Chapter 7
Signaling Determinants of Glioma Cell Invasion

Aneta Kwiatkowska and Marc Symons

Abstract Tumor cell invasiveness is a critical challenge in the clinical management of glioma patients. In addition, there is accumulating evidence that current therapeutic modalities, including anti-angiogenic therapy and radiotherapy, can enhance glioma invasiveness. Glioma cell invasion is stimulated by both autocrine and paracrine factors that act on a large array of cell surface-bound receptors. Key signaling elements that mediate receptor-initiated signaling in the regulation of glioblastoma invasion are Rho family GTPases, including Rac, RhoA and Cdc42. These GTPases regulate cell morphology and actin dynamics and stimulate cell squeezing through the narrow extracellular spaces that are typical of the brain parenchyma. Transient attachment of cells to the extracellular matrix is also necessary for glioblastoma cell invasion. Interactions with extracellular matrix components are mediated by integrins that initiate diverse intracellular signalling pathways. Key signaling elements stimulated by integrins include PI3K, Akt, mTOR and MAP kinases. In order to detach from the tumor mass, glioma cells secrete proteolytic enzymes that cleave cell surface adhesion molecules, including CD44 and L1. Key proteases produced by glioma cells include uPA, ADAMs and MMPs. Increased understanding of the molecular mechanisms that control glioma cell invasion has led to the identification of molecular targets for therapeutic intervention in this devastating disease.

Keywords Glioblastoma • Invasion • Migration • Rho GTPase • PI3K • Akt • Protease

Abbreviations

ATX	Autotaxin
BEHAB	Brain-enriched hyaluronic acid binding protein
DG	Dentate gyrus
DOCK180	Dedicator of cytokinesis 180
ECM	Extracellular matrix

A. Kwiatkowska (✉) • M. Symons
Center for Oncology and Cell Biology, Laboratory for Brain Tumor Biology,
Feinstein Institute for Medical Research, 350 Community Dr, 11030 Manhasset, NY, USA
e-mail: akwiatkows@nshs.edu; msymons@nshs.edu

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ELMO1	Engulfment and cell motility-1
Gab1	Grb-2 associated binder-1
GAP	GTPase activating protein
GBM	*Glioblastoma multiforme*
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
HGG	High grade glioma
LGG	Low grade glioma
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
MCP-1	Monocyte chemotactic protein-1
mDia	Mammalian homolog of Drosophila diaphanous
MMP	Matrix metalloproteinase
MT1-MMP	Membrane type metalloproteinase 1
NB	Non-neoplastic brain
NSC	Neural stem cell
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PDK1	PI3K-dependent kinase 1
PH	Pleckstrin homology domain
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
$p130^{Cas}$	Crk-associated substrate
ROCK	Rho-associated coiled-coil forming kinase
RTK	Receptor tyrosine kinase
SPARC	Secreted protein acidic and rich in cystein
SVZ	Subventricular zone
TAM	Tumor associated macrophage
TGFβ-1	Transforming growth factor-β-1
TIMP	Tissue inhibitor of metalloproteinases
TN-C	Tenascin-C
TSP-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1

7.1 Invasiveness of Glioma Cells

Malignant gliomas are characterized by a high proliferation rate, increased angiogenesis and diffusive growth. There is rarely a clear border between the tumor and the surrounding brain parenchyma. This complicates complete surgical resection, and as a consequence, usually within months after surgery, recurrent neoplasms are established in the proximity of the resection zone.

The pattern of glioma cell migration in the brain is not random. Tumor cells infiltrate the brain parenchyma as individual cells or isolated clusters, distributed mainly along blood vessels (perivascular zone), fiber tracts and subependyma (Farin et al. 2006; Giese et al. 2003; Scherer 1940). Although it has been shown that C6 rat glioma cells can intercalate between endothelial cells and astrocyte end feet, or in some cases displace astrocytes from entothelial cells, they rarely invade the blood vessel lumen (Farin et al. 2006). This is consistent with the well-established clinical observation that gliomas hardly metastasize to other organs or the spinal cord (Armstrong et al. 2011; Birbilis et al. 2010; Gotway et al. 2011; Schonsteiner et al. 2011).

In the resting adult brain, migrating neural stem cells (NSC) mainly originate from two niches, the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus. From there, these neural progenitors migrate towards the olfactory bulb (OB) or granular cell layer of the DG, respectively. There is evidence that the adult SVZ continues to generate glial progenitor cells. However, the vast majority of these progenitor cells reside outside the neurogenic niches and usually do not migrate (Cayre et al. 2009). Progenitor cell migration can be stimulated however by pathological conditions such as inflammation or stroke (Cayre et al. 2009; Zhang et al. 2005). Time-lapse microscopy analysis of neural and glial progenitor migration revealed that these cells are moving in a unique two-step process: continuous extension of long leading protrusions followed by saltatory movement of the cell body (Bellion et al. 2005; Cayre et al. 2009; Kakita and Goldman 1999).

Interestingly, the pattern of glioma cell migration strongly resembles the pattern of glial progenitor cell migration during normal brain development (Beadle et al. 2008; Farin et al. 2006; Kakita and Goldman 1999). Moreover, it was shown that the saltatory mode of migration reflects the requirement for the nucleus to squeeze through the small extracellular spaces that characterize the brain parenchyma (Beadle et al. 2008). This nuclear squeezing is dependent on myosin-based contractility, as it is inhibited by both, blebbistatin, an inhibitor of myosin II, and Y27632, a small molecule inhibitor of Rho-associated coiled-coil forming kinase (ROCK), a Rho effector protein that controls myosin II activation. Thus, studying the mechanisms that drive progenitor cell migration during brain development should facilitate our understanding of the signaling pathways that are involved in glioma cell dissemination.

There is growing evidence that antiangiogenic therapy prolongs progression-free survival (Norden et al. 2009). Unfortunately, treatment with bevacizumab (an antibody against vascular endothelial growth factor, VEGF) or cediranib (a VEGF receptor tyrosine kinase inhibitor) have resulted in little improvement in overall survival (de Groot et al. 2010). It has been reported that bevacizumab treatment results in a shift to more infiltrative tumor growth (de Groot et al. 2010; Lucio-Eterovic et al. 2009). This behavior is recapitulated in experiments using a model of primary human glioblastoma cells in the rat, in which treatment with bevacizumab was accompanied by a strong increases in the number of invading glioblastoma cells and in the distance that they travel from the tumor core (Keunen et al. 2011). Thus, combining bevacizumab treatment with an anti-invasion therapy may be beneficial.

