysis software. The image is representative of 10 slices for each cell lines obtained from two different mice. The images at 6 dpi were captured using tile scanning method to cover the wide area of dispersal of tumor cells.

Conclusion

Glioblastoma is a devastating diagnosis with a median survival span of approximately one year for patients (Suzuki et al. 2010). The transformed cells acquire unusual migration ability leading to the diffuse invasion in the brain tissues. However, the inability of these cells to penetrate the blood vessel's wall (Bernstein and Woodard 1995) has instigated much interest in finding whether glioma cells are deficient in certain motility factors or proteolytic enzymes that would normally enable tumor cells to intravasate the blood vessels.

This study shows that the knockdown of calpain 2 was significantly effective to restrict the dispersal of tumor cells away from the original site of injection in the zebrafish brain. Control glioblastoma cells rapidly aligned along the nearby blood vessels and migrated up to 450µm into the brain within 6 dpi. In contrast, the majority of the knockdown cells remained confined as focal mass and infrequent cases of migration to a maximum of 250µm were recorded. This observation is important because inhibition of the tumor dispersal is the primary requirement for the effective surgical resection of the tumor.

Consistent with previous reports, we did not find any clear indication of glioblastoma cell penetration into the blood vessels; however, the ortho analysis of the tumor cells distribution along the blood vessels in the x-z and the y-z plane showed that cells closely associate with the outer surface of blood vessels during migration. Angiogenesis is considered as an important aspect of tumor progression and invasion.

The ability to visually monitor the reorganization of the existing vessels or angiogenesis induced by tumor cells motivated us to use the transgenic *Tg(fli1:egfp)* zebrafish. Although we observed that knockdown of calpain 2 decreased the frequency of angiogenesis, however, tumor cells preferably migrated along the surface of host's blood vessels. The decreased rate of angiogenesis in knockdown cells compared to control cells could possibly be due to the down regulation of the secretion of the angiogenic factor VEGF, but the definitive conclusion requires further investigations. The comparable results of glioblastoma cell invasion between zebrafish brain and organotypic mouse brain slices strengthens the validity of using zebrafish as an orthotopic model for brain tumor cell invasion and angiogenesis studies. CMDiI staining of cells has a great advantage in that it can be readily used for any type of cells without any adverse effect on the morphology or interaction of cells with the extracellular matrix. However, the dilution of fluorescence intensity over time and weak fluorescence intensity from scattered individual cells are the biggest limitation. This prohibited the monitoring of invading tumor cells or the remodeling of blood vessels in the brain for extended time. The efforts are underway in our lab to prepare glioblastoma cells with alternative markers, for example, stable expression of GFP.

Calpain 2 has been implicated in different invasion-related processes such as regulation of MMPs and formation of invadopodial projections in different types of cancer cells; however, besides modulating extracellular matrix, as above processes are involved in, a necessary feature required for the efficient invasion of tumor cells is the

flexibility of the membrane and deformability of the cell shape that enables the cell to cruise through the narrow pores of extracellular matrix. 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. Cell migration in the three dimensional matrix is highly regulated by the orthogonal networks of actin filaments at the cell periphery and filamin is the only known actin-associated protein that cross-links branched actin filaments into orthogonal networks. We propose that the regulated proteolysis of filamin by calpain is a potential mechanism for regulating glioblastoma cell invasion which needs to be verified.

Materials and Methods

Reagents

DMEM media and Trypsin/EDTA was from Mediatech, Inc. (Manassas, VA). L-glutamine, Geneticin, D-PBS and CMDiI were purchased from Invitrogen (Eugene, OR). FBS was purchased from Sigma (St. Louis, MO). Matrigel was from BD Biosciences (Bedford, MA) and transwell permeable support (6.5mm diameter, 8.0µm pore size) was purchased from Corning (Corning, NY). CIM-16 plate was ordered from Roche Applied Sciences. FuGENE HD was purchased from Roche (Indianapolis).

Cell culture and labeling

Human U87MG glioblastoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM media containing 1% L-glutamine and 10% fetal bovine serum (FBS) at 37°C and the standard conditions of 100% humidity, 95% air and 5% CO_2. Control and calpain 2-specific shRNA was purchased from SA Biosciences (Frederick, MD). After transfection with the shRNA plasmid using FuGENE HD, glioblastoma cells with stable incorporation of control and calpain 2 specific shRNA in the genome were selected with 600µg/ml Geneticin and maintained in 400µg/ml Geneticin. The nucleotide sequence of calpain 2-specific shRNA was 5'-GGGCTGAAGGAGTTCTACATT-3' and the control shRNA was 5'-

GGAATCTCATTCGATGCATAC-3'. For transplantation in the zebrafish brain and mouse brain tissues, glioblastoma cells were grown to 80-90% confluency and labeled with cell tracker CMDiI dye from Invitrogen (Eugene, OR). The acronym CMDiI stands for the carboxymethylbenzamido (CM) derivative of the dialkylcarbocyanine (DiI) fluorescent probes. This is an amphiphilic molecule that comprises a charged fluorophore that localizes the probe at the membrane's surface and 18 carbon long lipophilic aliphatic tail that inserts into the membrane bilayer and thus anchors the probe to the membrane. The excitation and emission wavelength of CMDiI is 553nm and 570nm respectively with the molar extinction coefficient of 134000 $cm^{-1}M^{-1}$. Labeling of cells was performed according to manufacturer's protocol. A vial of CMDiI containing 50µg lyophilized powder was thawed at room temperature (RT) for 10 min and suspended in 50µl sterile dimethyl sulphoxide (DMSO) in the cell culture hood to prepare the stock (1 mg/ml). Immediately before labeling, the final working solution of 2µM was prepared by diluting 30µl of 1mg/ml stock in 15 ml filter sterile Dulbecco's phosphate buffered saline (D-PBS) from Invitrogen. Cells were washed once with sterile DPBS and 7.5 ml of 2 µM dye solution was added to uniformly cover the layer of ~90% confluent adherent cells in a 75 cm^2 flask. Cells were incubated at 37°C for 5 min and the staining was continued for additional 15 min at 4°C which enhances the incorporation of the probe in the plasma membrane and slows down endocytosis reducing dye localization into cytoplasmic vesicles. Post staining, cells had rounded up but were attached to the plastic surface. Cells were harvested using 0.8

ml trypsin-EDTA and collected by centrifugation at 1200 rpm for 2 min. The cell pellet was recovered in 5 ml media to make single cell suspension before counting using hemocytometer. The final concentration of 5cells/nl was prepared for transplantation experiments. During entire period of transplantation, stained cell suspension was maintained at 37°C.

Real-Time invasion assay

The real time monitoring of glioblastoma cell invasion was performed using xCELLigence from Roche Applied Sciences. This system used a specialized transwell apparatus CIM plate 16 (Cell Invasion and Migration) and the RTCA DP (Real Time Cell Analyzer-Dual Plate) instrument for performing electrical measurements. The lower side of the upper chamber of CIM plate contains an integrated microelectrode sensor made of gold coating which measures real time changes in the electrical impedance and provides quantitative information about the number of cell, cell viability and quality of cell attachment to the membrane. In this study, three different concentrations of Matrigel were used in the upper chamber of each well to make the extracellular matrix of varying density. Since Matrigel polymerizes at RT, the tools for Matrigel handling which included Matrigel, upper chamber of CIM plate, wide bore pipette tips, tubes and serum free media, were precooled at 4°C for one day before the experiment. The assay was performed according to manufacturer's protocol. Twenty microliter of Matrigel diluted in serum free media at concentrations 0.8mg/ml,

0.4mg/ml and 0.2mg/ml were coated in the upper chamber of the transwell and polymerized at 37°C for 4hrs. After polymerization, media was added to the lower chamber and it was assembled with the upper chamber to allow the microelectrode membrane surface to reach equilibrium. Cells were harvested with trypsin-EDTA and finally suspended in serum free media. Immediately before adding cells, the background impedance measurement of the media was recorded. Fifty thousand cells were added to the upper chamber of each well and the lower chamber contained DMEM media with or without FBS as chemoattractant. Cells were allowed to settle down for 30 – 45 min at RT and the CIM plate assembly was placed in the RTCA DP analyzer to record the electrical impedance of the membrane for 20 hrs at intervals of 15 min. The invasion data was analyzed using the RTCA software. The relative change in the electrical impedance between measurement at any time (t) and the background value (t_0) is expressed as Cell Index (CI) for each well. Therefore, CI reflects the number of cells reaching to the membrane and the quality of cell attachment with the membrane. The slope of cell index curve was calculated for each well between the time points of 50 min (the period between background check and initial invasion measurement) and 20 hr when the curves approached saturation and the experiment was ended. The slope describes the changing rate of cell index value and is correlated with rate of cell invasion. The standard deviation is calculated for the cell index values for each individual well and is represented as the error bar on the slope graph.

Zebrafish handling and cancer cell transplantation

Zebrafish 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 (*Danio rerio*) were kept at standard laboratory conditions of 28°C on a 14 hr light/10hr dark photoperiod in fish water consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®). Adult zebrafish were group spawned and embryos were collected and staged as described (Kimmel et al. 1995). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC). For the glioblastoma cell dispersal assay, tropical 5D zebrafish embryos were xenotransplanted at 4 dpf (Fig 2) whereas Tg(fli1:egfp) fish were transplanted with glioblastoma cells at 14 dpf (Fig 3,4,5) and 4dpf (Fig 6). Glioblastoma cells in culture were labeled with CMDiI dye as described above and suspended in media at $5x10^6$cells/ml. Zebrafish were anesthetized with 0.004% Tricaine in fish water and oriented in a small drop on a dry 2% agarose plate so that the dorsal surface of the head is exposed facing up to allow injecting cells into the brain. The needles for injection were prepared by pulling a borosilicate glass micropipette with internal diameter of 0.5mm and external diameter 1.4mm using a glass micropipette puller (Sutter Instrument Co., Novato, CA). The cell suspension (20µl) was mixed with phenol red (2µl) and the mixture (8 µl) was loaded in the needle which was placed on the ASI MPP1-2 air driven pressure injector. Cells were injected at 30psi with adjusted pulse durations to inject 50-200 cells. Post injection,

fish were immediately transferred to fish water in a separate container. After overnight recovery from injection, fish were imaged for the distribution and morphology of injected cells in the brain and the selected animals were transferred to individual wells of a 6-well plate containing 10 ml fish water. Animals were maintained at 29°C and starting at 5 days post fertilization (dpf), fish were kept on 3 times feeding schedule per day with the flake food developed at SARL.

Organotypic brain slice invasion assay

Brain tissue slices were obtained from 9-week-old B6SJL female mouse generously provided by Dr. Joseph Beckman's lab, Oregon State University. Animals were anesthetized with small amount of isoflurane and the brain was immediately obtained by decapitation. After washing the brain 2 times in 4% low melting agarose solution in PBS (pH 7.4), it was places in a container with buffered agarose solution to make a solid block. The block was attached to the stage of the vibratome (Leica VT 1000S) using glue and the cerebrum was further supported by adding molten 4% agarose solution around the base of the glued block on the stage. The 400-µm-thick horizontal slices were prepared starting from the anterior end of the brain reaching the posterior end with the vibratome set at frequency of 85 Hz and the blade speed at 0.2 mm/sec. The tissue sections were transferred to the six-well plate containing 500µl 10%FBS-DMEM supplemented with 600 µg/ml Geneticin and 2x Pennicilin/Streptomycin antibiotics. The brain slice culture was incubated at 37°C

under standard conditions of 100% humidity, 95% air and 5% CO_2. Before the transplantation of glioblastoma cells, the tissue sections were transferred to the upper chamber of a transwell cell culture insert (0.4µm pore size, six-well format) from BD Biosciences. Next, 500µl of medium was added to the upper chamber and 1.5 ml to the lower chamber.

Control and calpain 2 knockdown glioblastoma cells were stained with CMDiI dye and suspended to the final concentration of 10^7cells/ml in 10%FBS media as described above in this section. A 10µl Hamilton syringe was used for the transplantation of cells in each slice. Before placing cells on the brain slice, the media from the upper chamber of the transwell insert was aspirated to make the upper surface of the tissue semi-dry. This is important to avoid the dispersion of cells over the slice. Next, 10^4 cells were gently placed on top of the tissue in 1 µl transfer volume in 1-2 min and tissues were left undisturbed for 10 min to allow settling of the cells in the tissue followed by addition of 500µl media. Images were captured with a LSM510 confocal microscope 3 hrs post injection to record the initial distribution of cells on the tissue slices. Images were further captured on 3dpi and 5dpi to monitor the invasion of cells in the brain tissue. Area of the tumor cell dispersal was measured using LSM510 software.

Microscopy and data analysis

For the images shown in fig 2.2 and 2.6, zebrafish were anesthetized with 0.004% tricaine in fish water and imaged using Zeiss Axiovert 100 fluorescence microscope with a 10x objective lens and the CCD camera at zoom 0.4. Since CMDiI dye dilutes with each cell division, the exposure time was adjusted to capture all the labeled cells on different days of imaging. The area of tumor cell dispersal was measured at 1, 3 and 6dpi using the distance calibration tool of MetaMorph 6.2 image analysis software. To measure the initial area post injection, the fish which had received localized cell mass injection with minimal scattering were chosen to allow the better monitoring of cell dispersal over time. The fluorescent staining in the brain with a minimum diameter of 10µm were considered as a single cell and this criterion was based on the diameter of a round stained cell measured under cultured condition as shown in fig 2.2D. Also, the zebrafish which failed to orient identically on successive days of imaging were excluded from quantification to avoid any misrepresentation of the area occupied by cells. The images presented in fig 2.3, 2.4, 2.5 and 2.7 were captured using a LSM510 confocal microscope with 10x objective lens. Multi-track configuration was chosen for the image acquisition and HeNe1 and Argon lasers were used to excite CMDiI at wavelengths 543nm and GFP at 488nm respectively. Fish were anesthetized using 0.004 - 0.006% tricaine for imaging.

For the graph shown in fig 2.4B, the association of tumor cells with blood vessels was quantified using co-localization measurement tool of the LSM image

analysis software. Quantification was performed on approximately 15 individual slices from the z-stacks (ranging from 60-150μm in different fish) showing clearly visible cells, to measure the co-localization coefficient which represents the ratio of colocalized pixels to the total pixels in red channel, representing tumor cells. The value from individual slices were averaged to determine the overall percentage of tumor cells co-localized with blood vessels for control and calpain 2 knockdown cells at 1 and 6 dpi. Identical threshold intensity was applied for all the calculations to avoid background pixels. Student's paired t-test was performed for the statistical analysis of the data.

Acknowledgements

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PI3 kinase activity is required for filamin proteolysis by calpain 2 in glioblastoma cells

Chapter 3

Abstract

Filamin A (filamin) is the primary actin cross-linking protein which produces orthogonal networks of actin filaments at cell periphery. With more than 70 binding partners (Popowicz et al. 2006), the role of filamin as the dynamic scaffold protein has received much attention in regulating many cellular processes. Filamin has been implicated as a requirement of cell migration but the contradictory evidences presented by many groups in recent years have opened the case of 'filamin in cell migration and invasion' for debate and further investigations (Li et al. 2010).

