Published in final edited form as:
Mol Cancer Res. 2013 October ; 11(10): . doi:10.1158/1541-7786.MCR-13-0236.

The pathobiology of collagens in glioma

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Abstract

Malignant gliomas are characterised by diffuse infiltration into the surrounding brain parenchyma. Infiltrating glioma cells exist in close proximity with components of the tumour microenvironment, including the extracellular matrix (ECM). While levels of collagens in the normal adult brain are low, in glioma, collagen levels are elevated and play an important role in driving the tumor progression. In this review, we provide a comprehensive overview of the nature of collagens found in gliomas and offer insights into the mechanisms by which cancer cells interact with this ECM via receptors including the integrins, discoidin domain receptors and Endo180. We further describe the major remodelling pathways of brain tumour collagen mediated by the matrix metalloproteinases and highlight the reciprocal relationship between these enzymes and the collagen receptors. Finally, we conclude by offering a perspective on how the biophysical properties of the collagen ECM, in particular, mechanical stiffness and compliance may influence malignant outcome. Understanding the complex interactions between glioma cells and the collagen ECM may provide new avenues to combat the rampant tumor progression and chemoresistance in brain cancer patients.

Keywords

Collagen; Signal Transduction; Integrins; Discoidin Domain Receptors; Endo180; Glioblastoma; Matrix Metalloproteinases; Mechanotransduction

1. Introduction

Molecular profiling of tumors has led to an unprecedented understanding of the mechanistic basis of glioma initiation and progression. Significantly, through the efforts of The Cancer Genome Atlas (TCGA) consortium, we now have a comprehensive molecular portrait of glioblastoma multiforme (GBM-WHO grade IV), the most aggressive form of brain tumors in adults, ranging from defined genomic and epigenetic features to protein level signaling measurements (1). Despite the importance of these studies in illuminating glioma biology, much of the characterization performed thus far has largely ignored the heterogeneous nature of these tumors. Gliomas are composed of a complex milieu of tumor cells interacting in close proximity with the components of the tumor microenvironment including stromal cells and extracellular matrix (ECM). Interactions between tumor cells and their microenvironment are critical for driving cancer cell proliferation and invasion into the brain parenchyma, two characteristic hallmarks of GBM (2). In particular, the composition of the ECM is critically important in modulating tumor cell responses to exogenous cues such as growth factor activation or chemotherapy. The ECM of brain tumors consists of the basement membrane components, collagen IV, laminin and fibronectin lining the blood

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vessels as well as collagen I, tenascin-C, vitronectin and hyaluronan surrounding the tumor (3-5). In this review, we will discuss the role of collagen and its receptors in driving glioma progression. We further elaborate on the effects of matrix metalloproteinases on the collagen ECM and how modifications in the biochemical and biophysical properties of collagen can modulate tumor cell behavior. We conclude by providing a perspective on collagen receptors as potential therapeutic targets for disease.

2. Collagen and gliomas

Collagen consists of a right-handed triple helical structure composed of three polypeptide chains with a recurring Gly-X-Y sequence (where X and Y are usually proline and hydroxyproline respectively) (6). The polypeptide chains that contribute to the collagen structure can either be homo- or hetero-trimeric in nature and the resultant triple helix is known as the collagenous (COL) domain. In addition to the COL domain, there are other domains on the collagen chains that are referred to as non-collagenous (NC) domains. There are 29 different collagen types which are classified into three main classes. The fibrillar collagens are the most common class of collagens in the body and are typified by collagen type I. Type I collagen is synthesized in the cell as procollagen and secreted into the extracellular matrix (7). There it is subjected to processing by proteases where the N- and C-terminal propeptides are cleaved to form mature triple helical chains. This processed collagen then comes together to form higher order fibrils or macromolecular fibers that confer tensile strength to tissues. Type I collagen contains telopeptides in its N- and C-terminal domains which are susceptible to crosslinking by enzymatic and non-enzymatic means. Crosslinking of collagen I is critical for maintaining collagen structure and function and defects that inhibit this process are associated with a number of severe hereditary diseases including Marfans syndrome (8).