7.2 Factors That Control Glioma Invasion

Both the development of glioma as well as their invasive behavior is strongly controlled by the local microenvironment. Factors secreted by tumor cells diffuse into the peritumoral stroma affecting the local tissue. In response, cells in brain parenchyma secrete ligands that stimulate enhanced glioma invasion and/or change the local microenvironment into a more permissive one for tumor progression (Hoelzinger et al. 2007).

7.2.1 Autocrine Factors

Cells residing in the brain are embedded in extracellular matrix (ECM) primarily composed of hyaluronan and proteoglycans. The latter include brevican (brain enriched hyaluronic acid binding protein), neurocan, as well as the glycoproteins SPARC (secreted protein acidic and rich in cystein, also known as osteonectin), tenascin-C (TN-C) and thrombospondin-1 (TSP-1). Collagens, laminins and fibronectins, which are widely found in other tissues, are present only in the proximity of blood vessels

in the brain (Bellail et al. 2004). Importantly, overexpression of hyaluronan, vitronectin, osteopontin, tenascin-C and BEHAP correlates with tumor grade (Delpech et al. 1993; Higuchi et al. 1993; Jaworski et al. 1996; Mahesparan et al. 2003; Saitoh et al. 1995; Toy et al. 2009; Viapiano et al. 2003). ECM components play an important role in the regulation of signaling pathways that are responsible for tumor growth, proliferation, adhesion, migration and angiogenesis (Akiyama et al. 2001; Higuchi et al. 1993; Matusan-Ilijas et al. 2008; Zagzag et al. 1995, 1996). Therefore, it is likely that glioma progression is in part mediated by alterations in ECM composition. This is illustrated by the finding that experimental inhibition of osteopontin expression by U87 glioblastoma cells causes a significant reduction in the number of migrating cells *in vitro* and slower tumor growth *in vivo*, as knock down of osteopontin in U87 cells reduce the proliferation of cells within experimental glioma tumors (Lamour et al. 2010).

Glioma cells also secrete factors that, upon binding to their cognate receptor tyrosine kinases, contribute to enhanced tumor cell proliferation and motility. Important factors are epidermal growth factor (EGF), transforming growth factor α (TGFα), heparin-binding epidermal growth factor (HB-EGF), platelet derived growth factor (PDGF) and hepatocyte growth factor/scatter factor (HGF/SF) (Brockmann et al. 2003; Hoelzinger et al. 2007; Koochekpour et al. 1997; Ramnarain et al. 2006; Shih and Holland 2006). A detailed discussion about receptor tyrosine kinase signaling in glioma can be found in other chapters in this book (mainly in Chap. 8).

In addition to conventional autocrine signaling, recent data imply bioactive phospholipids in the regulation of glioblastoma dissemination. A good example is autotaxin (ATX), an enzyme with lysophospholipase D activity that converts lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA). Glioblastoma cells *in vivo* are exposed to various plasma components when the blood brain barrier (BBB) is disrupted (Seitz and Wechsler 1987; Wolff and Boker 1989). One such component is LPC, which is present in plasma at a high concentration (100–300 μM) (Kishimoto et al. 2002). Interestingly, in most analyzed glioblastoma tissues and glioma cell lines, autotaxin and LPA_1 receptor are highly expressed (Kishi et al. 2006). In addition, there is more ATX expressed in glioblastoma cells in the invading rim in comparison to those in the tumor core (Hoelzinger et al. 2005). Experimental overexpression of ATX enhances cell migration both *in vitro* and in *ex vivo* brain slices (Kishi et al. 2006; Hoelzinger et al. 2008). Conversely, inhibition of ATX expression leads to decreased invasiveness of cells in a three-dimensional collagen spheroid invasion assay in response to LPC (Hoelzinger et al. 2008). Taken together, these data strongly suggest a role for ATX in glioblastoma invasion.

7.2.2 Paracrine Factors

Microglia and macrophages can constitute up to 30 % of the total number of cells in glioblastomas, anaplastic astrocytomas and rodent gliomas (Badie et al. 2002; Badie and Schartner 2000; Charles et al. 2011; Roggendorf et al. 1996). For many years, tumor-associated macrophages (TAMs) were considered as a part of the immune response against the tumor or a nonspecific reaction evoked by local damage (Badie et al. 2002; Flugel et al. 1999; Watters et al. 2005). Although the role of microglia and TAMs in brain tumors is not fully understood, recent studies suggest that microglia/macrophages may be attracted by tumor-secreted factors such as monocyte chemotactic protein-1 (MCP-1) (Platten et al. 2003), in order to promote tumor growth and dissemination into the brain parenchyma (Badie and Schartner 2001; Charles et al. 2011; Markovic et al. 2009; Platten et al. 2003; Sliwa et al. 2007; Watters et al. 2005; Wesolowska et al. 2008; Zhai et al. 2011). It is thought that microglia, in response to glioma stimulation, produce diverse factors, including matrix metalloproteinases such as membrane type matrix metalloproteinase-1 (MT1-MMP) that contribute to ECM degradation and the processing of growth factors (Markovic et al. 2009). Moreover, microglia produce cytokines such as transforming growth factor β-1 (TGFβ-1) that promote tumor cell proliferation and

migration (Watters et al. 2005; Wesolowska et al. 2008). In addition, TAMs have been shown to activate NF-κB transcription factor-dependent production of interleukin-8 (IL-8) by gliomas (Hong et al. 2009). This chemokine also stimulates tumor cell migration (Wakabayashi et al. 2004). Interestingly, T cells are rarely seen in gliomas (Morimura et al. 1990) which correlates well with compromised microglia-mediated antigen presentation in these tumors (Badie et al. 2002; Flugel et al. 1999). In conclusion, the current literature suggests that glioblastoma tumors re-educate microglia/macrophages from an inflammatory phenotype to an anti-inflammatory and pro-tumor phenotype (Gabrusiewicz et al. 2011).

The presence of a neoplasm in the brain affects the function of parenchymal cells. As mentioned above, glioma cells stereotactically inoculated into the rat or mouse brain accumulate around blood vessels, where they displace astrocytic end feet from the endothelial cells. Astrocytes that withdraw their processes from the vascular wall become reactive (Nagano et al. 1993; Zagzag et al. 2000). Reactive astrocytes have been shown to secrete urokinase-type plasminogen activator (uPA) *in vitro*. uPA is a serine protease that converts plasminogen produced by glioma cells into active plasmin. Plasmin, in turn, activates pro-metalloproteinase-2 (pro-MMP-2) that is secreted by astrocytes (Le et al. 2003), thereby increasing local proteolytic activity. Thus, brain tumor dissemination is complex and relies on interactions between several cell types in a way that is not readily recapitulated using *in vitro* assays.