Excessive infiltration of glioblastoma cells into the brain tissue has stimulated research to determine the mechanisms regulating glioblastoma cell migration and invasion. In this study, we report that filamin expression suppresses the invasion of glioblastoma cells. Knocking down filamin demonstrated 3-fold increase in the invasion of glioblastoma cells compared to control cells. We asked the question that with this restrictive nature of filamin, how glioblastoma cells invade in the brain microenvironment. The cleavage of filamin protein would be a mechanism to regulate the cyclic turnover of actin networks promoting cell migration. With this hypothesis, we studied calpain 2 proteolysis of filamin and the results show that PI3 kinase regulates filamin proteolysis by calpain 2 in glioblastoma cells. This also provides a link to the possible mechanisms for calpain 2 regulation of glioblastoma invasion. The results from proteolysis assays revealed that the binding of PtdIns (3,4,5)-P_3 to filamin stimulates calpain 2 cleavage at a novel susceptible site after actin binding domain

which remained undetected in the literature. Cleavage at this site has the potential to regulate the function of filamin during cell migration and invasion.

Introduction

Cell migration, the limiting step for tumor invasion, is an actin dependent process and involves an orchestrated series of events which includes extension of membrane protrusions, stable attachments with the extracellular matrix and translocation of the cell body aided by tail retraction (Pollard and Borisy 2003; Vicente-Manzanares et al. 2005). Actin filaments are organized into short bundles, long stress fibers and three dimensional networks by actin binding proteins. Many of these proteins that facilitate polymerization, depolymerization, branching, bundling and cross-linking of actin filaments have been implicated in controlling migration of a cell (Ayscough 1998). Filamin (FLN) make up one important family of actin cross-linking proteins comprised of three highly conserved isoforms, FLNa, FLNb and FLNc in mammalian system (Gorlin et al. 1990; Hartwig and DeSisto 1991).

Filamin is a dimeric protein in which the dimerization domain at the carboxy terminus is separated from the actin binding domain at the amino terminus by 23 repeat units. Filamin cross-links actin filaments branched at higher angles (>70°) into the orthogonal networks at the leading edge of migrating cells (Stossel et al. 2001). These networks primarily provide the mechanical and elastic strength required for membrane protrusions and deformation resistance against the cytoplasmic sheer forces generated during migration (Tseng et al. 2004). Filamin binds to more than 70 partners, including transmembrane receptors and signaling proteins, and assembles them in proximity to each other, facilitating enhanced signal transduction in the cell

(Zhou et al. 2007). These functions are crucial for cell migration in response to microenvironmental stimuli.

Although filamin was long held as a requirement for cell migration, the contradictory evidences presented by many research groups have opened the role of filamin in cell migration for debate and further investigations. Cunningham et al first showed that melanoma cells lacking filamin expression have impaired migration, which was rescued by the re-expression of filamin protein (Cunningham et al. 1992). In the same study, they also noticed that the increased expression of filamin resulted in the inhibition of migration rather than stimulation. Neuronal cells expressing a dysfunctional mutant filamin fail to migrate to the cortical region of the brain leading to ventricular nodule formations and the disease periventricular heterotopia (PVH) (Fox et al. 1998). However, duplication of the filamin gene, instead of loss of function mutation, was identified in a male patient with severe PVH (Fink et al. 1997). A filamin interacting protein FILIP was reported to control neuronal migration by regulating filamin levels (Nagano et al. 2002). Further, strong binding of filamin with β-integrins in the cell membrane resulted in decreased cell migration (Calderwood et al. 2001). Recently, Baldassarre et al proposed a partial role of filamin in cell migration, reporting that filamin is needed to initiate migration but not for the maintenance of migration once started (Baldassarre et al. 2009). Together, these mixed reports suggest that cell migration is impaired by both complete absence as well as

over-expression of filamin protein, which indicates a delicate regulation of filamin function in the process of migration.

During migration in a three dimensional space (3D), the cell body needs to modify its shape and stiffness when moving through the extracellular matrix, the assembly of focal adhesive interactions is less-complete and the migration is mostly sustained by a predominantly cortical actin cytoskeleton (Friedl and Wolf 2003). The lack of filamin would result in the absence of actin orthogonal networks which provides elasticity and mechanical strength to the membrane, and on the other hand, overexpression of filamin would lead to overly stabilized actin orthogonal networks and cell-ECM attachments causing compromised flexibility and reduced detachment needed for translocation of the cell body. Therefore, a balance in the form of cyclic turnover of actin networks and cell-ECM attachments is essential for productive cell migration which can be facilitated by the regulated proteolysis of filamin in migrating cells.

Filamin has been shown to be cleaved by calpain, a calcium-activated cysteine protease which catalyzes proteolysis of many cytoskeletal proteins involved in cell migration (ref). The first evidence of calpain proteolysis of filamin was presented by Dr. Pastan's group (Davies et al. 1978), showing that cleavage affects actin cross-linking but not the actin binding ability of filamin. Human blood platelet filamin was also reported to be cleaved by calpain (Fox et al. 1985). The site of calpain cleavage on human filamin was first mapped between residues 1761 and 1762 in the hinge I

region giving rise to ~190 kDa and ~100 kDa fragments (Gorlin et al. 1990). All the reported works for calpain cleavage of filamin has been performed on purified proteins, but the physiological relevance of this process is not yet understood. Although, filamin has been established as a substrate of calpain (Kiema et al. 2006; Mammoto et al. 2007), the mechanisms regulating filamin dynamics in migrating cells is not understood.

Recently, we reported that shRNA mediated knockdown of calpain 2 resulted in the decreased proteolysis of filamin in glioblastoma cell lysate, identifying the isoform of calpain responsible for filamin cleavage in these cells (Jang et al. 2010). Further, our results showed that calpain 2 is required for invasion, but not migration, of glioblastoma cells in transwell assays which points to the involvement of filamin proteolysis in the invasion of glioblastoma cells (Jang et al. 2010). However, the role of filamin expression in glioblastoma cell invasion has not been studied. Persuaded with the goal to understand the mechanisms controlling invasion of glioblastoma cells, we got interested to determine how calpain proteolysis of filamin is regulated in glioblastoma cells.

Calpain 2 is ubiquitously expressed in mammalian system and has been implicated in various cellular processes and in numerous pathological conditions (Goll et al. 2003). However, the fact that it requires millimolar concentration of calcium ions for half maximal activity, compared to nanomolar range in the cells, has stimulated much investigation for other factors involved in the activation of this protease. Besides

a localized transient burst in the calcium concentration and the enzyme activation by interacting partners, another proposal to facilitate calpain proteolysis is the ways to modulate substrate recognition and susceptibility such as by phosphorylation or phosphoinositides binding. The report from our lab showed that binding of phosphoinositides, specifically PtdIns (3,4,5)-P_3, to alpha actinin, modulates its structural conformation and regulates its proteolysis by calpain (Full et al. 2007; Sprague et al. 2008). Another study presented a correlation between overexpression of PI3 kinase and increased calpain proteolysis of fodrin protein (Aki et al. 2002). Also, phosphoinositides have been recognized to bind to and regulate the structure and function of various cytoskeletal and adhesion proteins (Yin and Janmey 2003). With respect to filamin, Furuhashi et al provided evidence that filamin also binds with phosphoinositides, which affect the actin-gelling activity of filamin in vitro (Furuhashi et al. 1992b). Together, these studies form the ground for the hypothesis that phosphoinositide binding to filamin regulates its proteolysis by calpain 2.

Glioblastoma cells demonstrate constitutively elevated levels of PtdIns (3,4,5)-P_3 in their membrane and the autocrine signaling by glutamate is a continuous source of calcium ion influx in these cells. The exact downstream target of calcium influx is not known, however, calpain is a potential candidate. In this study, we determined that filamin expression was inhibitory to the invasion of glioblastoma cells. It was confirmed when knockdown of filamin increased the rate of invasion of these cells through Matrigel basement matrix as determined by real time invasion analysis.

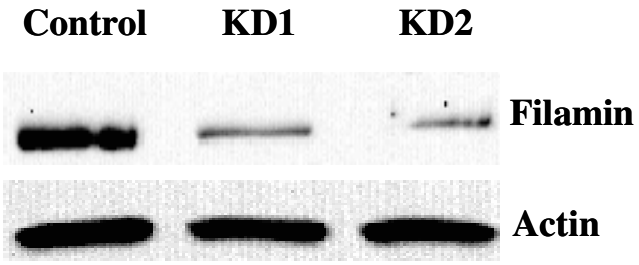
Further, we provide evidence that PtdIns (3,4,5)-P_3 binding to filamin regulates its proteolysis by calpain 2.

Results and Discussion

Inhibition of filamin expression enhances glioblastoma cell invasion in real time

Previously, the connection between filamin and cell invasion was reported by Varambally et al when they observed a significant decrease in the expression of filamin protein in advanced stages of metastatic prostate cancer (Varambally et al. 2005). Recently, a similar inverse correlation between filamin expression and invasiveness was documented for breast cancer cells (Xu et al. 2010). To identify its role in glioblastoma cell invasion, we stably knocked down the cellular expression of filamin protein using sequence specific shRNA as described in materials and methods. Out of two shRNA sequences showing knockdown, the cell line with 80% knockdown of filamin compared to the control cells was selected for further invasion analysis (Fig 3.1). Visual examination by phase contrast microscopy showed no differences in cell shape, spreading and process formations between control and filamin knockdown glioblastoma cells. In addition, proliferation of control and knockdown cells was measured by cell counting of continuous cultures over time with no difference observed between the two cell lines. Invasion was examined through Matrigel matrix

(A)


Control
KD1
KD2
Filamin
Actin

(B)

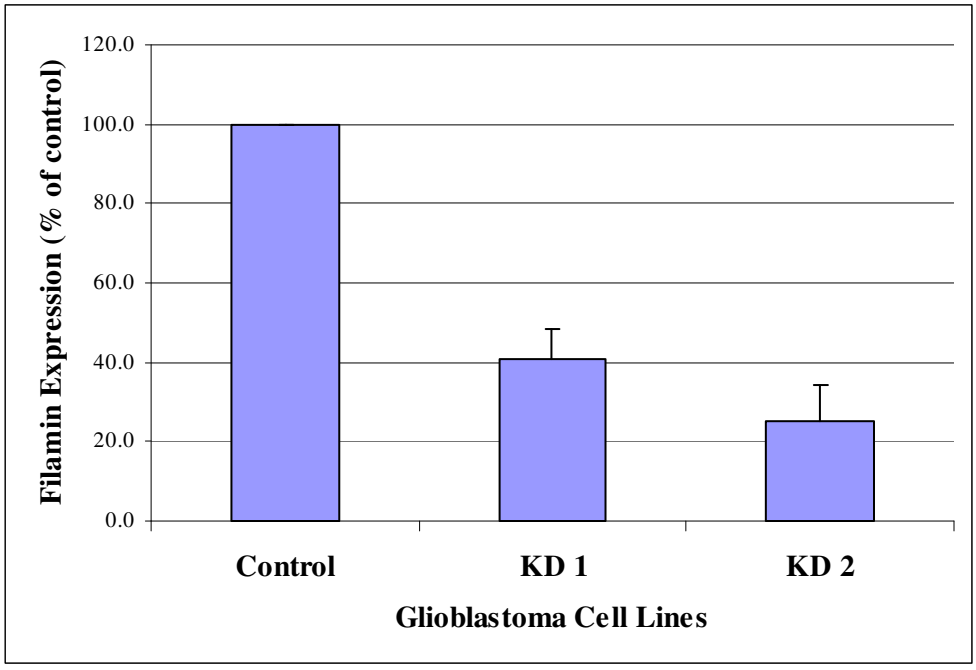

Filamin Expression (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Control
KD 1
KD 2
Glioblastoma Cell Lines

Fig 3.1: Knockdown of filamin expression in glioblastoma cells using sequence specific shRNA.

U87MG glioblastoma cells were transfected with plasmids encoding control or filamin specific shRNA (KD) followed by selection for stable knockdown cells with 1 µg/ml puromycin.

(A) The expression level of filamin protein was determined by western blotting of total cell lysates using monoclonal anti-filamin antibody. Actin was immonoblotted as the loading control.

(B) Filamin expression was quantified by densitometry using Kodak Image Station 440CF and Molecular Imaging Software. n= 4 ± SEM

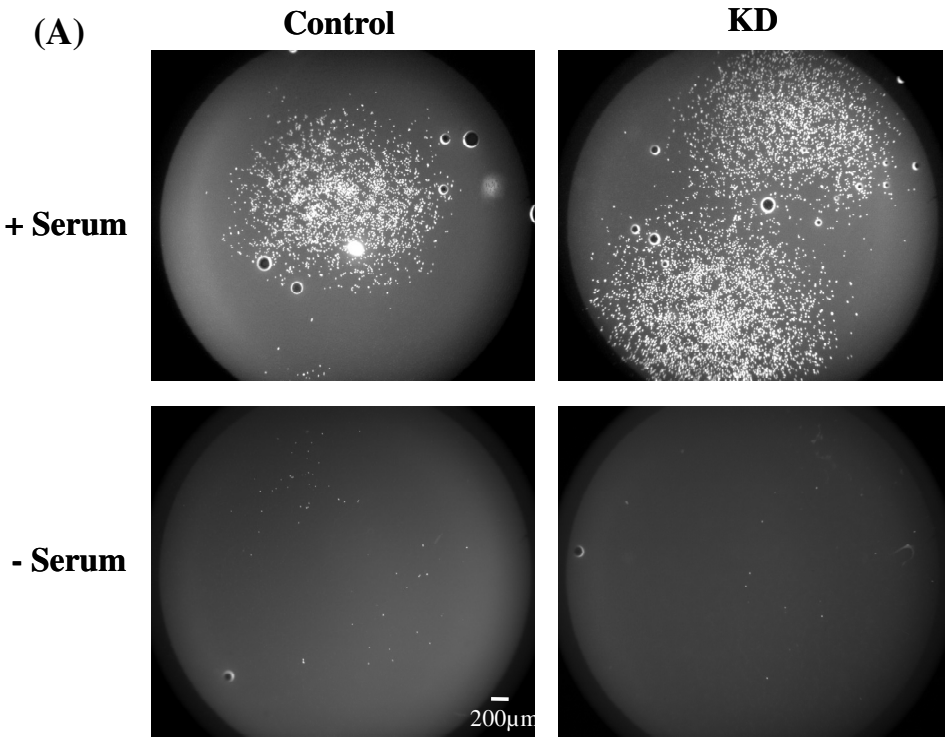
by conventional transwell assay as well as the rate of invasion was determined by real time analysis using xCELLigence system.