Collagen type I is the most studied collagen molecule and its role in maintaining tissue structural integrity is well established. Collagen I has additional roles in driving cellular function and signaling via its interactions with a wide variety of different proteins (6, 9). Through extensive database searching, a map of functional binding sites on collagen I has been assembled. It has been proposed that there are two mutually exclusive domains, the “cell interaction domain” and the “matrix interaction domain” (6). The “cell interaction domain” is thought to be responsible for the dynamic functions of collagen including signal transduction and ECM remodeling. This domain contains binding sites for receptors (integrins, discoidin domain receptors), enzymes (matrix metalloproteases) and matricellular proteins (SPARC). The “matrix interaction domain” is composed of binding sites for proteoglycans such as keratin sulphate and dermatan sulphate and is key to conferring structural properties to collagen fibrils. The contribution of protein interactions in these domains to collagen receptor signaling and modulation of cancer cell behavior will be discussed in subsequent sections of this review.

The second class of collagens is the network forming collagens including collagen type IV. The distinguishing characteristic of these collagens is the presence of NC-domains at the N- and C-terminal ends of the collagen α-helical chains (10). These domains are responsible for mediating interactions that drive the formation of collagen sheets and is a major component of the basement membrane. The final class of collagens are the fibril-associated collagen with interruptions in their triple helices (FACITs) (11). The FACITs are the largest class of molecules in the collagen superfamily. These collagens are characterized by the presence of an interrupted triple helix with a pair of conserved Cys residues separated by four amino acids at the C-terminal end. They also contain a thrombospondin domain in the N-terminal region and depending on the collagen type may additionally contain von Willebrand factor A-like domains and fibronectin type III repeats in this NC-region. However, the functional

roles of these FACITs are not well established and they are thought promote ECM stabilization through protein-protein interactions via the NC-domains, for instance interactions with fibrillar collagens to facilitate both intra- and inter-fibril associations (11).

While fibrillar collagens are the most abundant proteins in the body and are highly expressed in the interstitial matrix of many organs, the normal adult brain is unique in that it contains very limited amounts of collagen, accounting for its soft consistency. The major form of collagen in the brain is collagen IV which is present in the basement membrane surrounding vascular endothelial cells. Collagen IV levels are upregulated in gliomas and is localized to the basement membrane lining the vessel walls in astrocytomas of all grades including GBM (12). The presence of fibrillar collagens in gliomas is more contentious and it has been reported that GBMs do not express intratumoral fibrillar collagen (13). To resolve this issue, Huijbers et al. employed a combination of different approaches (including quantitative PCR, Masson's trichrome staining and collagen I-specific immunofluorescence staining) to demonstrate that a subset of glioma tissues and cancer cells contain appreciable levels of fibrillar collagens both within the tumor mass and in the surrounding stromal tissue (5). The identity of the collagen producing cells is still unresolved but glioma cells can synthesize their own complement of ECM, including collagens I and IV (14-15). In addition, similar to many growth factors and paracrine ligands, the stromal cells and normal brain parenchyma surrounding the tumor contribute to the complex ECM in the tumor microenvironment, including the production of collagen type IV (16).

Collagen XVI, a member of the FACIT class of collagens, is highly upregulated in gliomas compared to normal brain tissue (17). Immunofluorescence studies indicate that Collagen XVI is present in the tumor cells and also in the tumor vessels underlying collagen IV. Consistent with this observation, a number of primary and immortalized glioma lines were found to express and secrete collagen XVI *in vitro* (18). Interestingly multiple truncated forms were detected in addition to the full length protein suggesting that proteolytic cleavage of this collagen occurs in brain tumors. The functional significance of these truncated forms is unknown.