Expression of chemokine receptor 4 (CXCR4) and its ligand chemokine ligand 12 (CXCL12, also known as SDF-1) is increased in human astrocytomas (Barbero et al. 2002; Bajetto et al. 2006) and significantly more CXCR4 is expressed in invasive tumor foci as compared to the non-invasive tumor core (Ehtesham et al. 2006). Moreover, SDF-1 produced by the endothelium has been shown to stimulate U87 glioblastoma invasion *in vitro*. Under these conditions, glioblastoma cells produce more MMP-9 and cathepsins (another class of proteases, see below Sect. 7.4) (Kenig et al. 2010), enzymes that promote glioma cell invasion by cleavage of ECM components as well as activation of pro-enzymes present in the extracellular space (Kobayashi et al. 1991; Mai et al. 2002). The observations that inhibiting CXCR4, either by CXCR4-neutralizing antibodies, CXCR4-directed siRNA technology or AMD3100, a CXCR4-specific inhibitor, impaired glioma cell invasion *in vitro*, strongly supports a role for SDF-1 in the invasive behavior of glioma (Ehtesham et al. 2006; Hong et al. 2006).

7.3 Signaling Mechanisms That Control Glioma Invasion

7.3.1 Integrins

Cell migration requires anchoring of the leading edge to the ECM and release of cell attachment at the rear (Ridley et al. 2003). Integrins are heterodimeric transmembrane receptors, composed of α and β subunits. There are 8 β and 18 α subunits, combinations of which determine substrate specificity (D'Abaco and Kaye 2007). Integrins participate in bidirectional signaling across the plasma membrane. Integrins can be stimulated to bind ligands by intracellular signaling (inside-out signaling) or become activated upon interaction with extracellular ligands (outside-in signaling). Conformational changes within integrins enable association with and activation of diverse cytoplasmic adaptor proteins (Fagerholm et al. 2004; Hynes 2002). Integrins lack catalytic activity, and recruitment of these adaptor proteins is thought to be regulated by phosphorylation of the integrin cytoplasmic tails (D'Abaco and Kaye 2007; Fagerholm et al. 2004).

Integrins have been proposed to play a key role in glioma biology including cell migration (D'Abaco and Kaye 2007). Many studies have shown overexpression of the β1 subunit in malignant gliomas in

comparison to normal brain tissue and β1-blocking antibodies decrease glioma cell migration and invasion *in vitro* (Paulus et al. 1993; Rooprai et al. 1999; Tysnes et al. 1996). Experimental overexpression of α6 subunit in U87 glioma cells bearing a high level of β1 subunits increases glioma cell migration and invasion *in vitro*, enhances dissemination of glioma cells *in vivo* and the formation of infiltrative foci at the margin of tumors established in nude mice (Delamarre et al. 2009). In addition, immunohistochemistry studies have demonstrated that αvβ3 and αvβ5 are overexpressed in both glioma cells and tumor vasculature and that their expression is correlated with tumor grade (Bello et al. 2001a; Stupp and Ruegg 2007). Interestingly, stimulation of human glioblastoma cells with either TGF-β1 or TGF-β2 leads to an increase of αvβ3 at the cell surface and enhanced glioma cell migration (Platten et al. 2000). TGF-β-dependent stimulation of glioblastoma cell migration was shown to be abrogated by echistatin (a 49 amino acid peptide that binds integrins and blocks downstream signaling) or an αvβ3 neutralizing antibody (Platten et al. 2000).

αvβ3 and αvβ5 are the first integrins targeted to suppress tumor angiogenesis. Three classes of integrin inhibitors are under investigation: monoclonal antibodies targeting the extracellular domain of αvβ3 (e.g. vitaxin, phase II clinical trials completed) (Stupp and Ruegg 2007; Tucker 2006), synthetic peptides containing an RGD sequence recognized by both αvβ3 and αvβ5 (e.g. cilengitide, phase II clinical trials completed) (Gilbert et al. 2012; Reardon et al. 2008; Tucker 2006), and an RGD peptidomimetic antagonist of αvβ3 (e.g. S247) (Abdollahi et al. 2005). Clinical evidence shows modest antitumor activity of cilengitide (Gilbert et al. 2012; Reardon et al. 2008). However, as for other antiangiogenic drugs, the targeting of integrins may be most effective in combination with other therapeutic modalities, such as radiotherapy, especially as αvβ3 expression in endothelial cells is increased by radiation (Abdollahi et al. 2005; Gilbert et al. 2012; Stupp and Ruegg 2007).

7.3.2 Rho GTPases

Rho GTPases constitute a family of 22 members in humans and regulate a large number of the cellular functions, such as actin organization, cell migration, invasion (Burridge and Wennerberg 2004; Bustelo et al. 2007; Schmitz et al. 2000), cell proliferation and survival (Croft and Olson 2006; Gomez del Pulgar et al. 2005). They switch between the GTP-bound active state and the GDP-bound inactive state. The GTPase cycle is tightly controlled by three groups of regulators: guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Rossman et al. 2005; Schmidt and Hall 2002). Rho GTPases are activated by GEFs which promote exchange of GDP to GTP. Subsequently, GTPases are inactivated by interaction with GAPs, that stimulate intrinsic GTP hydrolysis (Moon and Zheng 2003). RhoGDIs play a dual role in the regulation of Rho proteins (Garcia-Mata et al. 2011). On the one hand, they inhibit spontaneous release of GDP, thus clamping GTPases in the inactive state, on the other, by binding to the prenyl groups of GTPases, they prevent association with and facilitate extraction of GTPases from membranes. Interaction with additional proteins, called "GDI displacement factors" releases the GDI from the GTPase, thereby facilitating access to GEFs (Dransart et al. 2005).

RhoA, Rac1 and Cdc 42 are the best characterized of the Rho GTPases (Heasman and Ridley 2008; Raftopoulou and Hall 2004). Rac1 controls the formation of lamellipodia, which are flat, actin-rich membrane protrusions at the cell periphery. RhoA regulates actomyosin contractility, formation of focal adhesions and stress fibers, and retraction of the tail of the cell during cell migration. Cdc42 regulates the formation of filopodia (thin, finger-like membrane protrusions) and is a key control element in the regulation of cell polarization.