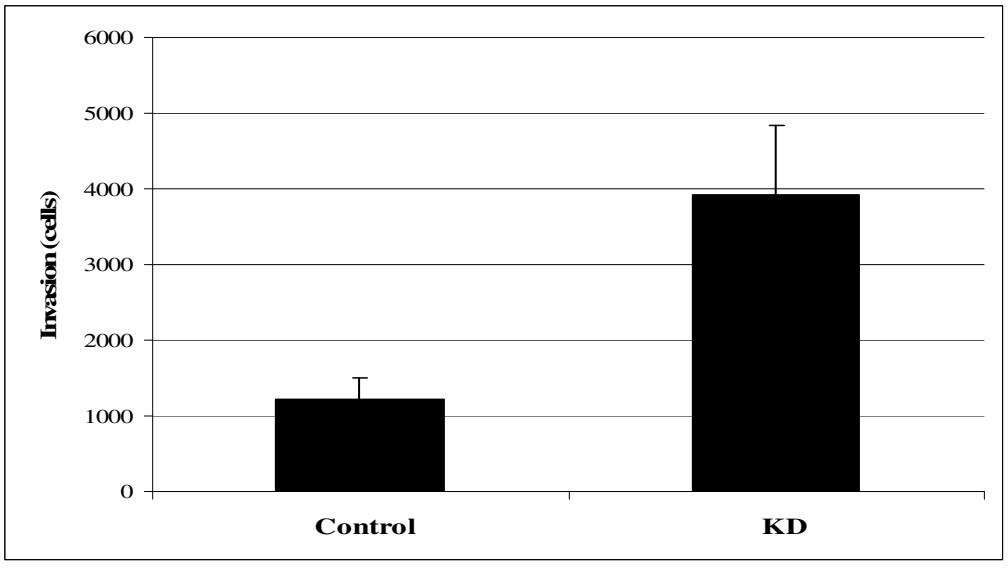
In the transwell assay, 2mg/ml Matrigel was polymerized in the upper chamber of each well to make a dense three dimensional matrix. Control and filamin knockdown cells (50,000) were suspended in serum free media and added to the Matrigel surface. Cells were allowed to migrate for 3 days through the matrix, stimulated with 10% FBS as chemoattractant present in the lower chamber of wells. Filamin knockdown cells demonstrated a 3-fold increase in invasion compared to control glioblastoma cells suggesting to a restrictive role for filamin in glioblastoma cells (Fig 3.2A&B). A Transwell invasion assay, like all conventional methods to test migration, has the following limitations: single end point data measurement, labeling of cells post experiment, and manual data analysis. It also lacks any quantitative measurement of cell behavior or invasion patterns in real time.

Considering the invasion process in 3D, the biophysical properties such as density and rigidity of extracellular matrix have been strongly correlated with the rate of motility of tumor cells (Zaman et al. 2006). To investigate the effect of filamin knockdown and density of extracellular matrix on the invasiveness of glioblastoma cells, we performed a real time invasion assay with increasing concentration of Matrigel. The upper chamber of the CIM assay plate was coated with 0.4 and 0.2 mg/ml Matrigel and polymerized as described in the Materials and Methods. Fifty thousand control or filamin knockdown cells were added on top of the Matrigel layer

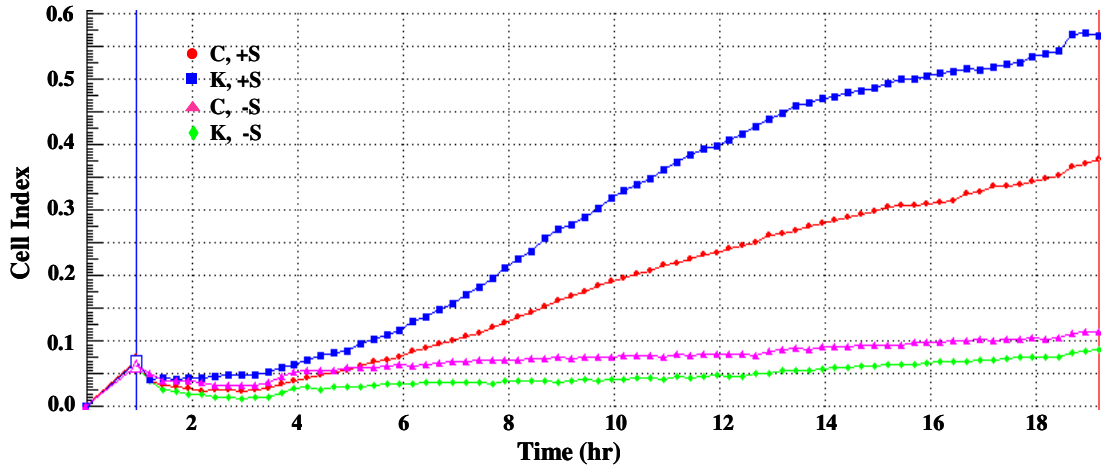
and the number of cells on the bottom side of the membrane was recorded every 15 min for 19 hrs. The number of invading cells, reaching the lower surface of the membrane, was represented as the cell index (CI) and the rate of invasion was determined by the slope of each invasion curve. Cell index reflects the change in the electrical impedance of the microelectrode integrated on the bottom of the transwell, and takes into account the number of cells and the quality of cell attachment on the membrane.

Filamin knockdown cells demonstrated consistent increased ability to invade through all the concentrations of Matrigel, compared to control cells (Fig 3.2C). The up regulation of invasion by filamin knockdown was more pronounced at higher densities of matrix and the difference between control and knockdown cells were minimal at 0.2 mg/ml Matrigel, the lowest concentration tested. Determined by the slope of cell index curves, we observed that knockdown cells demonstrated 62% and 26% increase in the rate of invasion compared to control cells at 0.4 and 0.2 mg/ml Matrigel (data not shown). This was in agreement with the 220% increase in invasion observed for knockdown cells using 2 mg/ml Matrigel when assessed after 3days in conventional transwell assay. These results suggest that filamin expression prevents glioblastoma cells from navigating through dense extracellular matrixes. Moreover, we observed that irrespective of filamin expression, the rate of invasion was lower, for both cell lines, through high density matrix and a gradual increase in the invasion was observed with decreasing extracellular matrix barriers. The prevailing concept in


(A)
Control
KD
+ Serum
- Serum
200µm


(B)
Invasion (cells)
6000
5000
4000
3000
2000
1000
0
Control
KD

(C)


C, +S
K, +S
C, -S
K, -S
Cell Index
Time (hr)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
2
4
6
8
10
12
14
16
18

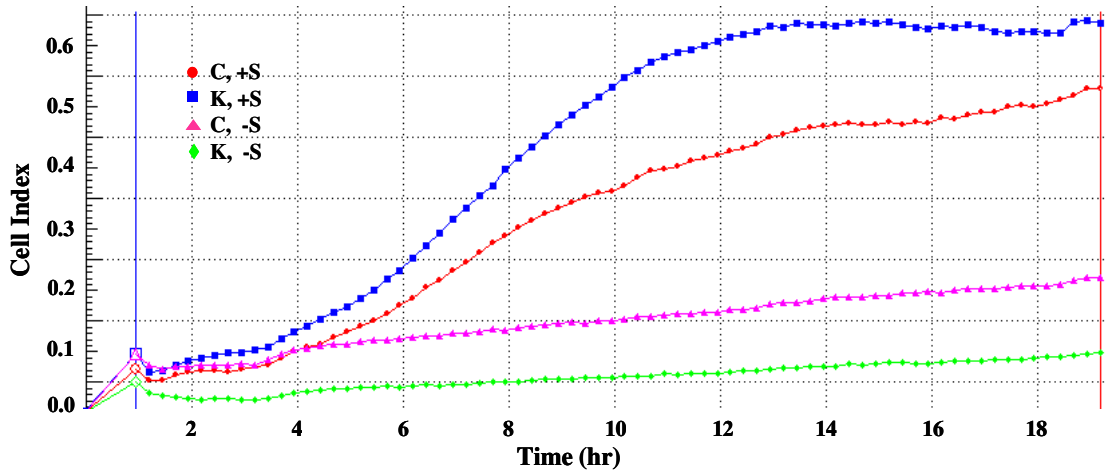

C, +S
K, +S
C, -S
K, -S
Cell Index
Time (hr)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
2
4
6
8
10
12
14
16
18

Fig 3.2: Knockdown of filamin expression enhances glioblastoma cell invasion.

Control and filamin knockdown (KD) cells were examined for invasion through the Matrigel extracellular matrix.

(A) Transwells with 8.0 µm pores were coated with 2 mg/ml Matrigel and 50,000 cells were added to the upper chamber in serum free media. Cells were stimulated to invade with ± 10% FBS as chemoattractant in the lower wells. After 3 days of incubation, cells on the bottom surface of the membrane of the transwells were fixed and stained using DAPI.

(B) Stained cells were quantified using MetaMorph 6.2 and graph was plotted for the number of cells that invaded through Matrigel in the presence of 10%FBS. n = 3 ± SEM.

(C) Control and KD cells were monitored in real time as described in Materials and Methods. The upper chamber was coated with 0.4 mg/ml and 0.2 mg/ml concentrations of Matrigel and the lower chamber received DMEM ±10% FBS as chemoattractant. In serum free media, 50,000 cells were added to the upper chamber of each well. The cell index represents the number of cells that have migrated through the Matrigel to reach the bottom surface of transwells containing the integrated microelectrode. The curves show the cell index readings measured every 15 min for control and knockdown cells invading through 0.4 mg/ml (top) and 0.2 mg/ml (bottom) Matrigel. Legends: C-control; K-knockdown; S-10% FBS. Plots are representative of two independent experiments.

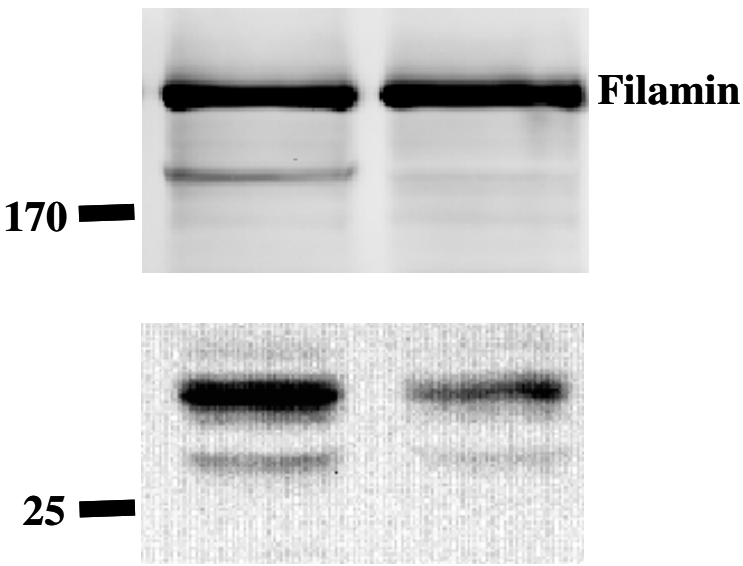
tumor cell invasion is that cells invade neighbor areas by the proteolytic degradation of extracellular matrix mainly by secreting MMPs (Rao 2003). However, both clinical and basic research has also shown that different cell types retain capacity to migrate in the absence of MMPs (Wolf et al. 2003) which suggests for the involvement of other mechanisms regulating cellular movement through the extracellular matrix. Migration through dense surroundings with smaller pore sizes would require a high degree of flexibility to allow deformability of the cell shape and rapid re-organization of cytoskeletal assembly.

Since filamin is critical to provide mechanical stability to cortical actin filaments (Flanagan et al. 2001), the knockdown of filamin would correlate with less stable orthogonal networks and cell-ECM attachments which can be an advantage to improve the plasticity of the cell membrane and help in the rapid cytoskeleton contractility to facilitate increased invasion. However, in tumor cells which express filamin at normal levels, the similar effect might be achieved by the proteolysis of filamin protein leading to enhanced turn over of actin networks in a migrating cell. Previously, we reported that filamin is a potential substrate of calpain 2 in glioblastoma cells (Jang et al. 2010), however, the mechanisms regulating filamin proteolysis are not clear.

Calpain 2 cleavage produces novel breakdown products of filamin in glioblastoma cells

It is well established that calpain catalyzes selective proteolysis of substrates and, rather than complete degradation, produces functional breakdown products (Goll et al. 2003; Wang et al. 2007). To determine the pattern of filamin proteolysis, cell extracts from control and calpain 2 knockdown glioblastoma cells were analyzed for filamin breakdown products by Western blotting. Previous studies have reported that calpain cleaves filamin in the hinge 1 region generating a ~190kDa fragment from the amino terminus and a carboxy terminal product of ~100 kDa (Gorlin et al. 1990; Mammoto et al. 2007). Consistent with these studies, we observed the breakdown products of 190 and 100 kDa, however, modification of the protocol to enhance identification of low molecular weight proteins revealed a novel breakdown product of ~30 kDa (Fig 3.3). Furthermore, the 30 kDa breakdown product was significantly reduced in the lysates from calpain 2 knockdown cells suggesting it's generation by calpain 2 proteolysis. The 30 kDa fragment was recognized with an antibody specific for the N-terminal residues (1-50) of filamin suggesting that the fragment contained the actin binding domain. Cleavage at this site would represent a novel mechanism for the regulation of filamin function modulating the turnover of actin filament networks during cell migration.

(A)



Immunoblot: anti-filamin

(B)

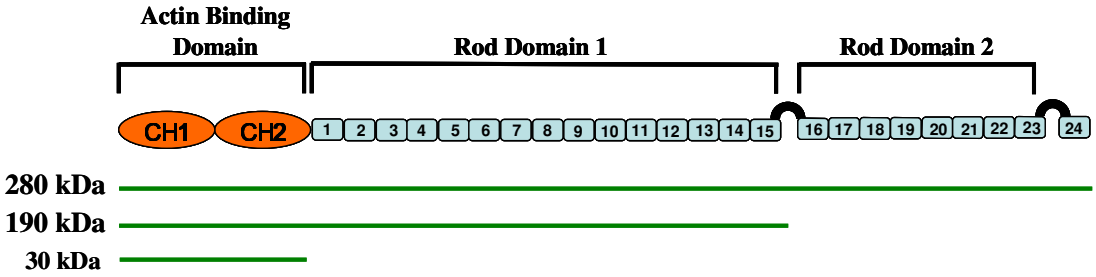


Fig 3.3: Calpain 2 cleavage produces a novel breakdown product of filamin in glioblastoma cells.

(A) The total cell lysate of control and calpain 2 knockdown (KD) cells (75000 cells per lane) was immunoblotted using monoclonal antibody against N-terminal residues of filamin. The samples were transferred and developed separately under the conditions best suited for detecting large and small breakdown fragments. Lower blot reveals the novel band that remained undetected in the literature.

(B) The schematic diagram of a filamin monomer showing locations of the actin binding domain, rod domains and the c-terminal dimerization domain. The bars correlate the relative molecular sizes of the fragments to the structural locations on the full length protein.