Analysis of molecular datasets also highlights the importance of collagens in gliomas. An integrated computational analysis of mutations, miRNA and mRNA expression based on the TCGA dataset for GBM patients revealed interesting insights into collagen regulation in glioma (19). Analysis of gene expression levels identified the collagen genes COL3A1, COL4A1 and COL5A2 as highly enriched in GBM versus normal tissue samples. Using algorithms to infer gene coexpression networks, the study found a functional module in GBMs centered around COL4A1. The authors further established COL3A1 as an important genetic node within the GBM network that when removed had the one of the greatest effects on disrupting the connectivity of gene co-expression networks. Integrated analysis of miRNA and mRNA profiles led to the identification of mir-29c as a potential tumor suppressor in GBM. COL4A1 and COL4A2 are targets of mir-29c and depletion of this miRNA results in an upregulation of collagen genes. In addition to mRNA and miRNA expression studies, sequencing analysis performed by the TCGA and International Cancer Genome Consortium (ICGC) have identified mutations in more than 20 different collagen genes. These mutations occur at low frequency (<1%) in both GBM tumors and cell lines (Catalogue of Somatic Mutations in Cancer – COSMIC database). It remains unclear if these mutations are non-functional passenger mutations or drivers that confer a selective survival advantage to tumor cells and further work is required to establish the role of these mutations in cancer.

3. Collagen receptors in gliomas

Collagen has three primary functions in brain tumors. One of its main roles is to act as a scaffold and provide sites of adhesion for cells. These attachment sites are critical for cellular processes such as glioma cell migration along collagen tracks and contractility in response to external forces. The second function of collagen is to serve as a reservoir of matricellular proteins, proteoglycans and growth factors (6). Through a balance of collagen synthesis, posttranslational modifications and degradation, collagen can be tuned to store or release specific factors in a temporal or dose-dependent manner in response to changing biological context. The third role of collagen is as a ligand for the activation of signal transduction networks required for tumor growth, differentiation and invasion (9). These functions of collagen are mediated through the action of collagen binding receptors and in this section we will discuss the importance of the integrins, discoidin domain receptors and Endo180 in glioma biology.

3.1 Collagen binding integrins

The integrins are a class of transmembrane receptors that are composed of a heterodimer of α and β subunits. In mammals, there are 18 α and 8 β subunits that together form 24 known integrin pairs (9). The integrins are specialized adhesion receptors that bind a variety of different ECM ligands with distinct heterodimers dictating the nature of ligand specificity. There are four known collagen binding integrins: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$ in which the alpha subunit has an inserted domain (I-domain) that is the collagen recognition site (20). These receptors exhibit cell type specificity with $\alpha_1\beta_1$ being primarily expressed in mesenchymal cells and $\alpha_2\beta_1$ in epithelial cells. $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$ expression is restricted to bone, cartilage and fibroblasts and will not be discussed further in this section. The first fibrillar collagen motif identified to be a ligand for both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ was GFOGER, where O is hydroxyproline (21). Further work isolated additional binding sites and established a consensus motif of GXX'GEX" where X is a hydrophobic residue or R and X" is often R but cannot be K (9).

The integrins do not possess intrinsic catalytic activity and mediate their signaling effects through protein-protein interactions that result in the formation of a large adhesion complex known as the adhesome (Figure 1). Many of the protein components in these complexes have been identified and include talin, kindlins, focal adhesion kinase (FAK), Src and paxillin amongst others (for a comprehensive review see (22) and references therein). An interesting and unique characteristic of integrin biology is its ability to transduce bidirectional signaling. Binding of the integrins to collagen results in conformational changes that lead to the separation of the cytoplasmic tails in the α and β subunits, exposing the sites required for protein binding and adhesome formation (23). In addition, collagen contains multiple integrin binding motifs which promote receptor clustering and ligand avidity that activates downstream signal transduction events (24). This mode of signaling is known as outside-in signaling. Integrins are intimately associated with the intracellular components of the cell, such as the actin cytoskeleton through the interaction with talin and the microtubule network via paxillin. These interactions allow for an alternative form of signaling known as inside-out signaling where changes in the intracellular compartment result in the modulation integrin affinity for its ECM ligand (25). In this manner, the integrins can readily respond and adapt to alterations in both the extracellular environment as well as intrinsic cellular events.