Rac proteins (Rac1, Rac2 and Rac3) are highly homologous, but differ in their tissue distribution (Heasman and Ridley 2008). Rac1 is ubiquitously expressed, Rac2 is specific for hematopoietic cells and Rac3 is abundantly expressed in neural tissues (Burridge and Wennerberg 2004). Although no

significant change was observed in Rac1 mRNA expression levels across astrocytoma grades, in a large set of glioblastoma tumors, Rac1 mRNA was shown to be elevated in tumors of patients with shorter survival (Salhia et al. 2008). Interestingly, immunohistochemical analysis revealed a strong increase in Rac1 protein expression with tumor grade (Salhia et al. 2008). These findings are consistent with an earlier proteomic study showing that Rac1 protein levels are increased in high-grade (85 %) versus low-grade (20 %) gliomas and correlate with poor survival (Iwadate et al. 2005). Taken together, these data suggest that Rac1 is regulated at the translational and/or protein stability level. Notably, Rac1 has been shown to display marked plasma membrane localization in a fraction of glioblastoma tumor samples, but not in low grade astrocytomas (Salhia et al. 2008). Plasma membrane localization of Rac1 reflects a high activation state, suggesting that this GTPase may contribute to the malignant behavior of glioblastomas.

siRNA-mediated depletion of Rac1 or Rac3 significantly decreases glioblastoma cell invasion in a Matrigel invasion assay (Chan et al. 2005). Interestingly, in contrast to depletion of Rac1, Rac3 depletion only slightly inhibits glioblastoma migration, implying that Rac1 and Rac3 may be involved in different mechanism that contribute to cell invasion (Chan et al. 2005). Depletion of Rac1 also significantly inhibits glioblastoma cell invasion in *ex vivo* brain slices (Chuang et al. 2004), underlining the importance of this GTPase in glioblastoma invasiveness.

There are a number of Rac effectors (proteins that bind to active Rac and relay its functions) that control cell migration and invasion (Burridge and Wennerberg 2004), although their role in glioma invasion still largely remains to be characterized. The Rac effector synaptojanin 2 (Malecz et al. 2000), a phosphatidylinositol 5-phosphatase, has been shown to regulate glioblastoma cell migration and invasion *in vitro*. Synaptojanin 2 localizes to both invadopodia and lamellipodia and is thought to control the formation of these structures (Chuang et al. 2004).

In addition to overexpression of Rho GTPases, aberrant expression or genetic alterations of upstream regulators has been detected in a variety of human cancer types (Gomez del Pulgar et al. 2005). In particular, the Rho GEFs Ect2, Vav3, Trio and SWAP-70 display increased expression at the message level in brain tumors when compared to normal brain tissue and expression is correlated with poor patient survival (Salhia et al. 2008; Seol et al. 2009; Tu et al. 2010). Notably, depletion of any of these GEFs significantly inhibits glioblastoma cell migration and invasion (Salhia et al. 2008; Seol et al. 2009).

Dedicator of cytokinesis 180 (DOCK180) and engulfment and cell motility 1 (ELMO) form a bipartite GEF that activates Rac proteins (Cote and Vuori 2007; Lu and Ravichandran 2006). Depletion of either ELMO1 or DOCK180 strongly reduces Rac1 activation and glioblastoma cell invasion (Jarzynka et al. 2007). Interestingly, both DOCK180 and ELMO proteins display increased expression in the invading tumor rim compared to the tumor core of human glioma specimens.

An additional mechanism that could contribute to an increase in Rho GTPase activation levels in cancer is the downregulation of GAPs. One example in gliomas is the Rac GAP β2-chimaerin, which shows high levels in normal brain and low-grade astrocytomas in comparison to malignant gliomas (Yuan et al. 1995). Thus, loss of β2-chimaerin may contribute to the increase in Rac activation levels in glioblastoma.

There are two major effector proteins of RhoA, RhoB and RhoC GTPases: mammalian homolog of Drosophila diaphanous (mDia) and Rho-associated coiled-coil forming kinase (ROCK) (Narumiya et al. 2009; Wheeler and Ridley 2004). mDia belongs to the formin protein family and catalyzes actin nucleation and polymerization. mDia depletion in glioma cells interferes with microtubule stabilization, cell polarization, focal adhesion turnover and results in attenuated cell migration (Yamana et al. 2006). Analysis of the underlying mechanism revealed that mDia controls actin-dependent c-Src recruitment to focal adhesions, phosphorylation of the adaptor protein Crk-associated substrate ($p130^{Cas}$), Rac activation and subsequent focal adhesion disassembly. mDia also promotes the accumulation of Cdc42 and adenomatous polyposis coli (APC) at the front of the cell, which may provide a mechanism for the role of mDia in cell polarization.

ROCK phosphorylates and inactivates myosin phosphatase and directly phosphorylates myosin light chain. These two actions of ROCK stimulate actomyosin contractility. ROCK also phosphorylates and activates LIM kinase that in turn phosphorylates and inactivates the actin filament severing protein cofilin. As mentioned above, ROCK has been implicated in nuclear squeezing and glioblastoma cell invasion in *ex vivo* brain slices (Beadle et al. 2008). ROCK is also necessary for glioma cell migration along myelinated retinal axons *in vitro*, where ROCK inhibition was shown to result in shorter lamellipodia and non-polarized extension of filopodia (Oellers et al. 2009). In contrast, inhibition of ROCK has been shown to increase the migratory behavior of glioma cells under less physiological conditions, such as a two-dimensional cell migration in a radial migration assay and a transwell Matrigel invasion assay (Salhia et al. 2005), underlining the importance of physiologically relevant experimental conditions to assess the role of actomyosin contractility in cell invasion.

7.3.3 PI3K and Phospholipid Signaling

Phosphatidylinositol 3-kinases are lipid kinases that phosphorylate the 3 position of the inositol ring of phosphatidylinositols and phosphoinositides (Endersby and Baker 2008). Class IA PI3Ks are heterodimers composed of a regulatory subunit (five isoforms encoded by three genes p85α, p55α, p50α (*PIK3R1*), p85β (*PIKR2*) and p55γ (*PIKR3*)) and a catalytic subunit (p110α, p110β and p110δ). The three catalytic subunit isoforms are encoded by the *PIK3CA*, *PIK3CB* and *PIK3CD* genes (Furnari et al. 2007).