PtdIns (3,4,5)-P_3 binding to filamin enhances the susceptibility to proteolysis by calpain 2

To understand the generation of the 30 kDa breakdown product in glioblastoma cells, which remained undetected in previous studies of other cell types, we revisited the unique characteristics of these cells. Glioblastoma cells possess the constitutively higher levels of PtdIns (3,4,5)-P_3 in their membranes which is attributed to the lack of the PTEN phosphatase and enhanced activity of PI3-kinase (Kanu et al. 2009a). Further, data from our lab have shown that PtdIns (3,4,5)-P_3 binding to α-actinin in the CH2 domain of the actin binding region modulates its structure (Fraley et al. 2005; Full et al. 2007), function (Corgan et al. 2004) and increases its proteolysis by calpain 2 (Sprague et al. 2008). Since, the actin binding domain are highly conserved in the spectrin family of actin binding proteins which includes α-actinin and filamin, we tested for the regulation of filamin proteolysis by PtdIns (3,4,5)-P_3 binding. To enhance detection of calpain 2 proteolysis in the amino-terminal region, assays were carried out using filamin purified from chicken gizzard which does not have hinge 1 region between repeats 15 and 16 (Barry et al. 1993; Ohashi et al. 2005). However, the amino acid sequence of human non-muscle filamin and chicken gizzard filamin is 76% identical over entire protein and 85% identical in the actin binding domain (Ohashi et al. 2005). Purified filamin was incubated with PtdIns (3,4,5)-P_3 to allow binding before calpain 2 was added and the catalysis was triggered by adding $CaCl_2$. The proteins were analyzed after separation by SDS-PAGE. Minimal cleavage

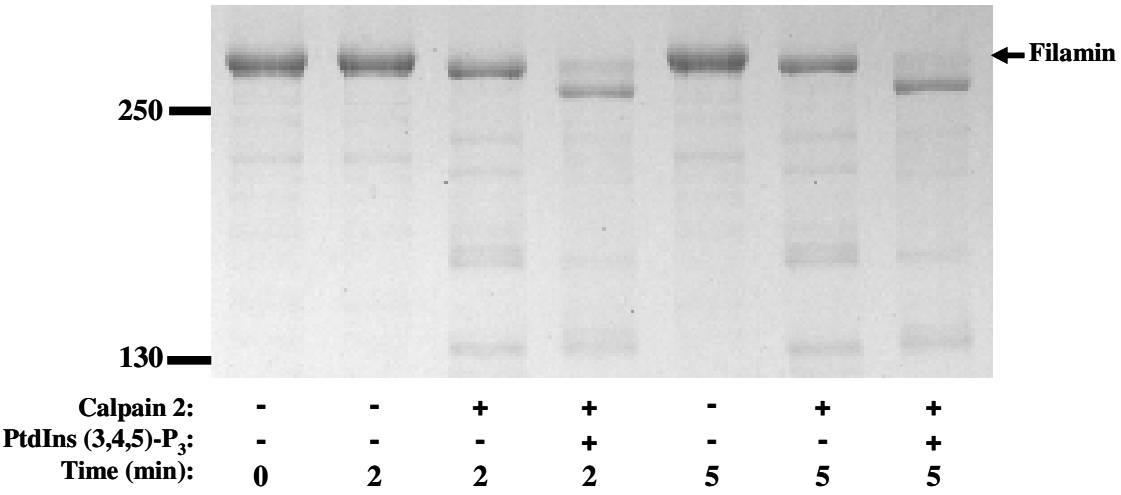


Fig 3.4: PtdIns(3,4,5)-P_3 binding increases the susceptibility of filamin to proteolysis by calpain 2.

Filamin (2 μM) was pre-incubated in the presence or absence of PtdIns(3,4,5)-P_3 (50 μM) for 15 min at 30°C for 15 min. Calpain (0.17μM) and $CaCl_2$ (1 mM) were added and the incubation was continued at 30°C. Samples were removed at 0, 2 and 5 min and the reaction was stopped with 2x SDS buffer containing EDTA and EGTA. After boiling for 2 min at 100 ^{0}C, the proteins were separated on a 7.5% gel by SDS-PAGE and stained with GelCode Blue. n = 6.

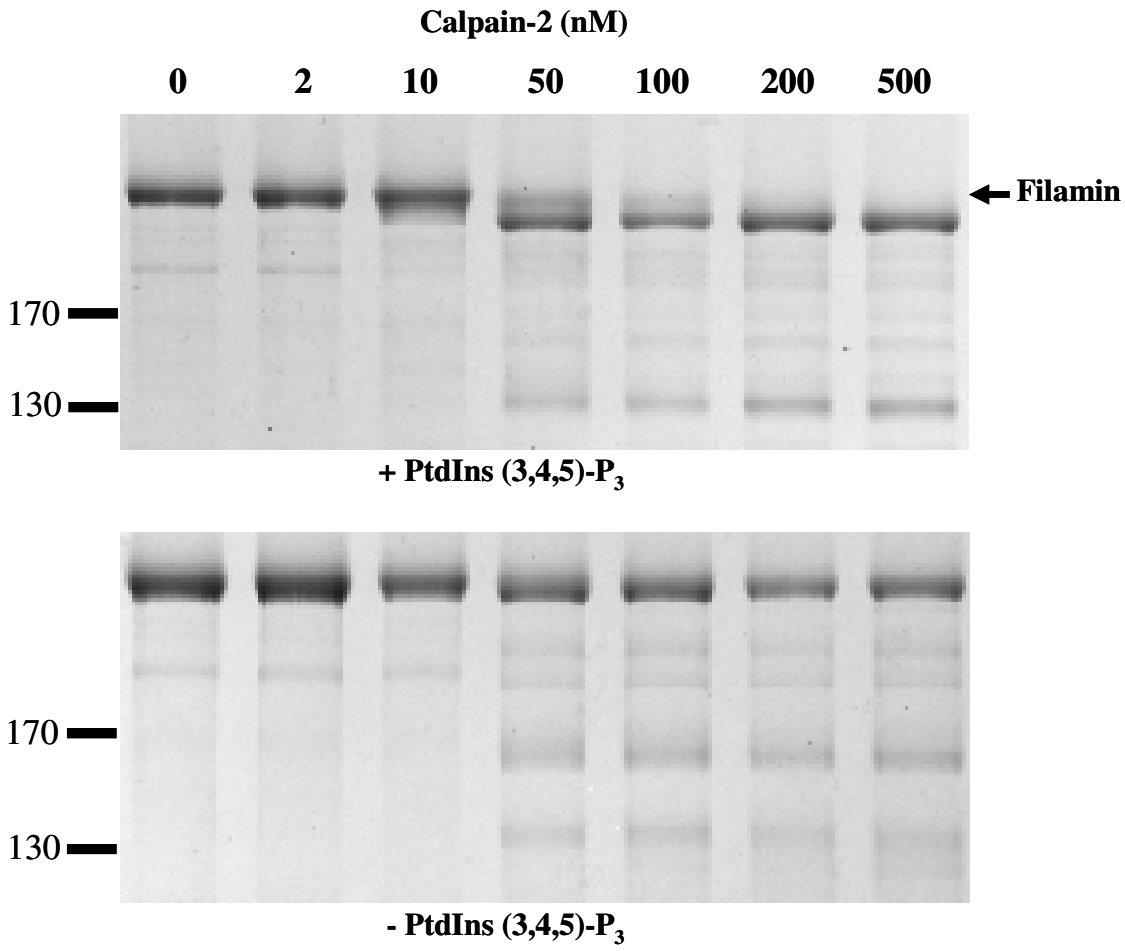


Fig 3.5: PtdIns(3,4,5)-P_3 binding increases the sensitivity of filamin to low nanomolar concentrations of calpain 2.

Filamin (2 µM) was pre-incubated with or without PtdIns(3,4,5)-P_3 (50 µM) at 30°C for 15 min. Incubations were further continued for additional 5 min at 30°C in presence of increasing concentrations of calpain 2 and 1mM $CaCl_2$. Reactions were stopped with 2x SDS buffer containing EDTA and EGTA. After boiling for 2 min at 100 ^{0}C, the proteins were separated by SDS-PAGE and stained with GelCode Blue. n = 3.

was observed in the absence of PtdIns (3,4,5)-P_3; however, when PtdIns (3,4,5)-P_3 was bound to filamin, almost complete loss of full length protein was observed in first 2 min producing a stable breakdown product migrating at molecular weight of approximately 250kDa (Fig 3.4). When filamin proteolysis was compared in presence of PtdIns (4,5)-P_2 or PtdIns (3,4,5)-P_3 binding, our results showed a significantly higher effect of PtdIns (3,4,5)-P_3 binding to increase the filamin cleavage (data not shown). This indicates for a role of PI3-kinase in regulating filamin proteolysis in cells. Moreover, when subjected to proteolysis at different concentrations of calpain 2, filamin was very sensitive to the enzyme at as low as 50nM, when bound to PtdIns (3,4,5)-P_3, whereas, no cleavage was observed even at ten fold higher concentration of calpain in the absence of PtdIns (3,4,5)-P_3 (Fig 3.5). This result suggests that binding of PtdIns (3,4,5)-P_3 enhances proteolysis of filamin by increasing its sensitivity to calpain. We propose that PtdIns (3,4,5)-P_3 binding modulates the three dimensional conformation of filamin exposing a site highly susceptible to calpain 2.

PtdIns (3,4,5)-P_3 mediated calpain cleavage abolishes actin binding ability of filamin

The effect of calpain 2 cleavage on the function of filamin was determined by assaying the actin cross-linking activity of filamin following proteolysis in the absence or presence of PtdIns (3,4,5)-P_3. The proteolysis incubations were performed as described in materials and methods and the reactions were stopped with EGTA

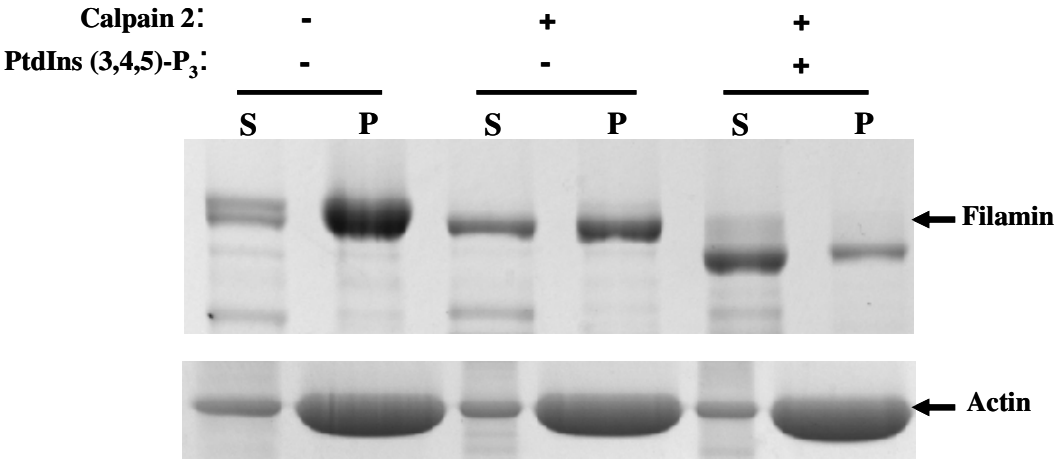


Fig 3.6: PtdIns(3,4,5)-P_3 mediated calpain 2 cleavage inhibits actin binding ability of filamin.

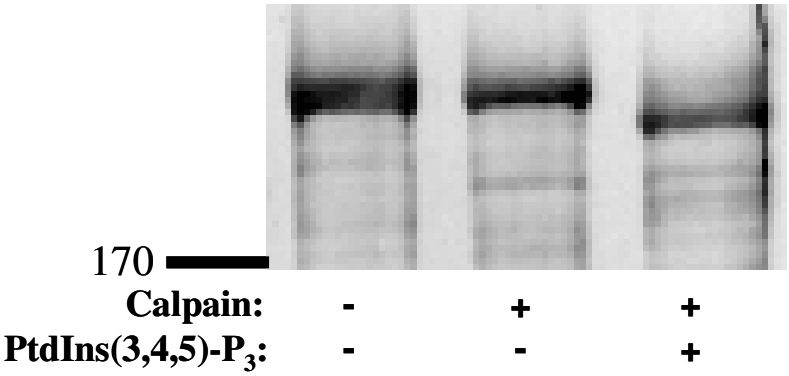
Filamin was proteolysed with calpain 2 for 5 min in presence and absence of PtdIns(3,4,5)-P_3 as in Fig 4 and the reaction was stopped with 4 mM EGTA. Actin filaments (10 µM) were added and the solution was incubated at RT for additional 30 min. Samples were centrifuged at 100,000 x g for 30 min at RT. Proteins in the supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and stained with GelCode Blue. n=3.

followed by addition of actin filaments to the mixture. The binding of filamin to actin was assessed by centrifugation at 10,000 x g, optimal to sediment actin filaments, assuming that binding partners of actin would settle down in the pellet with actin filaments leaving non binding proteins in the supernatant phase. When proteolyzed in the presence of PtdIns (3,4,5)-P_3, the stable breakdown product of filamin (~250 kDa) was mostly located in the supernatant fraction suggesting inability to bind to actin filaments (Fig 3.6). In contrast, filamin proteolysed with calpain 2 in the absence of PtdIns (3,4,5)-P_3, settled down in the pellet with actin. Similar result was observed for the actin cross-linking ability of filamin when proteolysed in presence and absence of PtdIns (3,4,5)-P_3. To further characterize the loss of actin binding ability of the filamin breakdown product, immunoblotting of the fragments was performed using antibody against the carboxy terminal residues of filamin. The full length protein and the stable breakdown product of filamin produced in the presence of PtdIns (3,4,5)-P_3 was recognized by the antibody suggesting that the c-terminal region of filamin remained intact upon cleavage by calpain 2 (Fig 3.7A). Together, these data confirms that calpain 2 cleaves filamin in the N-terminal region, thereby, interfering with the actin binding ability of the fragments.

Calpain 2 proteolysis removes the actin binding domain of filamin

To precisely understand the regulation of filamin proteolysis by PtdIns (3,4,5)-P_3, we sought to identify the location of the highly sensitive calpain cleavage site on

(A)


170
Calpain: - + +
PtdIns(3,4,5)-P3: - - +

(B)

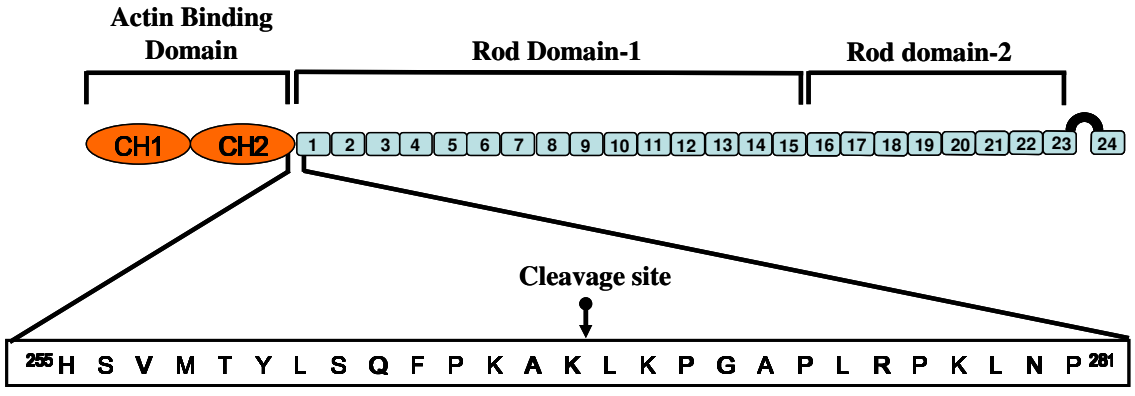

Actin Binding Domain
Rod Domain-1
Rod domain-2
CH1
CH2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Cleavage site
255 H S V M T Y L S Q F P K A K L K P G A P L R P K L N P 281

Fig 3.7: Calpain 2 cleaves PtdIns(3,4,5)-P_3 bound filamin after Lys 268.