Unlike other integrins such as αvβ3, α5β1 and β8, the collagen binding integrins do not appear to be the dominant class of integrins basally expressed in GBM (26). The $\alpha_2\beta_1$ receptor was found in a limited number of GBM cases and present in only a small number of tumor cells (27). A subset of GBM cell lines express the α_2 subunit and blocking antibodies

directed against this subunit were able to reduce cancer cell invasion into rat brain aggregates (26, 28). Instead expression of α2β1 in GBM appears to be context dependent and inducible in response to transforming growth factor beta (TGF-β). TGF-β is a secreted factor that regulates cellular proliferation, apoptosis and differentiation. It has been shown that TGF-β is expressed in glioma cells and confers poor prognosis in GBM patients (29-30). Treatment of GBM cells with TGF-β modulates ECM composition by increasing collagen synthesis (30). In addition, in T98G GBM cells, TGF-β enhances α2 subunit expression which results in increased lattice contraction in a 3-dimensional collagen gel (31). These observations may have implications in therapy as GBM cell lines subjected to CyberKnife irradiation displays elevated TGF-β and β1 subunit expression which is accompanied by increased cell invasion (32). Taken together, the data indicate that TGF-β plays an important role in regulating both the collagen and integrin receptor levels in GBM.

3.2 Discoidin Domain Receptors

The discoidin domain receptors (DDRs) are a class of receptor tyrosine kinases (RTKs) that are activated by collagen rather than classical growth factor ligands (33). There are two family members with DDR1 being primarily expressed in epithelial cells and DDR2 in mesenchymal cells. The receptor is composed of an N-terminal extracellular DS homology domain, a single transmembrane region, a juxtamembrane domain and a C-terminal catalytic tyrosine kinase domain (9). The cytoplasmic domains of DDR1 and DDR2 have tyrosine residues that serve as potential phosphorylation sites for receptor activation. Collagen binding leads to a delayed phosphorylation response that takes several hours to achieve full activation but remains sustained for days without significant downregulation (33-34). DDR1 is activated by both fibrillar and basement membrane collagen while DDR2 binds exclusively to fibrillar collagens and collagen X. Using a triple helical collagen peptide toolbox, it has been determined that these receptors bind with high affinity to a GVMGFO motif within collagens I, II and III (9). The 2 members of the DDR family have been associated with cell proliferation, adhesion, migration and extracellular remodeling (33).

DDR1 is expressed in multiple brain tumor types. DDR1 mRNA was enriched in pediatric brain tumors including GBM, astrocytomas, ependymoma and primitive neuroectodermal tumor (PNET) (35). A follow-on study showed that DDR1 was expressed in 27 of 29 high-grade primary and metastatic brain tumors (36). DDR1 was found to be expressed exclusively in the tumor cells and not in the blood vessels, inflammatory cells or normal brain tissue surrounding the tumor. In another study of 29 brain tumors, high DDR1 mRNA expression was negatively correlated with survival in GBM patients (37). The predictive ability of DDR1 for patient outcome was further confirmed in an independent cohort of 19 glioma samples. Similarly, a study by Ram et al. showed that DDR1 was overexpressed in glioma tissues using gene expression arrays, immunohistochemistry and immunoblotting analysis (38). Using retroviral tagging in a murine model of glioma, Johansson et al. identified DDR1 as a candidate brain tumor loci gene that potentially cooperates with PDGF in gliomagenesis (39). They showed that the expression of DDR1 was enriched in both GBM and oligodendroglioma-like tumors that arise in this mouse model. These data demonstrate that DDR1 is highly expressed in GBM and may play a role in promoting gliomagenesis and tumor progression.

The functional role of DDR1 in driving GBM was confirmed with RNA interference in a panel of GBM cell lines (37). DDR1 knockdown resulted in a significant decrease in GBM cell growth and invasion. Alternative splicing has also been suggested to regulate DDR signaling and function in GBM. DDR1 has 5 isoforms formed by alternative splicing (DDR1a-e; Figure 2) (33). DDR1c is the longest form and represents the intact full-length version of the protein. DDR1a and b both lack 6 amino acids in the kinase domain, while DDR1a also lacks 37 amino acids in the juxtamembrane domain. DDR1d and e are truncated