PI3Ks are regulated through the inhibitory effect of the regulatory subunit on the catalytic subunit (Yu et al. 1998). Upon direct binding of the regulatory subunit to phosphorylated receptor tyrosine kinases (RTKs), including c-Met, VEGFR and PDGFR (Escobedo et al. 1991; Igarashi et al. 1998; Ponzetto et al. 1993), p110 inhibition is released (Bader et al. 2005).

PI3K is also regulated by Src family kinases. An interesting example is that binding of CD95L (also known as FasL) to CD95 on glioblastoma cells recruits Yes (a member of Src kinase family) to the receptor. Yes, in turn, recruits the p85 subunit of PI3K leading to PI3K activation and enhanced glioblastoma cells migration *in vitro*. Interestingly, neutralization of CD95L in a murine intracranial model of GBM as well as treatment of C6 glioma cells with FasL Interfering Protein (FIP) reduces the number of invading cells (Kleber et al. 2008; Wisniewski et al. 2010). It is remarkable that CD95L, which promotes apoptosis in many cells, also stimulates their invasiveness. Notably, in general, glioblastoma tumors are resistant to CD95-induced apoptosis and even induce CD95L expression in the surrounding tissue. These findings suggest that targeting the CD95L/CD95 system may be beneficial in the treatment of glioblastoma tumors, as no CD95 is expressed in the healthy brain (Kleber et al. 2008).

Class I PI3Ks preferentially convert phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) to phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) (Martelli et al. 2010). The formation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ triggers the recruitment of proteins with pleckstrin homology (PH) domains to the plasma membrane, including PI3K-dependent kinase 1 (PDK1), Akt (Stambolic and Woodgett 2006) and GEFs (Fleming et al. 2000; Shinohara et al. 2002). A negative regulator of PI3K signaling is phosphatase and tensin homolog deleted on chromosome ten (PTEN). PTEN-mediated hydrolysis of $P(3,4,5)P_3$ (Stambolic et al. 1998) counteracts PI3K-dependent stimulation of many cellular functions including cell survival, proliferation and invasion.

Deregulation of PI3K function leads to tumorigenesis (Jaiswal et al. 2009). This observation is important in glioma biology as RTKs are often overexpressed or mutated in gliomas (Kapoor and O'Rourke 2003; Parsons et al. 2008; TCGA 2008). PTEN is also extensively deregulated in glioblastomas. PTEN mutations occur in 25 % of patients and loss of 10q, which includes PTEN, occurs in 70 % of patients (Endersby and Baker 2008). Epigenetic gene silencing by methylation of the PTEN

promoter also has been reported (Baeza et al. 2003). Notably, GBM patients with inactivated PTEN have a shorter survival time (Ermoian et al. 2002).

Additionaly, PI3K signaling is deregulated in glioblastomas as a consequence of genetic aberrations within the *PI3K* gene. Mutations in *PIK3CA* have been described in primary human glioblastomas from both adult and pediatric patients with a frequency between 7 and 20 % (Gallia et al. 2006; Parsons et al. 2008; TCGA 2008). Several of these mutations have been shown to be kinase activating in other human malignancies. Interestingly, mutations in the *PIK3R1* gene have been found in 8–10 % of studied glioblastomas (Parsons et al. 2008; TCGA 2008). The clustering of these mutations around residues that serve as contact points with p110 strongly suggest that these mutations may relieve the inhibitory effect of p85α on p110α, thereby rendering PI3K constitutively active (TCGA 2008).

Importantly, a chemical inhibitor of PI3K (PX-866) has been shown to suppress glioblastoma cells invasiveness and VEGF secretion and diminished tumor growth, thereby prolonging animal survival (Koul et al. 2010). Thus, PI3K inhibitors may be beneficial for patients who overexpress the PI3K or carry an activating mutation in *PIK3CA* or *PIK3R1* genes.

7.3.4 Akt Kinase

The Akt family of kinases comprises Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) that are encoded by three independent genes. All isoforms of Akt kinases are activated in a PI3K-dependent manner (Matheny and Adamo 2009). $PI(3,4,5)P_3$ and $PI(3,4)P_2$ recruit Akt from the cytoplasm to the plasma membrane via the N-terminal PH domain of the kinase. Membrane recruitment of Akt results in conformational changes that permit its subsequent phosphorylation and full activation (Franke et al. 1995; King et al. 1997; Matheny and Adamo 2009; Milburn et al. 2003). First, mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Ser-473 in the activation loop of Akt (Huang and Manning 2009; Partovian et al. 2008; Sarbassov et al. 2005), that facilitates subsequent PDK1-dependent phosphorylation of Thr-308 (Wick et al. 2000). mTOR participates in two signaling complexes, mTORC1 and mTORC2.

Interestingly, Akt signaling is governed by negative feedback loop interactions. Akt activates mTORC1 by phosphorylation and inactivation of tuberus sclerosis 2 (TSC2). mTORC1 in turn stimulates the activity p70S6 kinase (p70S6K), that can inhibit Akt by a dual mechanism. First, p70S6K phosphorylates insulin receptor substrate 1 (IRS-1), leading to its proteasomal degradation. IRS-1 is an adaptor protein of the insulin receptor and the insulin-like growth factor-1 receptor (IGF-1R), both of which are major activators of the PI3K/Akt pathway. Thus, p70S6K-mediated inhibition of IRS-1 results in inhibition of Akt signaling (Easton et al. 2006; Martelli et al. 2010). A second negative feedback loop involves p70S6K-stimulated phosphorylation of Rictor, the core component of mTORC2, which negatively regulates mTORC2 dependent activation of Akt (Dibble et al. 2009). Thus, inhibition of mTOR can enhance Akt kinase activity (O'Reilly et al. 2006), which could explain the moderate antitumor activity of mTOR inhibitors (Chang et al. 2005; Galanis et al. 2005). Figure 7.1 shows model of Akt activation and mTORC1-dependent negative feedback mechanisms.

The kinase activity of Akt may be additionally increased via direct interaction with phosphatidylinositol 3-kinase enhancer A (PIKE-A), a protein that is amplified in variety of human cancer cells, including glioblastomas. PIKE-A preferentially binds to activated Akt, and siRNA-mediated depletion of PIKE-A diminishes Akt phosphorylation (Ahn et al. 2004).

Akt activation is terminated via dephosphorylation by protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPPα) (Brazil and Hemmings 2001; Fayard et al. 2005; Gao et al. 2005; Meier et al. 1998; Millward et al. 1999). Interestingly, expression of PP2A regulatory subunit Aα has been shown to be reduced in a significant fraction of both glioblastomas and oligodendrogliomas, thereby deregulating this phosphatase (Colella et al. 2001).