(A) Filamin proteolysis by calpain 2 was performed in the presence of PtdIns (3,4,5)-P_3 as described above and proteins were separated by SDS-PAGE. Immunoblotting with the anti-filamin antibody specific for C- terminal residues recognized the stable calpain 2 cleaved product (~250 kDa) suggesting for the intact C-terminal end.

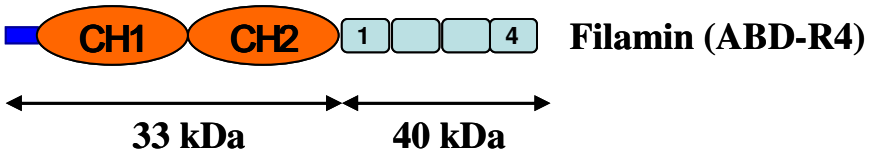
(B) The susceptible site of cleavage (marked with the arrow) was determined by the N-terminal sequencing of the breakdown product. The sequence shows a segment of the linker region between the actin binding domain and the rod domain 1. n=3.

filamin. The N-terminal sequencing of the stable breakdown product of filamin produced by calpain 2 revealed that PtdIns (3,4,5)-P_3 binding induced cleavage after the amino acid lysine 268 (Fig 3.7B), which is located in the linker region between the actin binding domain and the rod domain I. This finding explains why the stable breakdown product of filamin lost the ability to bind actin filaments and suggests a potential mechanism for the functional regulation of filamin in cells.

PtdIns (3,4,5)-P_3 binding induces calpain 2 cleavage of human filamin after the actin binding domain

To determine whether PtdIns (3,4,5)-P_3 binding regulates proteolysis of human filamin after lysine 268, a truncation fragment of the human filamin encoding the actin binding domain and repeats one to four of the rod domain 1 (ABD-R4) was constructed and purified as hexa-histidine tagged protein as described in the Materials and Methods. The cDNA sequence alignment of filamin from human and chicken gizzard finds 100% identity in the region (30 residues) flanking lysine 268, the site of cleavage, which suggests for the similarity of the local structure in the folded proteins. Calpain 2 proteolysis of truncation proteins was performed identically as described above for the full length protein and the breakdown products were analyzed by SDS-PAGE followed by staining with GelCode Blue. The complete loss of the full length protein (77 kDa) was observed in the presence of PtdIns (3,4,5)-P_3 generating a breakdown product running at ~40kDa. This product was not identified with an

(A)


CH1
CH2
1
4
Filamin (ABD-R4)
33 kDa
40 kDa

(B)

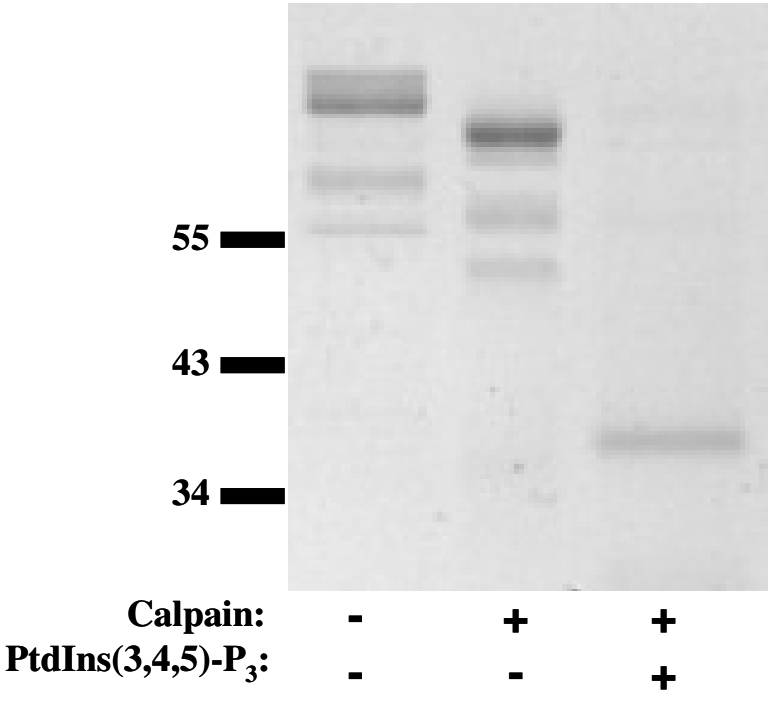

55
43
34
Calpain: - + +
PtdIns(3,4,5)-P3: - - +

Fig 3.8: Binding of PtdIns(3,4,5)-P_3 to human filamin induces cleavage by calpain 2 after the actin binding domain.

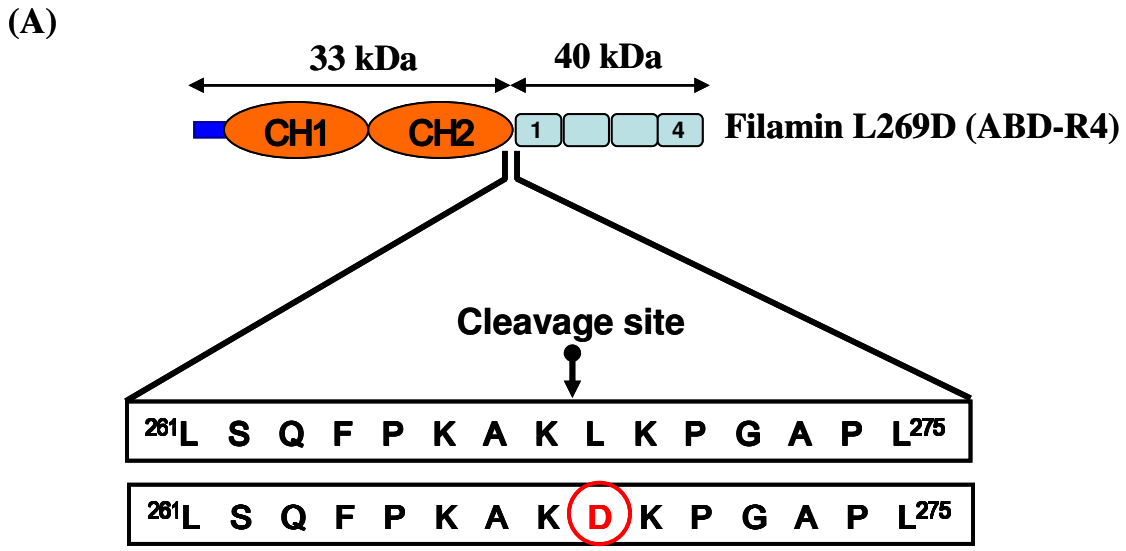
(A) The diagram of filamin truncation protein representing the relative molecular sizes of the actin binding domain (CH1 and CH2) and repeats 1-4. The molecular weight includes the hexa-histidine tag shown as the blue rectangle before the actin binding domain.

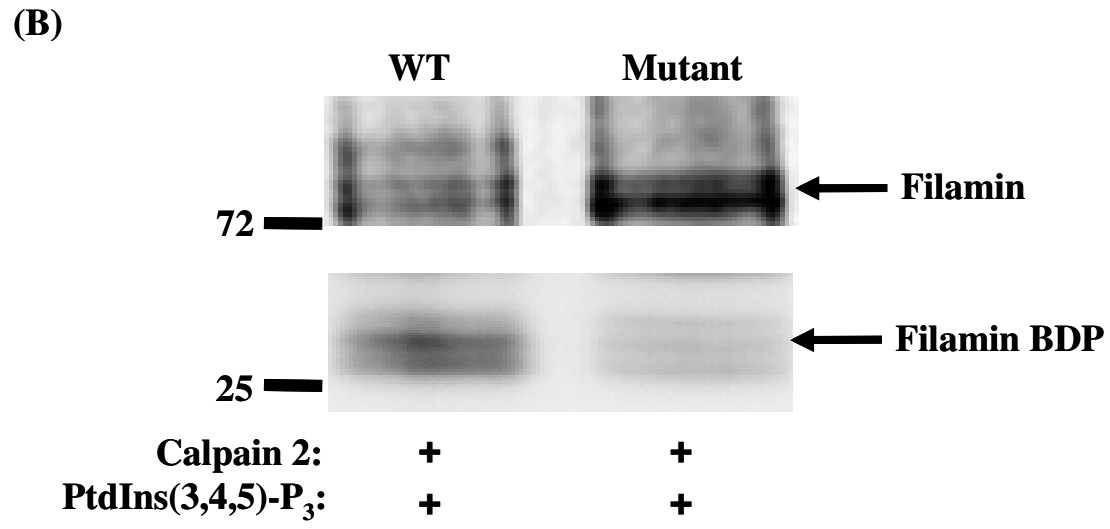
(B) Filamin (2μM) was pre-incubated with or without PtdIns(3,4,5)-P_3 (50 μM) at 30°C for 15 min. Calpain 2 (0.17μM) and $CaCl_2$ (1 mM) were added and the incubation was continued at 30°C. Samples were removed at 0, 2 and 5 min and the reaction was stopped with 2x SDS buffer containing EDTA and EGTA. The proteins collected at 5 min timepoint were separated by SDS-PAGE and stained with GelCode Blue. n = 3.

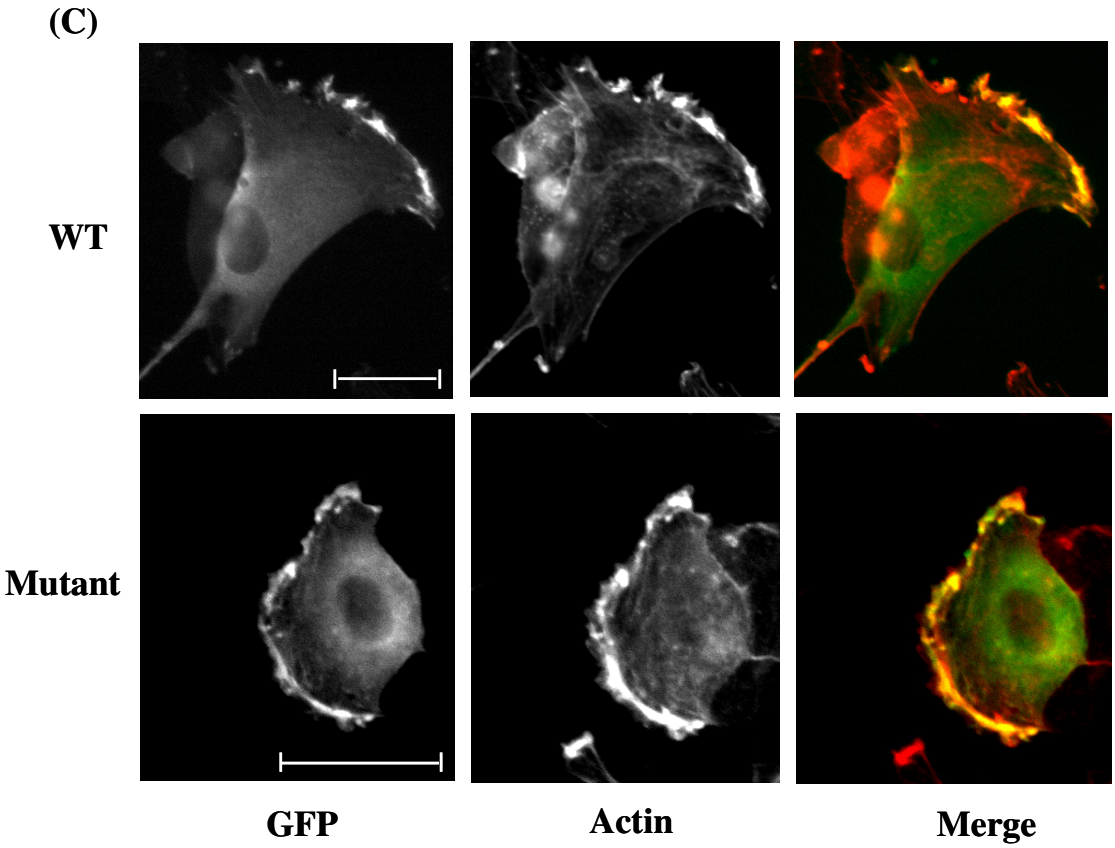
antibody specific for the amino-terminal residues of filamin suggesting it to be the C-terminal fragment of the protein after cleavage at Lys268 (Fig 3.8). The Western blotting of the fragments with anti-hexa-histidine antibody recognized a stable breakdown product running close to the full length truncation protein in the absence of PtdIns (3,4,5)-P_3 suggesting that calpain 2 cleaved at the c-terminal end leaving the amino-terminus intact (data not shown). The 40kDa fragment was not recognized by the anti-hexa-histidine antibody further confirming it's generation from the c-terminal end of the protein. Together, these observations suggest that human filamin protein also possesses a calpain susceptible site in the linker region between the actin binding domain and the Rod domain 1.