forms that do not exhibit kinase activity. In the case of DDR1d, the entire kinase domain is absent, while DDR1e contains a frame-shift resulting in inactivation of the kinase domain. A previous study in glioma cells showed that DDR1a and 1b differ in their biological properties (38). While both DDR1a and 1b increase cellular adhesion to collagen, only DDR1a cells showed enhanced invasion via the secretion of high levels of matrix metalloproteinase-2 (MMP-2). Both inhibition of DDR1 using blocking antibodies or MMP inhibition diminished DDR1a mediated cancer cell invasion. DDR1a lacks the ShcA phosphotyrosine binding motif present in DDR1b which may result in distinct pathway activation between the two splice variants. Additionally, DDR1a uniquely binds to the fibroblast-growth factor receptor substrate-2 (FRS2) through its juxtamembrane domain (33). Collectively, these data suggest that different splice variants of DDR1 exhibit distinct oncogenic properties which may manifest through the activation of unique signal transduction pathways in glioma cells.

The comprehensive signaling networks activated downstream of DDR1 in glioma are not well established and readers are directed to recent excellent reviews for details of known DDR pathways (9, 33). Here we will summarize the main signaling pathways activated by DDR1 in other biological systems. A number of signaling proteins and adaptors bind directly to DDR1 including phosphoinositide-3-kinase (PI3K), signal transducer and activator of transcription 5 (STAT5), SHP-2, NCK2 and SHC (Figure 1). The C-terminal of DDR1 kinase domain contains the YELM binding motif for association with the p85 subunit of phosphoinositide-3-kinase (PI3K). The p85 subunit of PI3K associates with DDR1 in the human breast cancer cell line T47D (40). Studies using mammary epithelial cell lines revealed that DDR1 transiently interacts and phosphorylates STAT5 in conjunction with prolactin-induced JAK2 activity (41). Phosphorylation of DDR1 at Y703 and Y796 promotes the interactions with the SH2 and PTP domains of the SHP-2 tyrosine phosphatase forming a complex (42). DDR1 cytoplasmic domain has also been reported to interact with the SH2 domain of non-catalytic region of tyrosine kinase 2 (NCK2), an adaptor protein which promotes cell migration and invasion (43). A recent proteomic study of DDR1 interactors has confirmed many of these binding events and uncovered novel DDR1 binders including RasGAP, SHIP1 and SHIP2 (44). Since these experiments were performed under supraphysiological levels of DDR1 phosphorylation, it remains to be determined if these new DDR1 binders are observed *in vivo*.

3.3 Endo180

Endo180 is a member of the mannose receptor family that have roles in the uptake of extracellular proteins. Members of this family are characterized by an extracellular modular domain architecture that consists of an N-terminal cysteine-rich domain, a fibronectin type II (F2) domain and series of C-type lectin-like domains (eight in Endo180) (45). They also have a transmembrane region and a short cytoplasmic tail that is responsible for providing signals to the intracellular endocytic machinery. The primary function of Endo180 is to mediate the endocytosis and degradation of collagen and this receptor plays an important role in collagen remodeling, acting as a potential negative regulator to the integrins and the DDRs.

The F2 domain of Endo180 is responsible for binding to both triple helical as well as denatured collagen (gelatin). Endo180 has been shown to bind to Collagen I, IV and V, although there is evidence that there may be a preferential binding to the gelatin form of collagen (45). After collagen binding, the receptor is internalized and the collagen fragments are routed to the lysosome for degradation (Figure 1). The internalization signals are mediated by a dihydrophobic motif in the cytoplasmic tail of the receptor and the receptor undergoes constitutive recycling between the plasma membrane and the endosomal compartments (46). Depletion of Endo180 in a transgenic mouse model of breast cancer

(PymT) resulted in an accumulation of collagen levels in the stroma surrounding the tumor cells (47), suggesting that this receptor is important for ECM remodeling of the tumor microenvironment.