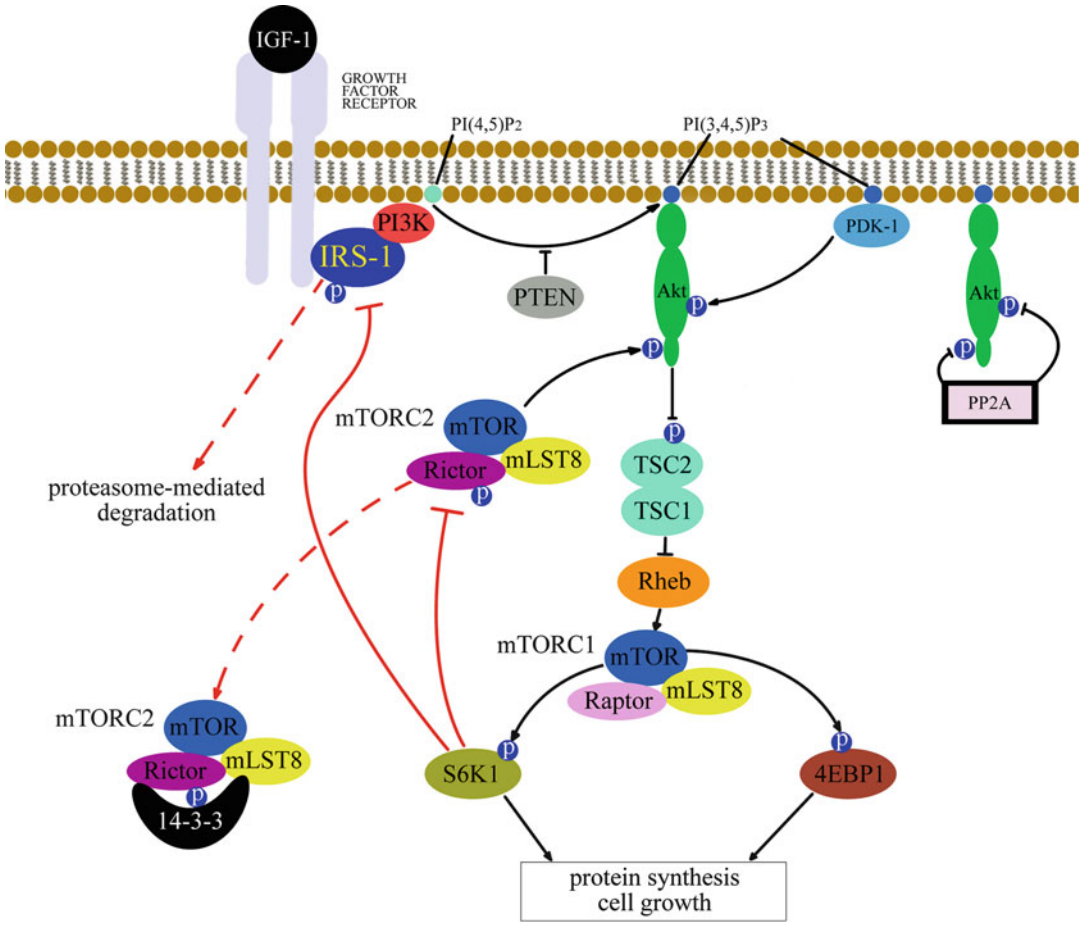

Fig. 7.1 Model of Akt kinase activation and mTORC1-dependent negative feedback mechanisms. Ligand binding induces IGF-1R-dependent association of IRS-1 with PI3K, thereby stimulating production of $PI(3,4,5)P_3$ by PI3K. $PI(3,4,5)P_3$ recruits Akt and PDK1. Subsequently, Akt is phosphorylated by mTORC2 and PDK1, leading to full activation. Akt phosphorylates and inhibits TSC2, leading to Rheb- dependent activation of mTOR. In turn, mTORC1 phosphorylates S6K1 kinase and 4EBP1, stimulating protein synthesis. Activation of mTORC1 stimulates two parallel negative-feedback loops (*red pathways*) that inhibit Akt. S6K-1 phosphorylates IRS-1 (*red line*) and directs IRS-1 to proteasomal degradation (*red dashed arrow*). A second negative loop was proposed where S6K1 phosphorylates the Rictor subunit of mTORC2, thereby promoting 14-3-3 binding to Rictor. S6K-1-dependent phosphorylation of Rictor negatively regulates the ability of mTORC2 to phosphorylate Akt. Akt activation is terminated via dephosphorylation by PP2A

Initially, Akt kinase was known primarily for its role in regulation of cell survival and cell cycle progression (Brunet et al. 1999; Datta et al. 1997; del Peso et al. 1997; Diehl et al. 1998; Kennedy et al. 1999; Li et al. 2002; Shin et al. 2002; Viglietto et al. 2002; Zhou et al. 2001). However, evidence has accumulated that Akt plays a key role in regulation of the invasive glioma phenotype (Molina et al. 2010; Pu et al. 2004). Indeed, invasive glioblastoma cells have a higher level of phosphorylated Akt in comparison with cells isolated from the tumor core in a model of human invasive GBM established in the mouse brain (Molina et al. 2010).

The molecular mechanisms that mediate Akt-stimulated cell migration are still being explored (Stambolic and Woodgett 2006). Phosphorylated Akt localizes to lamellipodia of moving cells,

where it colocalizes with Rac and Cdc42. Constitutive activation of Rac1 or Cdc42 increases Akt phosphorylation in fibroblasts, and inhibition of Akt inhibits cell migration stimulated by Rac or Cdc42 (Higuchi et al. 2001), thus Akt may be activated via GTPase-dependent PI3K activation (Murga et al. 2002). Akt can stimulate cell migration in a number of ways (Stambolic and Woodgett 2006), including the role of p70S6K in actin reorganization (Qian et al. 2004) and direct phosphorylation of Girdin/Akt phosphorylation enhancer (APE) protein (Enomoto et al. 2005; Zhang et al. 2009). Girdin can crosslink actin filaments and anchor cortical actin to the plasma membrane. Phosphorylated Girdin relocalizes to the leading edge of moving cells and promotes short-branched actin filaments (Enomoto et al. 2005).