Mutation of leucine to aspartic acid at residue 269 makes filamin resistant to cleavage by calpain 2

To determine the role of PtdIns (3,4,5)-P_3-induced cleavage of filamin in glioblastoma cell invasion, we generated a mutant form of filamin resistant to calpain 2 proteolysis. Site directed mutagenesis of GFP-tagged filamin was performed to substitute the amino acid leucine (L) at position 269 with aspartic acid (D). To confirm that the mutant protein is resistant to cleavage by calpain 2, a truncation fragment encoding the sequence from amino acids 1 to 660 (Fig 3.9A), which includes the actin binding domain and repeats one to four of the rod domain 1 (ABD-R4), was cloned by


(A)
33 kDa
40 kDa
CH1
CH2
1
4
Filamin L269D (ABD-R4)
Cleavage site
261L S Q F P K A K L K P G A P L275
261L S Q F P K A K D K P G A P L275


(B)
WT
Mutant
72
Filamin
25
Filamin BDP
Calpain 2: + +
PtdIns(3,4,5)-P3: + +


(C)
WT
Mutant
GFP
Actin
Merge

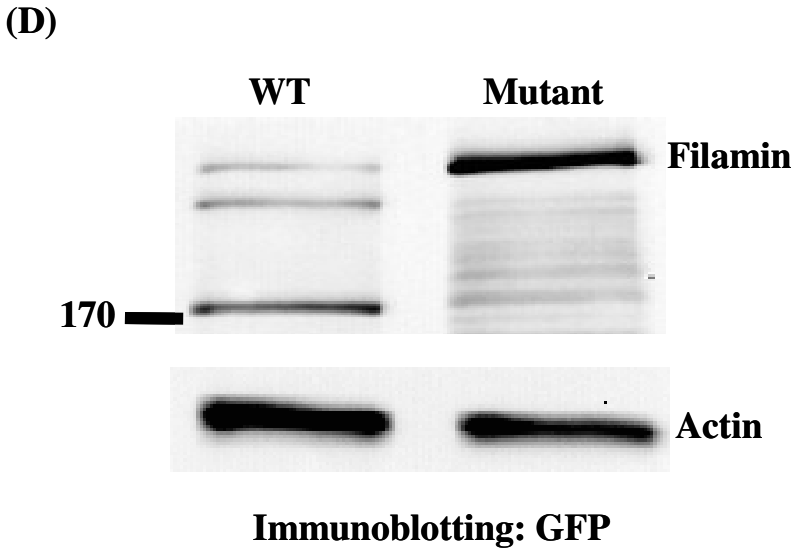

(D)
WT
Mutant
Filamin
170
Actin
Immunoblotting: GFP

Fig 3.9: Point mutation of filamin at position 269 allows normal localization in cells but prevents cleavage by calpain 2.

The cDNA of GFP-filamin was subjected to site-directed mutagenesis using PCR to generate a clone of L269D mutant GFP-filamin which was further verified by sequencing.

(A) The diagram shows the truncation form of the mutant filamin sequence cloned and purified as hexa-histidine tag protein. Identical to the WT protein (Fig 3.8), the truncation fragment consisted of the actin binding domain and repeats 1 to 4. The depicted molecular weight includes the hexa-histidine tag shown as the blue rectangle before the actin binding domain.

(B) Wild type (WT) and mutant filamin truncation proteins (2μM) were pre-incubated with PtdIns(3,4,5)-P_3 (50 μM) at 30°C for 15 min. Proteolysis was initiated by adding calpain 2 (0.17μM) and $CaCl_2$ (1 mM) and the incubation was continued at 30°C. Samples were removed at 5, 15 and 30 min and the reaction was stopped with 2x SDS buffer containing EDTA and EGTA. The proteins collected at 30 min were separated by SDS-PAGE and Western blotting was performed using anti-filamin antibody. The transfer and immunoblotting conditions were modified in order to detect the small breakdown products of filamin.

(C) The human glioblastoma cells stably expressing GFP-filamin wild type (WT) or L269D mutant, maintained at 400μg/ml Geneticin, were fixed on glass coverslips using formaldehyde and costained for actin filaments with Rhodamine Phalloidine. No adverse effect of GFP expression or the point mutation was observed on the cellular morphology and both WT and mutant filamin demonstrated normal co-localization with actin filaments at the peripheral cell membrane. The yellow regions in the merged images represents colocalized actin (red) and GFP-filamin (green). Scale bar =25μm.

(D) The cleavage of L269D mutant filamin in glioblastoma cells was determined by the Western blotting of total extract from cells stably expressing WT or mutant proteins (75000 cells per lane) using monoclonal antibodies against GFP and N-terminal residues of filamin. N=3.

PCR and purified as a hexa-histidine tagged protein as described in the Materials and Methods. In the presence of PtdIns (3,4,5)-P_3, almost complete proteolysis of the wild type ABD-R4 was observed. In contrast, little cleavage of the L269D mutant ABD-R4 was observed by calpain 2 in the presence of PtdIns (3,4,5)-P_3. The antibody specific to the amino-terminal residues of filamin identified the expected ~30 kDa breakdown fragment from the wild type protein and not from the L269D mutant, which indicated that calpain 2 cleaved at the susceptible site (Fig 3.9B). Results of the assays with ABD-R4 showed that the L269D mutant filamin is resistant to PtdIns (3,4,5)-P_3-induced calpain 2 proteolysis.

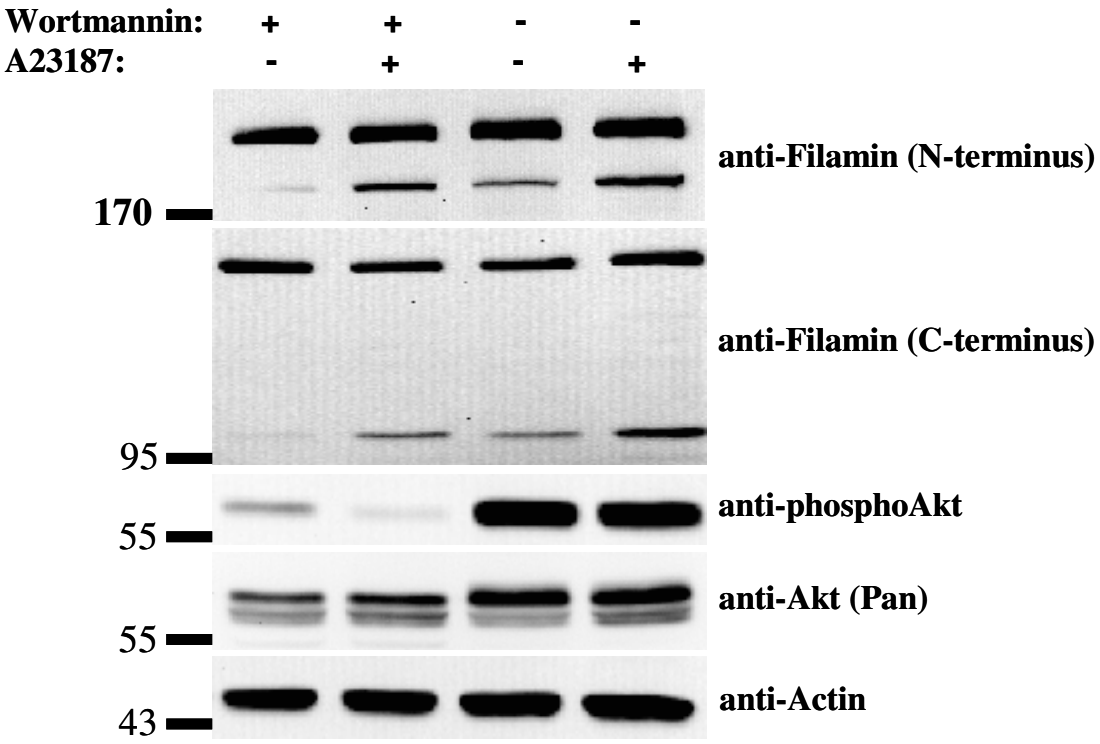
Stable clones of glioblastoma cells expressing GFP-filamin wild type or the calpain 2-resistant L269D mutant were prepared to assay cellular function. The sub-cellular localization of wild type GFP-filamin appeared consistent with endogenous filamin along actin filaments in the membrane ruffles at the cell periphery, suggesting that the GFP tag did not alter the localization of filamin in glioblastoma cells. The L269D mutant filamin also localized with the actin filaments in the membrane ruffles, however, the filamin staining at the cell periphery appeared to be more intense for calpain 2 resistant L269D mutant filamin compared to the wild type protein (Fig 3.9C). The difference in intensity between GFP-filamin wild type and L269D mutant localized to membrane ruffles may have been due to decreased susceptibility of the mutant to proteolysis.

The proteolysis of GFP-filamin proteins in glioblastoma cells was investigated by immunoblotting the total lysates from cells stably expressing wild type or mutant proteins. A thick band representing the full length L269D mutant filamin was observed, whereas full length wild type filamin was almost completely lost, suggesting that the L269D filamin is resistant to proteolysis in glioblastoma cells (Fig 3.9D). With the anti-GFP antibody, we expected to observe the breakdown fragments of ~57 kDa and ~217 kDa from the wild type GFP-filamin, based on the cleavage after lysine 268 (30 kDa) and in the hinge-1 region (~190 kDa) respectively. However, the molecular weight of the major breakdown products indicated that filamin was cleaved at ~143 kDa and ~223 kDa from the amino-terminal end of the full length protein, excluding the GFP-tag (~27 kDa). These breakdown products are not observed from the endogenous filamin in the lysate of untransfected glioblastoma cells. The excessive proteolysis of wild type GFP-filamin raises concern that the presence of the GFP-tag alters dynamics of the interaction with actin filaments increasing susceptibility to degradation in cell. Therefore, stable expression of GFP-filamin in the glioblastoma cells was not a valid approach for examining the filamin in invasion.

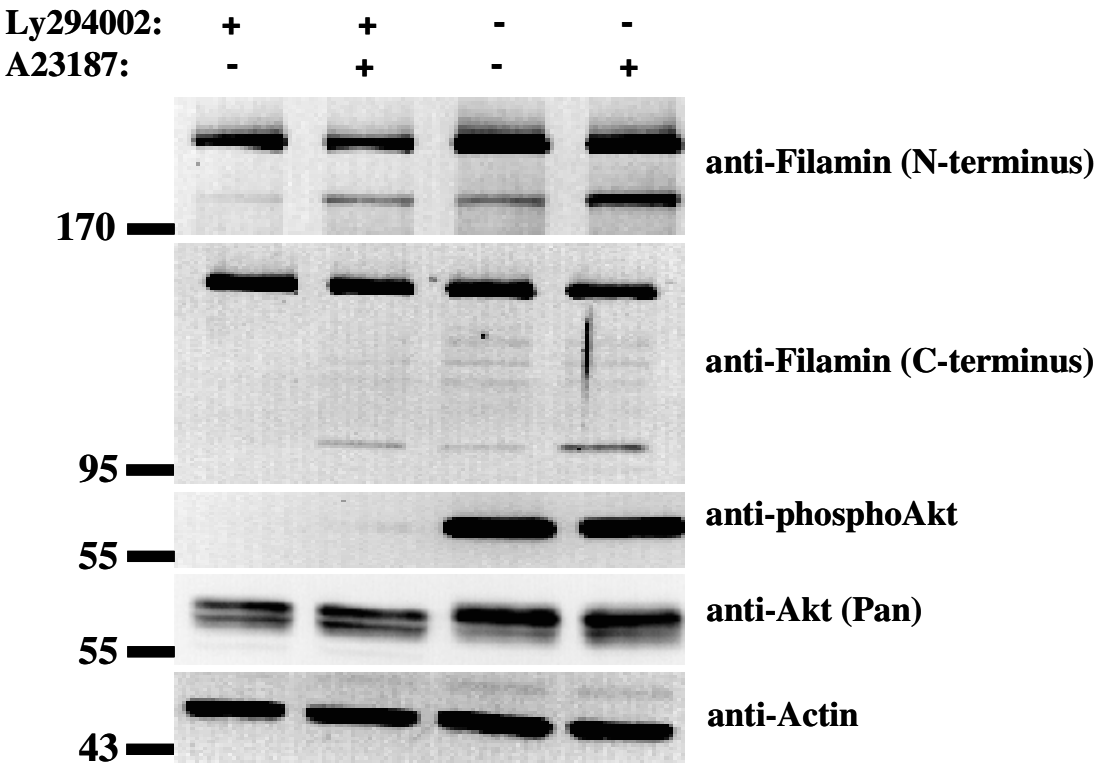
PI3 kinase activity is required for filamin proteolysis in glioblastoma cells

Our data, so far, strongly suggests that filamin is a substrate of calpain 2 in human glioblastoma cells and PtdIns (3,4,5)-P_3 binding to purified filamin induces calpain 2 proteolysis after the actin binding domain. Together, these results point to the possible involvement of PI3 kinase pathway in regulation of filamin cleavage in glioblastoma cells. To determine the regulation of human filamin proteolysis by PtdIns (3,4,5)-P_3 binding, glioblastoma cells were treated with the inhibitors of PI3-kinase, wortmannin (Fig 3.9A) and Ly294002 (Fig 3.9B), to deplete the PtdIns (3,4,5)-P_3 levels in the cell membrane. Further, cells were subjected to calcium ionophore, A23187, to induce calcium influx in cells intended to stimulate calpain activation. Cells were lysed in the SDS buffer containing EDTA and EGTA, proteins were separated by SDS-PAGE, immobilized onto the nitrocellulose membrane and proteins were identified using antibodies specific to amino or carboxyl terminus of filamin to verify the pattern of breakdown products. Our finding showed that the inhibition of PI3-kinase activity resulted in the reduction of filamin cleavage both under basal condition as well as when stimulated with calcium ionophore (Fig. 3.9). Before making conclusions, the depletion of PtdIns (3,4,5)-P_3 in the cell membrane, caused by wortmannin and Ly294002, was confirmed by assessing the levels of the phosphorylated form of Akt protein in cell lysates. Akt is a protein kinase which requires to bind PtdIns (3,4,5)-P_3 for its phosphorylation at serine 473 by downstream

(A)


Wortmannin: + + - -
A23187: - + - +
anti-Filamin (N-terminus)
170
anti-Filamin (C-terminus)
95
anti-phosphoAkt
55
anti-Akt (Pan)
55
anti-Actin
43

(B)


Ly294002: + + - -
A23187: - + - +
anti-Filamin (N-terminus)
170
anti-Filamin (C-terminus)
95
anti-phosphoAkt
55
anti-Akt (Pan)
55
anti-Actin
43

(C)

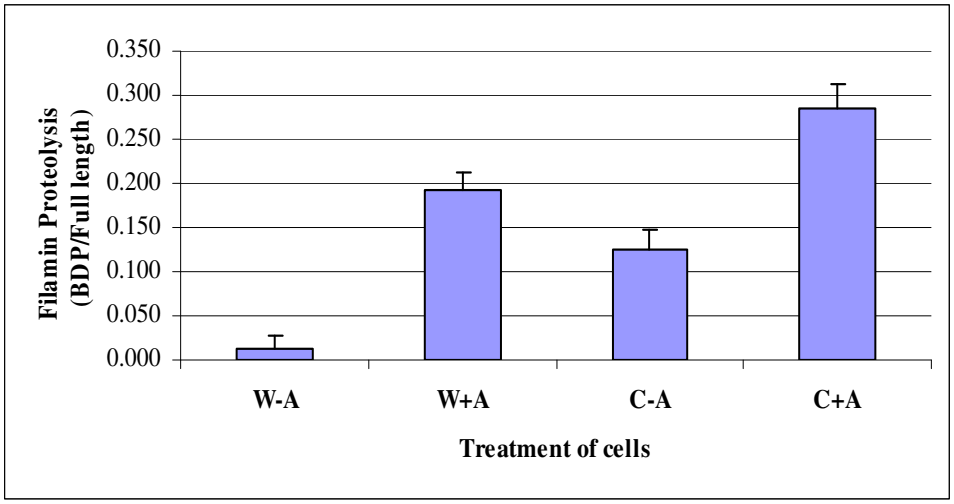


Fig 3.10: PI3-kinase activity is required for filamin proteolysis in glioblastoma cells.