Two studies independently identified Endo180 to be highly enriched in gene expression data of GBM versus lower grade astrocytomas or normal brain (5, 48). Immunohistochemistry studies confirmed this result with perinuclear staining of Endo180 in tumor cells in the majority of GBM sections examined versus grade III anaplastic astrocytomas or anaplastic oligodendroglioma (5). Interestingly, co-staining for the presence of collagen I showed a close association of Endo180 positive tumor cells with collagen I deposition, suggesting that Endo180 may be responsible for collagen remodeling in brain tumors. Consistent with this observation, depletion of Endo180 in glioma cells resulted in a marked reduction in collagen internalization. Furthermore, knockdown of Endo180 in glioma cells resulted in a dramatic decrease in cell motility and invasion with no effects on proliferation. The decrease in cell migration was accompanied with actin cytoskeletal rearrangements into stress fibers and loss of lamellipodia formation (48). Huijbers et al. additionally showed that TGF-β upregulated Endo180 expression levels through a TGF-β responsive element in Endo180 promoter region (5). Finally, gene expression analysis showed that Endo180 strongly correlated with the mesenchymal subclass of GBM which has been associated with aggressive disease and poor patient outcome, providing a rationale for the use of Endo180 as a surrogate marker for this subclass. These studies implicate Endo180 as an important regulator of GBM invasion but additional studies establishing the functional role of Endo180 in collagen remodeling and its effect on GBM progression are still required in order to understand its mechanism of action.

4. Matrix metalloproteinases and collagen remodeling in glioma

The ECM can act as both a substrate for and a barrier to invasion. *In vitro* studies have shown that tumor spheroid invasion in collagen gels is limited by available collagen concentration; while reduction in gel pore size is associated with a reduction in invasive distance (49-50). Unsurprisingly, glioma cells have the ability to extensively remodel their microenvironment both through deposition of new matrix components and degradation of existing ones (51). One key class of proteins responsible for regulating the turnover of collagens are the matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases comprising 23 members, which can be assigned to one of eight structural groups (Figure 3). The domain structure of the MMPs includes: a secretory signal sequence; a regulatory pro-peptide; a catalytic domain; and a C-terminal hemopexin domain linked to the catalytic domain *via* a hinge region (51-52). In addition, while five of the structural groups are secreted, three (the membrane-type or MT-MMPs) are associated with the cell membrane, either *via* a transmembrane domain or a GPI anchor (51). MMPs are first synthesized in an inactive pro-form in which interactions between a cysteine thiol in the pro-peptide domain and the catalytic zinc ion prevent substrate cleavage. Enzyme activation requires disruption of this inhibitory interaction, most commonly through protease-mediated removal of the inhibitory pro-peptide.

MMPs possess a broad range of overlapping substrate specificities and family members are able to degrade substrates as diverse as collagens, fibronectin, laminin, proteoglycans, cell surface proteins, and pro-forms of growth factors, including TGF-β. Both the catalytic and extra-catalytic domains play a role facilitating MMP-collagen interactions. For example the fibronectin repeats in MMPs-2 and -9 are involved in binding denatured collagen (52), while both catalytic and the hemopexin domains of MMP-1 are involved in collagen binding (53). Given the overlapping substrate specificities of the MMPs, it has been proposed that MMP compartmentalization may be important for tuning their activity. Accumulating evidence

suggests that both secreted and membrane type MMPs interact with cell surface-anchored proteins in order to direct their activity to distinct pericellular locations (52). The relative ability of different MMPs to cleave distinct substrates therefore appears to be defined by both the inherent ability of the enzyme to bind the substrate as well as the ability of the cell to concentrate the enzyme to specific sites where various substrates are present.

In the context of collagen turnover, 14 MMPs have been reported to degrade various collagens (Table 1). MMPs can both modulate the interaction between collagens and their cognate receptors, whilst themselves being regulated by pathways initiated by the collagen receptors. In the following section, we discuss the role of MMPs in glioma invasion and tumor progression, elaborate on the mechanisms by which MMPs facilitate these processes. We then review the role of collagen receptors in modulating the expression and activity of MMPs, and finally present some of the reciprocal relationships by which MMPs might serve to modulate interactions between collagens and their receptors.

4.1 MMPs in glioma progression and invasion

Extensive evidence supports a role for various MMPs in glioma pathophysiology and their expression and activity have been correlated with malignant progression in human glioma (54). Depletion of MMP-9 in glioma cell lines results in reduced adhesion to the ECM, diminished migration speed, and reduced invasion through type IV collagen (55). *In vivo* MMP-9 depletion reduced infiltration and proliferation of GBM cells in mouse xenografts (55). Similarly, the invasive capacity of different glioma cell lines through matrigel substrates in transwell migration assays has been correlated with levels of MMP-2 expression (56), and downregulation of MMP-2 has been reported to inhibit radiation enhanced invasiveness of glioma cells (57). Reducing MMP-2 expression in U251 glioma cells implanted into nude mice also diminished tumor growth (57).