A comprehensive study has evaluated the role of all three Akt isoforms in gliomagenesis using a model system driven by common glioma abnormalities: loss of function of PTEN and p53 protein and expression of EGFRvIII receptor in primary murine astrocytes (PMA). The results showed that Akt3 regulates anchorage-independent growth of transformed astrocytes and human glioma cells. Additionally Akt3, but not Akt1 or Akt2, knockdown reduces the ability of PMA to invade matrigel (Endersby et al. 2011). Previous studies discussed the critical role of Akt2 in regulation of glioma invasion (Pu et al. 2004; Zhang et al. 2009, 2010). Thus, depending on the glioma model used, the specific functions of the various Akt isoforms may vary. It also remains unclear which Akt isoform plays a major role downstream of PI3K in gliomas and whether the respective isoforms interact with distinct binding partners depending on the cell setting.

The expression of Akt1 is similar in gliomas and normal control tissue (Mure et al. 2010). Akt3 mRNA and protein decrease with increasing grade of malignancy (Mure et al. 2010), contrary to Akt2 expression that increases with tumor grade (Mure et al. 2010; Wang et al. 2010; Zhang et al. 2010). Although Akt3 expression levels in malignant glioma are significantly reduced compared to normal tissue, its kinase activity is equal to that of Akt2, and approximately 2-fold higher than that of Akt1 (Mure et al. 2010), thereby compensating for its decrease in expression.

7.4 Proteases

ECM proteins as well as parenchymal cells are natural obstacles for migrating tumor cells. In order to invade brain tissue, glioma cells detach from the tumor mass and reorganize the ECM by complex proteolytic mechanisms and expression of ectopic ECM components (Nakada et al. 2007). Glioblastomas overexpress a number of proteases, including uPA, matrix metalloproteinases (MMPs), ADAMs and cathepsins (Fillmore et al. 2001; Fukuda et al. 2005; Nakada et al. 1999; Pagenstecher et al. 2001; Rempel et al. 1994; Sivaparvathi et al. 1996a, b, c; Yamamoto et al. 1994; Zhao et al. 2008).

The regulation of proteolytic activity is complex and often involves cross-talk between different classes of proteases. One example is uPA, a serine protease that is synthesized as an inactive propeptide – zymogen (known as pro-uPA), which binds to the uPA receptor (uPAR) on the plasma membrane and becomes cleaved by active plasmin. uPA catalyses the conversion of nonactive plasminogen into plasmin, thus establishing a positive feedback loop. Notably, binding of uPA to its receptor provides localized proteolytic activity (Pillay et al. 2007). uPa directly cleaves and activates pro-MMP-9 in glioblastomas (Zhao et al. 2008) and experimental depletion of both uPA and uPAR from glioblastoma cells suppresses invasion *in vitro,* intracerebral tumor formation in nude mice as well as growth of subcutaneously pre-established tumors (Gondi et al. 2003). In summary, the uPA-uPAR axis controls many signaling pathways that contribute to the malignant behavior of glioblastoma. Importantly, uPA expression and activity are elevated in anaplastic astrocytomas (AA) and GBMs in comparison to non-neoplastic brain (NB) or low grade glioma (LGG) (Landau et al. 1994; Yamamoto et al. 1994). Thus, targeting these proteases may be therapeutically beneficial.

Interdependent activation of proteases also occurs in the family of matrix metalloproteinases, which comprises 25 enzymes that bind Zn^{2+} ions in their active site. Based on substrate specificity, structure and subcellular localization matrix metalloproteinases are grouped into collagenases, gelatinases, stromelysins and membrane metalloproteinases. Like uPA, metalloproteinases are synthesized in cells as zymogens that have to be proteolytically processed to become fully active. The number of active enzymes in the cell is regulated on the level of gene expression, protein secretion and activation or inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Ra and Parks 2007).

MT1-MMP is a key metalloproteinase that contains a transmembrane sequence and cytoplasmic domain (Ra and Parks 2007). The transmembrane/cytoplasmic domain is responsible for localizing the enzyme to invadopodia (Nakahara et al. 1997), actin-rich structures to which most proteolytic activity of invading cells is localized (Frittoli et al. 2011; Ridley 2011; Symons 2008). The MT1-MMP cytoplasmic domain is also necessary for dynamin-dependent removal of the enzyme from the cell surface via clathrin-mediated endocytosis (Jiang et al. 2001), an additional mechanism of MT-MMP-1 regulation.

Processing of pro-MMP-2 involves the formation of a MT1-MMP/TIMP-2 complex at the cell surface (Zucker et al. 1998). This complex binds pro-MMP-2 via interactions between C-terminal of TIMP-2 and the hemopexin C-domain of pro-MMP-2 (Overall et al. 2000). Subsequently, an additional molecule of MT1-MMP cleaves and activates MMP-2 (Fillmore et al. 2001; Ra and Parks 2007) (Fig. 7.2). This mechanism of activation requires a proper balance in the expression level of pro-MMP-2, TIMP-2 and MT1-MMP. In line with this mechanism is the finding that CD95L (FasL) stimulates MMP-2 activity in rat glioma cells via NF-κB-driven transcription of TIMP-2. Inhibition of TIMP-2 expression decrease overall MMP-2 activity and leads to accumulation of inactive pro-MMP-2 (Wisniewski et al. 2010). Alternative mechanisms of MMP-2 activation have also been suggested (Mazzieri et al. 1997; Monea et al. 2002; Morrison et al. 2001).

Interestingly, MMP-2 is subject to autocatalytic processing, resulting in the formation of the hemopexin fragment (PEX). As for MMP-2 itself, the level of PEX increases with tumor grade (Bello et al. 2001b). Paradoxically, PEX inhibits glioblastoma cell migration, invasion and proliferation and induces apoptosis. It also strongly inhibits angiogenesis, most likely, through its binding to αvβ3. Intraperitoneal administration of PEX inhibits tumor growth by 99 % in both subcutaneous and intracranial human glioma xenografts mouse models, with no sign of toxicity (Bello et al. 2001b), suggesting that PEX is a promising therapeutic candidate. The anti-tumor and anti-angiogenic effects of PEX are reminiscent to those of endostatin (Cao 2001) and suggest that the effects of PEX on the malignant behavior of glioblastoma is overridden by powerful proinvasive and proangiogenic factors.

MT1-MMP, MMP-2 and MMP-9 are three major MMPs overexpressed in glioblastoma in comparison with non-neoplastic brain (Fillmore et al. 2001; Nakada et al. 1999; Pagenstecher et al. 2001; Zhang et al. 2010). Upregulation of expression of those MMPs in HGG can be explained, at least in part, by hyperactivation of the PI3K/Akt signaling pathway in those tumors. In line with this, inhibition of PI3K or Akt reduces MMP expression in glioblastoma cells (Kwiatkowska et al. 2011). In addition, downregulation of Akt-dependent MMPs production is associated with inhibition of glioma cell invasion *in vitro* and a reduction in the number of satellite tumors and tumor volume in an *in vivo* model of glioma (Pu et al. 2004; Zhang et al. 2009; 2010).