U87MG glioblastoma cells were incubated with PI3-kinase inhibitors, (A) wortmannin (50nM) and (B) Ly294002 (20µM) for 1 hr in 0.2% FBS-DMEM at 37°C. After 1 hr, cells were treated with the calcium ionophore A23187 (10µM) for 5 min. DMSO was used as carrier for both the inhibitors and the ionophore. Reaction was terminated and cells were lysed using 2x SDS buffer containing EDTA and EGTA. Proteins in the lysates were separated by SDS-PAGE and transferred to the nitrocellulose membrane. Proteins were detected using antibodies against filamin (N-terminus), filamin (C-terminus), phospho Akt (S-473), Akt (Pan) and actin.

(C) The densitometry quantification was performed for the assessment of filamin cleavage. The bar graph shows the extent of proteolysis, in terms of the ratio of breakdown product (BDP) to full length protein, assessed from the immunoblot probed with anti-filamin (N-terminus) antibody in wortmannin and A23187 treated samples in (shown in the top panel of A). Legends: W-wortmannin; A-A23187; C-carrier. n = 3 ± SEM.

enzymes. We also verified that the reduction in Akt phosphorylation is not a result of the decrease in the full length protein level, by western blotting of cell lysates with Akt (Pan) antibody which recognizes both phosphorylated and unphosphorylated forms of Akt. The phosphorylation of Akt is also accompanied with a shift in its electrophoretic mobility to slower migrating species (top band) and is resolved from the unphosphorylated Akt proteins (Fig. 3.9). Immunoblotting for actin showed equal loading of protein samples. To further eliminate any variability between protein concentrations in the lysates, the cleavage of filamin was quantified as the ratio of breakdown product and the full length protein. Inhibition of PI3-kinase activity resulted in 90% reduction in the cleavage of filamin under basal condition. Stimulation with the calcium ionophore increased filamin breakdown in general; however, an even greater increased cleavage in the absence of wortmannin and LY294002 strongly suggests for the PI3 kinase regulation of the calcium activated calpain 2 proteolysis of filamin in glioblastoma cells. Importantly, the antibodies specific for the N-terminal and C-terminal residues of filamin primarily identified breakdown products of ~190 kDa and ~ 100 kDa molecular sizes respectively. Though, the generation of these breakdown products of filamin by calpain has been reported by previous research groups (Fox et. al, 1985; Gorlin et. al., 1992), the regulation of this proteolysis by PI3 kinase pathway has never been studied. Our study provides, for the first time, the molecular mechanism regulating calpain proteolysis of filamin in glioblastoma cells. Comparing the breakdown products from chicken gizzard and human filamin, it

appears that calpain 2 cleavage at lysine 268 might be secondary to that in hinge 1 region which can be explained based on the relatively unfolded structure of hinge regions. In summary, our data suggests that PI3-kinase activity is required for the proteolysis of filamin in glioblastoma cells.

Conclusion

This study focused to understand the molecular mechanisms regulating calpain proteolysis of filamin in glioblastoma cells. The unique capacity of glioblastoma cells to invade the brain tissues poses a challenge to the success of therapeutic strategies. This emphasizes the need to understand the molecular events regulating migration and invasion of these cells to be able to prevent tumor cell dispersal into the brain so that focal therapies such as surgical tumor resection and radiation treatments could be effectively applied. To facilitate their migration through the extracellular matrix posing immense degree of barriers and obstructions, cells demonstrate dynamic reorganization of actin cytoskeleton which is controlled by many structural and signaling proteins regulating the elongation, branching, cross-linking and attachment of actin filaments to the extracellular matrix (Lauffenburger and Horwitz 1996).

Filamin cross-links branched actin filaments into orthogonal networks which provide the viscoelastic resistance to cytoplasmic sheer forces and mechanical strength during cell migration. We present evidence that knockdown of filamin results in increased invasion of glioblastoma cells. Since glioblastoma cells express filamin at high level, we got interested in how these cells achieve immense invasiveness in the brain. Our observation that filamin is constitutively cleaved by calpain 2 in these cells suggests for a mechanism to facilitate migration through the rapid turn over of the orthogonal networks of actin filaments.

This study provides first report that PI3 kinase activity is required for filamin proteolysis by calpain 2 in glioblastoma cells. This finding provides an explanation to a previous report in which PI3 kinase activity was correlated with the increased invasion of glioblastoma cells (Kubiatowski et al. 2001). Further, by using purified full length filamin from chicken gizzard and a truncation form of human filamin protein purified from *E. coli,* we showed that PtdIns (3,4,5)-P_3 binding to filamin regulates its proteolysis by calpain 2 after the residue lysine 268. The cleavage at this site inhibits the binding ability of filamin to actin filaments suggesting a role of PI3 kinase in regulation of the function of filamin protein in cells. However, the breakdown products of filamin from experiments with purified proteins and from human glioblastoma cell lysates suggests that cleavage in the hinge 1 region, as previously reported (Gorlin et al. 1990) appears to be dominant in human filamin and the cleavage after the actin binding domain appears to be prominent in the absence of hinge 1 region (chick filamin).

It is still not known how does PtdIns (3,4,5)-P_3 binding, in correlation with the PI3 kinase regulation of proteolysis, modulates the structural conformation of filamin to facilitate the selective proteolytic cleavage. Further investigations are required to this end and the studies with the filamin mutants that cannot bind PtdIns (3,4,5)-P_3 and/or is resistant to proteolysis by calpain 2 at lysine 268 are currently underway in our lab. This has potential to reveal the precise molecular mechanism controlling filamin proteolysis and its role in glioblastoma cell invasion.

Materials and Methods

Antibodies and reagents

Filamin antibodies, anti-N-terminus monoclonal (E-3) and anti-C-terminus polyclonal (H-300), were from Santa Cruz Biotech (San Jose, CA); anti-calpain 2 was from Triple Point Biologics (Forest Grove, OR); anti-actin (clone AC-40) was from sigma; anti-phosphoAkt (S-473) and anti-Akt (Pan) antibodies were from Cell Signaling Technology (Danvers, MA) and peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Phosphoinositides were purchased from Matreya (State College, PA). Calpain 2 (rat recombinant; specific activity≥1500units/mg) was from Calbiochem. Calcium ionophore A23187 and PI3 kinase inhibitor LY294002 was purchased from Enzo life sciences (Plymouth, PA). GelCode Blue protein staining solution was from Pierce and the nitrocellulose membrane (0.45 μm) was purchased from BioRad. Wortmannin, FBS and Puromycin were purchased from Sigma (St. Louis, MO). DMEM media and Trypsin/EDTA was from Mediatech, Inc. (Manassas, VA). L-glutamine, Geneticin and DAPI were purchased from Invitrogen (Eugene, OR). Matrigel was from BD Biosciences (Bedford, MA) and transwell permeable support (6.5mm diameter, 8.0μm pore size) was purchased from Corning (Corning, NY). CIM-16 plate was ordered from Roche

Applied Sciences. Plasmid midipreparation kit was purchased from Qiagen and FuGENE HD was from Roche Diagnostics (Indianapolis, USA).

Cell culture and knockdown of filamin

Human U87MG glioblastoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM media supplemented with 1% L-glutamine and 10% fetal bovine serum (FBS). Control and filaminA specific shRNA were purchased from SA Biosciences (Frederick, MD). FilaminA (Gene sequence available from accession # NM_001456) target sequence 1, 5'–GGAGTGCTATGTCACAGAAAT-3' (KD1), target sequence 2, 5' –CAGCTCGAGTGCTTGGACAAT-3' (KD2) and scrambled sequence 5'-ggaatctcattcgatgcatac-3' are the nucleotide sequences used to prepare filamin knockdown and control glioblastoma cells. The stocks of high quality transfection grade plasmid of shRNA were prepared from *E. coli.* Briefly, the shRNA plasmids were transformed into competent *E. coli* cells of strain DH5α and the colonies were selected in the presence of ampicillin. Bacterial cultures were grown and plasmids were prepared according to the protocol of plasmid midipreparation kit from Qiagen. Parental U87MG glioblastoma cells were transfected with the control and filamin specific shRNA plasmids using Fugene HD transfection reagent and the polyclonal population of stably transformed cells were selected and maintained with 1µg/ml puromycin. The knockdown of filamin was verified by determining the expression

level of filamin protein in control and knockdown cells by western blotting of the total cell lysate with filamin specific antibodies.

Transwell invasion assay

The upper compartment of the transwell permeable supports with a porous membrane of 8.0µm pore size were coated with 50 µl of 2mg/ml Matrigel composition diluted in ice cold serum free DMEM to form a three dimensional extracellular matrix barrier. After 1 hr of polymerization at 37°C, Matrigel coated transwells were transferred to a 24 well plate containing 650µl of media with or without 10%FBS. Fifty thousand control and filamin knockdown cells, suspended in 100µl serum free media, were added to the upper compartment of the transwells and allowed to invade through the matrix for 3 days at 37°C. After 3 day incubation, the Matrigel and cells remaining in the upper compartment were removed using a cotton swab and the cells which had invaded through the pores on the bottom side of the membrane were fixed with methanol and mounted on a glass slide using ProLong Gold antifade reagent with DAPI (Invitrogen). Images were captured using a cooled CCD camera (CoolSNAP-HQ, Photometrics, Tucson, AZ) connected to the Axiovert 100 microscope (Carl Zeiss, Thornwood, NY) and the number of DAPI stained cells quantified using MetaMorph 6.2 software Molecular Devices, Downingtown, PA).

Real-Time transwell invasion assay

The real time monitoring of glioblastoma cell invasion was performed using xCELLigence from Roche Applied Sciences. This system used a specialized transwell apparatus CIM (Cell Invasion and Migration) plate 16 and the RTCA DP (Real Time Cell Analyzer-Dual Plate) instrument for performing electrical measurements. The CIM plate transwells have a membrane of pore size 8.0µm and the lower side of the upper chamber contains an integrated microelectrode sensor made of gold coating which measures the real time changes in the electrical impedance and provides quantitative information about the number of cells and quality of cell attachment to the membrane. In this study, three different concentration of Matrigel were used in the upper chamber of transwells to make the extracellular matrix of varying density. Since Matrigel polymerizes at RT, the tools for Matrigel handling which included Matrigel, upper chamber of CIM plate, wide bore pipette tips, tubes and serum free media, were precooled at 4°C for one day before the experiment. The assay was performed according to manufacturer's protocol. Twenty microliter of Matrigel diluted in serum free media at concentrations 0.8mg/ml, 0.4mg/ml and 0.2mg/ml were coated in the upper chamber of the transwells and polymerized at 37°C for 4hrs. After polymerization, media was added to the lower chamber and it was assembled with the upper chamber to allow the microelectrode membrane surface to reach equilibrium. Cells were harvested with trypsin-EDTA and suspended in serum free media. Immediately before adding cells, the background impedance measurement of the

media was recorded. Fifty thousand control and filamin knockdown cells were added to the upper chamber of each well and the lower chamber contained DMEM media with or without 10% FBS as chemoattractant. Cells were allowed to settle down for 30 - 45 min at RT and the CIM plate assembly was placed in the RTCA DP analyzer to record the electrical impedance of the membrane for 20 hrs at intervals of 15 min. The invasion data was analyzed using the RTCA software. The relative change in the electrical impedance between measurement at any time (t) and the background value (t_0) is expressed as Cell Index (CI) for each well. Therefore, CI reflects the number of cells invading through the matrix to reach the bottom surface of the membrane and the quality of cell attachment on the membrane. The rate of invasion was determined by calculating the slope of invasion curves between 1 hr, the point of initial measurement after the background reading and 19 hr when the curves attained saturation and experiment was ended. The slope describes the changing rate of cell index value and is correlated with rate of cell invasion. The standard deviation is calculated for the cell index values for each individual well and is represented as the error bar on the slope graph.

Purification of filamin; full length and truncation proteins

Filamin was purified from chicken gizzard as described previously (Feramisco and Burridge 1980). Purified protein was dialyzed in 20mM HEPES (pH 7.0), 50mM NaCl and 1mM EDTA. The cDNA of the wild type GFP-filamin protein with the GFP

tag at the amino-terminus was generously obtained from Dr. Thomas P. Stossel, Harvard Medical School. The site directed mutagenesis was introduced to substitute the amino acid leucine at position 269 with aspartic acid to prepare calpain 2 resistant mutant GFP-filamin proteins. The full length wild type and mutant protein were expressed in U87MG glioblastoma cells by transfection using FuGENE HD and the stably expressing clones were selected using 600 µg/ml Geneticin and the expression of GFP-filamin was maintained at 400µg/ml Geneticin. The mutant cDNA was commercially obtained from Biopioneer.Inc. For the preparation of truncation proteins, the sequence of the wild type and mutant protein coding for amino acids 1-660 was amplified from the full length cDNA of filamin by PCR. The forward primer of sequence 5'-ACAGGAATTCGATCCATGAGTAGCTCC-3' and the reverse primer reading 5'- ACAGAAGCTTCATGAAGGGGCTGAGGC-3' were used to amplify the desired region and the amplified sequence was cloned in the vector pPROEX HTb between EcoRI and HindIII as the restriction sites. The clone was verified by sequencing and the hexa-histidine tagged wild type and mutant proteins were overexpressed in the *E. coli* and purified using Ni-NTA agarose beads according to the manufacturer's protocol (Qiagen). The purified proteins were dialyzed in the same buffer used for the full length protein.

Calpain proteolysis assay

Filamin (1.2μM, dimers) was preincubated with phosphoinositides (50μM) for 15 min at 30°C. The samples for 0 min time point were saved and incubation was continued in the absence or presence of calpain 2 (168nM) and $CaCl_2$ (1mM). The reactions were stopped at defined time points with a modified gel loading buffer containing 250mM Tris-Cl (pH 6.8), 2% SDS, 5mM EGTA, 5mM EDTA, 25mM dithiothreitol, and 10% glycerol, followed by 2 min incubation at 100°C. The proteins were separated by SDS-PAGE and stained with GelCode Blue (Pierce). Images were captured on a Kodak Image Station 440CF.

For Western blotting, Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by the method of Towbin et al (Towbin et al. 1979). The blots were blocked for 1 hr with 5% non-fat dry milk in Tris-buffered saline (20mM Tris base, 137mM NaCl) containing 0.05% Tween-20. The blots were subjected to antibodies treatments diluted in the blocking buffer. The bands were visualized by chemiluminescence using Super Signal West ECL substrate (Pierce). Band intensities were quantified by densitometry using Kodak Image Station 440CF.

F-actin binding assay

The binding of filamin and breakdown products with actin filaments was examined by co-sedimentation assay. Filamin proteolysis by calpain in the presence or absence of PtdIns (3,4,5)-P_3 was performed as described above. After 5 min of

incubation with calpain 2 and $CaCl_2$, the catalytic reaction was stopped by adding 4mM EGTA to the reaction. Actin filaments (10µM) were added and incubation continued for 30 min at room temperature (RT) followed by centrifugation at 100,000 X g for 30 min at RT. The supernatant was separated from the pellet and proteins were suspended in gel loading buffer. Proteins were subjected to SDS-PAGE and stained with GelCode Blue.

PI3 Kinase activity assay

Human U87MG glioblastoma cells ($1.5x10^6$) were seeded in 100mm tissue culture dish and grown in 10% FBS-DMEM media to achieve to 80 to 90% confluency. After 24 hrs, the monolayer of cells was washed with 0.2%FBS-DMEM media and incubation was continued in 0.2%FBS media for additional 12 hrs. The serum deprived cells were subjected to PI3 kinase inhibitors; wortmannin was used at the final concentration of 50nM and Ly294002 at 20µM diluted in DMSO, and incubated for 1 hr at 37°C. The optimal concentration of inhibitor was determined by a dose curve of glioblastoma cells. Calcium ionophore, A23187, was added to the same media at the final concentration of 10µM and incubation was continued for additional 5 min. After gently aspirating the media, cells were lysed with 500 µl of modified gel loading buffer and harvested mechanically with a cell scraper. The suspension of cells was cleared by sonication followed by centrifugation to pellet down insoluble debris.

The clear lysates were boiled at 100°C for 2 min and proteins were analyzed by western blotting as described above.

Concluding discussion and future Studies

Chapter 4

Since its first identification in the late 19th century, glioblastoma has been recognized as diffusely infiltrating tumors (Klatzo 1952) and the invasion of tumor cells into the normal brain tissues is considered as the most insidious feature of this disease (Nakada et al. 2007). Based on the knowledge of genetic mutations and signaling pathways upregulated in glioblastoma cells, a range of individual or multimodal therapeutic approaches have been developed. However, even with the most aggressive therapy, survival for patients has only improved from an average of 10 months to 14 months in the last five years (Van Meir et al. 2010). The studies presented in this thesis aimed to understand the role of calpain proteolysis in regulating glioblastoma cell invasion. This chapter summarizes the main conclusions of this dissertation with an effort to describe how results presented in previous chapters are linked to answer-at least in part-the bigger question of how the invasion of glioblastoma cells is regulated.

Until recently, clinical therapies for glioblastoma have primarily targeted cell surface receptors of up regulated signaling pathways, angiogenesis or extracellular matrix degrading enzymes. However, the past few years have seen increased development of drugs aimed at intracellular signaling pathways such as inhibitors of the PI3 kinase-Akt pathway, mammalian target of rapamycin (mTOR), and histone deacetylase (Van Meir et al. 2010). Emerging evidence for the role of calpain 2 in human cancer cell migration and invasion makes it a potential therapeutic target.

Calpain 2 regulates glioblastoma cell invasion in vitro

This study presents strong evidence for the requirement of calpain 2 for the invasion of glioblastoma cells both *in vitro* and in living brain environment. As discussed before, preventing cellular invasion has the potential to make glioblastoma treatment curative. The *in vitro* real time analysis demonstrated that knockdown of calpain 2 decreases the rate of invasion of glioblastoma cells as a function of extracellular matrix density. The inhibition of calpain 2 resulted in 59%, 22% and 9% decrease in the rate of invasion at Matrigel concentrations of 0.8 mg/ml, 0.4 mg/ml and 0.2 mg/ml, respectively. This suggests that calpain 2 is essential for migration through the dense 3D extracellular matrix and not on two dimensional (2D) surfaces. In a previous report from our lab, we observed 90% decreased invasion for calpain 2 knockdown glioblastoma cells compared to control cells and it was correlated with 39% lower levels of extracellular MMP2 in knockdown cells. However, the drastically decreased ability of calpain 2 knockdown cells to invade high density ECM suggests that degrading ECM with MMPs are not the only cellular adaptations. This includes deformability of the cell shape to be able to squeeze through the narrow pores of the extracellular matrix.

These invasion experiments used Matrigel, an artificial extracellular matrix which is highly rich in fibrous components such as laminin and collagen (Kleinman and Martin 2005). The brain extracellular matrix is primarily composed of non-fibrous

components and the fibrous ECM in the brain is limited to the perivascular areas. Importantly, glioblastoma cells are well adapted to the microenvironment and do not metastasize outside of the brain. Hence, the invasion of glioblastoma cells could be truly validated when examined in brain microenvironment.

Calpain 2 is required for glioblastoma cell invasion in vivo

We used transparent zebrafish embryos to visually monitor the invading tumor cells in the living brain. When fluorescently labeled control and calpain 2 knockdown glioblastoma cells were transplanted into the zebrafish brain, knockdown cells showed limited dispersal away from the initial cluster of cells. In contrast, control cells started to detach and migrate away from the original mass as early as 1 day post injection (dpi) and a widespread distribution of cells was observed 6 dpi. Control cells demonstrated ~3 fold increase in the area of dispersal compared to knockdown cells at 6 dpi. To determine the preferred route of dispersal, we investigated tumor cell invasion in the brain of Tg(*fli*1:egfp) zebrafish, which express GFP in endothelial cells. Our data showed that glioblastoma cells migrated predominantly along the abluminal surface of blood vessels. The association of tumor cells with the blood vessels was quantified and control glioblastoma cells demonstrated 20% increase in the co-localization with blood vessels over 6 days post injection compared to only 6% increase in case of knockdown cells. Together, the results suggest that the expression

of calpain 2 is essential for glioblastoma cell invasion in the brain microenvironment However, further studies are required to determine if calpain 2 directly regulates the association and migration of tumor cells along the blood vessels. Also, investigation is needed to conclude if calpain 2 is required for the migration of glioblastoma cells along the axon fibers in the brain. To further explain their localization with blood vessels, we asked if calpain 2 plays role in angiogenesis of these tumors.

Calpain 2 enhances tumor cell angiogenesis in glioblastoma

Clinical trials of drugs targeting angiogenesis in glioblastoma tumors are underway, however, results have shown very little efficacy. Careful examination of tumor cells injected in the Tg(*fli*1:egfp) zebrafish brain revealed that knockdown of calpain 2 reduces the frequency of angiogenesis. Important to mention here is that vessel-cooption was observed to be the preferred path of dispersal over angiogenesis. Since, calpain 2 knockdown cells showed limited dispersal and mostly remained confined in clusters, more angiogenesis was predicted in the knockdown cells. However, control cells induced reorganization of host's blood vessels as well as angiogenesis more frequently than knockdown cells. This suggested the involvement of calpain 2 in the process of tumor cell angiogenesis. Further investigation is necessary to determine the mechanism involved and the regulation of angiogenesis factor VEGF secretion is a strong candidate for studies.

Invasion of tumor cells requires migration through the dense extracellular matrix of tissues which provides both the substrates for cell membrane attachments to generate traction, as well as a barrier towards the advancing cell body. Proteolytic remodeling of the ECM is considered to be critical for the invasion of tumor cells through the three dimensional matrix (Wolf and Friedl 2005; Zaman et al. 2006). Many studies have reported upregulated expression of matrix degrading proteases such as MMP, urokinase-type plasminogen activator (uPA) and cathepsins in gliomas and have correlated the activity of these enzymes with increased invasion of glioma cells (Nakada et al. 2007; Rao 2003). On the other hand, studies have also reported that matrix degradation is not an absolute requirement for tumor cell invasion and tumor cells demonstrate switching from proteolytic to non-proteolytic mode of invasion when treated with pharmacological inhibitors of MMP, uPA and cathepsins (Friedl and Wolf 2003; Wolf et al. 2003). Moreover that, the clinical trials of MMP and serine protease inhibitors have presented ineffective outcomes, showing significant progression despite inhibitor treatment (Friedl and Wolf 2003).

The protease-independent migration requires membrane flexibility, shape change and ability to squeeze through narrow matrix regions. Importantly, the proteolysis independent migration of tumor cells was shown to be mediated primarily by cortical actin cytoskeleton composed of orthogonal networks of actin. This observation gains further support from a study that suggests that lamellar actin networks provide stability to the membrane and control the productive migration of

the cell (Ponti et al. 2004). Hence, proper regulation of the orthogonal networks of actin filaments is essential for the membrane flexibility and shape deformability of cells during invasion. Since, filamin cross-links actin filaments into orthogonal networks, we became interested in determining the role of filamin in glioblastoma cell invasion.

Filamin expression is restrictive to glioblastoma cell invasion

The data presented in chapter 3 demonstrated that filamin expression is inhibitory to the invasion of glioblastoma cells when the knockdown of filamin resulted in ~3-fold increase in the invasiveness of cells through 2 mg/ml Matrigel. The increase in the invasion of knockdown cells over control cells showed a direct relationship with the density of the ECM. This could be explained by the loss-of-ability of control cells to traverse through the gradually narrowing pores of extracellular matrix with increasing concentration of Matrigel. We asked; if filamin restricts migration through dense ECM, then how do glioblastoma cells invade in the human brain?

Careful examination of filamin breakdown products in the cell extracts of control and calpain 2 knockdown glioblastoma cells identified a novel band running at ~30 kDa. Further, western blotting with the anti-filamin antibody indicated this fragment originated from the amino terminal end of filamin by calpain 2-mediated

proteolysis. Since the size of the fragment is close to that of the actin binding domain of filamin, cleavage at this site was anticipated to have a regulatory effect on the function of filamin. This got us interested in determining the regulation of proteolysis in the cell.

PI3 kinase regulates filamin cleavage by calpain 2

Calpain 2 proteolysis of purified filamin protein in the presence and absence of PtdIns (3,4,5)-P_3 showed that binding of PtdIns (3,4,5)-P_3 to filamin induces proteolysis by calpain 2 at the amino-terminal end producing a stable breakdown product of ~250 kDa with intact C-terminal residues. The sensitive site of cleavage on the full length protein was determined by subjecting the fragment to N-terminal sequencing and was mapped to be between residues Lys268 and Lue269 which lies in the linker region between the actin binding domain and the rod-domain 1. Together, the results show that PtdIns (3,4,5)-P_3 binding to filamin induces cleavage by calpain 2 which removes the actin binding domain and abolishes the actin binding ability of filamin. PtdIns (3,4,5)-P_3 mediated regulation of filamin proteolysis in human glioblastoma cells was tested by subjecting glioblastoma cells to PI3 kinase inhibitors and calcium ionophore was used to stimulate calcium-activated proteolysis. Comparing the lysates from glioblastoma cells treated with or without PI3 kinase inhibitors revealed that inhibition of PI3 kinase resulted in decreased cleavage of

filamin under basal condition as well as when stimulated with calcium ionophore. In the absence of PI3 kinase inhibitors, stimulation with the ionophore increased filamin breakdown products suggesting this to be calcium activated cleavage which indicates to calpain 2. These results show that PI3 kinase activity is required for the calpain 2 induced proteolysis of filamin in glioblastoma cells.

Finally, the results from chapter 2 showed that calpain 2 is required for the invasion of glioblastoma cells. Results presented in chapter 3 indicate that calpain 2 cleaves filamin which is regulated by the PtdIns (3,4,5)-P_3 binding. Revising the facts from chapter 1, glioblastoma cells demonstrates a net enhanced PI3 kinase activity which is attributed to the increased activation of EGFR and PI3 kinase and deletion of the antagonist PTEN gene. Therefore, the cell membrane of glioblastoma cells contains a constitutively increased level of PtdIns (3,4,5)-P_3. Further, a migrating cell has been shown to demonstrate a net increase in the PtdIns (3,4,5)-P_3 concentration at the leading edge making it available for interacting with cytoskeletal proteins such as filamin.

I propose, at the conclusion of my thesis, that PtdIns (3,4,5)-P_3 binding increases filamin proteolysis by calpain 2, which results in the reorganization and turnover of the actin networks at the leading edge making cells more flexible and allowing deformation of cell shape which leads to the increased invasion of glioblastoma cells through the pores of ECM.

Future Studies

The insights presented in this dissertation about the potential regulators of the glioblastoma invasion can be further built upon to precisely determine the underlying mechanisms which can be aimed for targeted therapy.

How does calpain 2 regulate tumor cell migration along blood vessels?

Although the literature had evidence for the glioblastoma cell spreading along the vascular tracts in the brain, the factors regulating this pattern was not known. We discovered that calpain 2 is required for glioblastoma cell dispersal in the brain and tumor cells migrate primarily along the surface of blood vessels. However, it is not clear if calpain 2 is essential for the association of tumor cells with the blood vessels which requires further investigations. Our lab reported a decrease in the MMP2 levels from calpain 2 knockdown cells in vitro (Jang et al. 2010), which provides the ground to determine if it is the MMP2-mediated ECM digestion that prevents invasion of glioblastoma cells in vivo. The experimental approach could use MMP inhibitors to monitor the effect of inhibiting pericellular proteolytic digestion on the association of tumor cells along the vascular surfaces. Tg(*fli*1:egfp). It would also be important to know if calpain 2 controls expression of the MMP 2 gene or activation of MMP in glioblastoma cells to design further strategies for inhibiting tumor cell invasion.

Another important area of investigation is the proteolysis of e-cadherin in regulation of tumor call invasion. The cadherin family of transmembrane protein mediates the cell-cell adhesions and has been shown to be a substrate of calpain. Inability of calpain 2 knockdown cells to invade could arise due to the increased intercellular adhesion and need verification. Yet another area of investigation would be to determine the calpain 2 regulation of VEGF in glioblastoma cells. Importantly, though, our results showed that blood vessel surfaces was chosen as the primary route of dispersal, further studies are needed to verify if calpain 2 controls cell migration along the axonal wall of neurons as well.

Does calpain 2 regulate PI3kinase/Akt pathway?

The current focus of our lab is in the regulation of Akt by calpain 2 in glioblastoma cells. As discussed in chapter 1, PI3 kinase activation is a frequent event in glioblastoma tumors and is a significant target in focus. Downstream of activation, PI3 kinase leads to the activation of Akt (protein kinase B) through phosphorylation. Akt has been found to be activated in invasive glioblastoma cells and its activation is controlled by the PHLPP phosphatase in vivo (Molina et al. 2010). Determination of the calpain 2 regulation of Akt pathway either by direct proteolysis of Akt or indirectly through phosphatases would have high impact in terms of advancing our understanding of glioblastoma invasion.

We presented that filamin proteolysis by calpain 2 is regulated by the binding of PtdIns (3,4,5)-P_3 to filamin and proposed this to be a mechanism for the turnover of actin networks at the leading edge. Mutant filamin proteins resistant to proteolysis by calpain 2 and filamin unable to bind PtdIns (3,4,5)-P_3 have potential to identify the cross-talk between calcium signaling through calpain activation and PI3 kinase induced filamin proteolysis. Live cell imaging and FRAP could be used on cells expressing wild type and calpain 2 resistant filamin to understand the calpain 2 regulation of lamellipodial flexibility and the turnover actin networks.

Is calpain 2 required for the invasion of all types of gliomas?

All the data presented in this dissertation comes from experiments performed on U87MG glioblastoma cells. Hence, further studies using different glioblastoma cell lines would be essential to establish that results presented in this report are not cell type specific.

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