Proteases also regulate glioma cell invasion by promoting receptor shedding from the plasma membrane (Nagano et al. 2004; Okamoto et al. 1999a, b; Yang et al. 2011). Notably, ADAM-10, which belongs to the multidomain membrane-anchored protein family called adamalysins, catalyses proteolysis of the L1 receptor. Notably, increased surface expression of ADAM-10 on migrating glioblastoma cells correlates with loss of surface L1 (Yang et al. 2011). Experimental attenuation of L1 expression in glioblastoma cells reduces migration velocity *in vitro* and suppresses invasion of tumor cells into chick embryonic brain. Migration can be restored upon adding L1 ectodomain to migrating cells (Yang et al. 2011). In addition, ADAM-10 and ADAM-17, in response to different stimuli, cleave the CD44 hyaluronan receptor. Depletion of either adamalysin suppresses CD44 ectodomain shedding and strongly inhibits cancer cell migration on hyaluronan (Nagano et al. 2004).

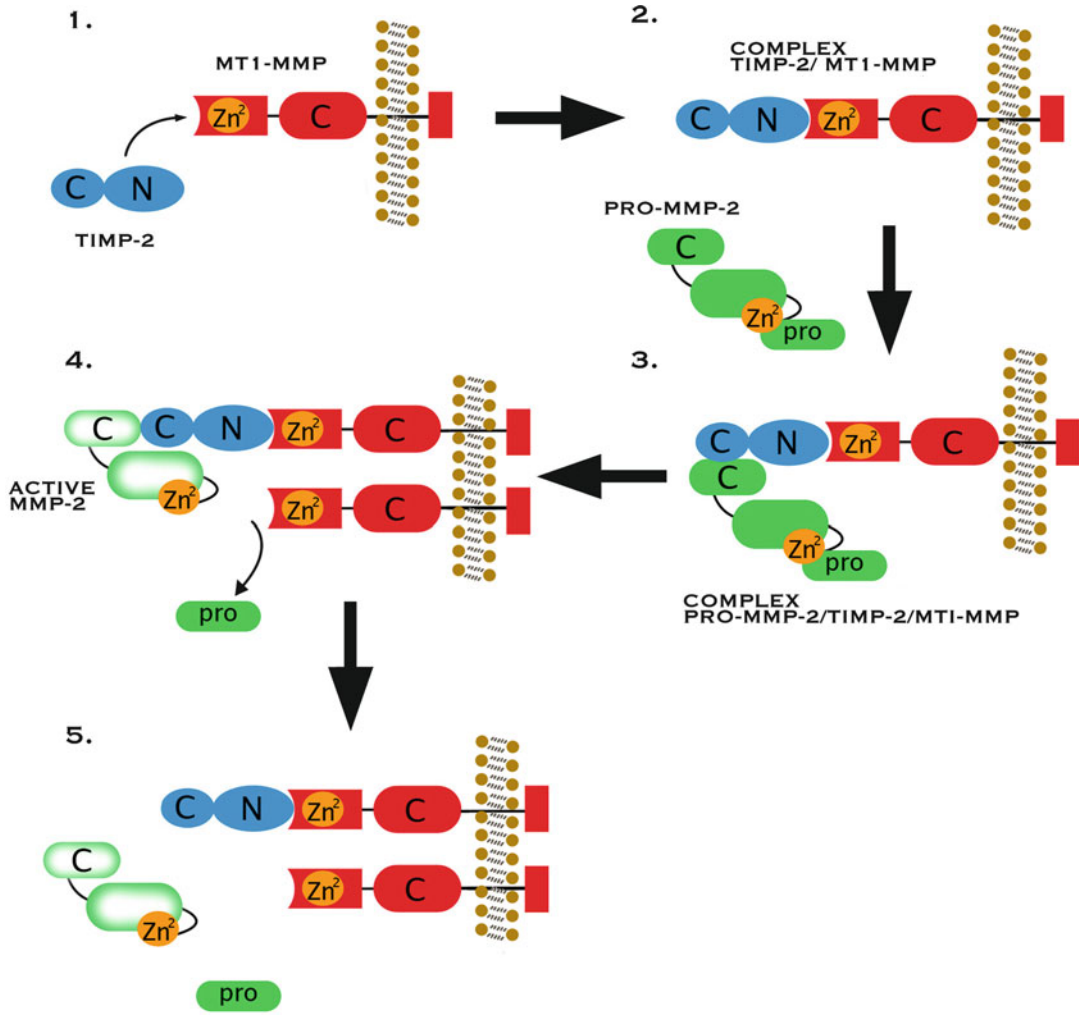

Fig. 7.2 Mechanism of MMP-2 activation. Processing of pro-MMP-2 requires formation of a MT1-MMP and TIMP-2 complex (*1–2*). This complex functions as a receptor for binding of pro-MMP-2 (*3*). Subsequently, an additional MT1-MMP molecule cleaves pro-MMP-2 (*4*). Active MMP-2 is released into the intercellular space (*5*). Modified from Brauer 2006

Moreover, overexpression of ADAM-17 in U87 glioma cells results in increased invasion into Matrigel and tumor growth, whereas inhibition of ADAM-17 reduces tumor growth. Interestingly, specific ablation of ADAM-17 decreases activation of EGFR/PI3K/Akt pathway, probably by decreasing TGFα shedding (Zheng et al. 2011). Thus, ADAM proteases stimulate cell invasion by multiple mechanisms, including ECM degradation, receptor shedding and growth factor activation.

7.5 Conclusions and Future Directions

Extensive *in vitro* and *in vivo* studies have revealed the complexity of signaling mechanism that drive dissemination of malignant glioma cells into surrounding brain tissue. The hope is that better understanding of critical signaling elements will help to identify molecular targets for therapeutic

intervention. In addition, we anticipate that specific targeting of the invasive behavior of glioblastoma will have limited therapeutic benefit and that anti-invasion strategies will have to be combined with additional therapeutic modalities, such as chemo- or radio-therapy. The discovery of genetically distinct subclasses within HGG patients is an important step toward introducing personalized therapies (Huse et al. 2011). Thus, we anticipate that specific combination regimens will be introduced to treat respective HGG subgroups.

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