While there is good evidence that MMPs -2 and -9 facilitate glioma progression and invasion, the role of other MMP family members is less obvious. Hagemann et al. compiled a list of MMPs that are likely to play important roles in GBM on the basis of both expression patterns reported in the literature, and their own PCR screens (51). The authors concluded that MMPs -1, -2, -7, -9, -11, -12, -14, -15 and -25 associate with glioma progression, while no evidence could be found to support a role for MMPs -3, -8, -10, -13, -16, -17, -20, -21, -23, -26, -27 and -28. Wang et al. recently demonstrated that increased MMP-13 expression correlated with glioma progression, with high expression levels being predictive of shorter overall survival (58). A similar correlation between elevated expression of MMP-19 and poor prognosis has also been shown (59). These studies suggest that MMP-13 and MMP-19 may also have potential functional roles in glioma progression.

Blocking MMP activity with MMP inhibitors or blocking antibodies reduces glioma cell invasion (55). However, there is some debate as to whether MMP-mediated collagen degradation is indeed truly essential for tumor invasion. Wolf et al. have described a compensatory mechanism by which cells grown in 3D collagen are able to maintain their ability to invade the surrounding matrix under conditions where cellular proteolysis is inhibited (60). In this study, proteolytically-competent HT1080 cells expressing MT-MMP1 displayed similar migration rates to those measured for the same cells treated with broad-spectrum protease inhibitor cocktails. However, while migration rates were unaffected by MMP inhibition, the mechanisms by which cells invaded the surrounding matrix were altered. In the absence of protease inhibitors, cells were observed to migrate through collagen matrices in a mesenchymal manner where cells are elongated, with the cell anterior binding to collagen fibers and generating traction forces. However, following protease inhibition, cells appeared to migrate with an amoeboid phenotype where cells exhibited a more rounded phenotype and traversed the network of collagen fibers by squeezing through

gaps between fibers without substantially remodeling the matrix. More recently, Sabeh *et* al. reported that the ability of cells to employ such a compensatory switch from a mesenchymal to an amoeboid mode of migration is context dependent (61). Specifically, the authors demonstrated that this compensatory mode of migration was only able to rescue the ability of cells to invade collagen matrices following MMP inhibition if the matrices were prepared from pepsin-extracted, (as opposed to acid-solublized) collagen. This effect was attributed to the fact that pepsin treatment removes collagen telopeptides and gels formed from acid-extracted collagen therefore contain cross-links that are absent from pepsin-extracted collagen gels, and as such are not permissive for protease-independent amoeboid cell migration.

In contrast to their findings for single-cell invasion, Wolf et al have reported that MMP activity is essential for collective cell migration from tumor spheroids embedded in collagen matrices even in gels prepared from pepsin-extracted collagen, which are permissive for MMP-independent invasion of single-cells (62). Overall, the available data suggest that MMP activity, and in particular MT-MMP1 activity, plays an important role in removing steric barriers to invasion, and facilitating tumor cell invasion through collagen matrices.

4.2 Reciprocal regulation of MMPs and collagen receptors

Remodeling of the ECM by MMPs is a tightly regulated process, and both transcriptional regulation and post-translational modulation of activity are ultimately important for controlling the rate of matrix turnover. At the transcriptional level, the DDRs have a role in modulating the expression of MMPs. For example, DDR1 overexpression in pituitary adenoma has been shown to induce cancer cell invasion through increasing MMP-2 and MMP-9 expression (63). In glioma cells DDR1a promotes invasion *via* an MMP-2-dependent mechanism and overexpression of DDR1 leads to increased production of pro-MMP-2 and MMP-9 (38). It is noteworthy that while expression of DDR1a and b in glioma cell lines resulted in the increased expression of both pro-MMP-2 and pro-MMP-9, activation of MMP-9 was not found under any conditions, and activation of MMP-2 was only observed following collagen activation of the receptor. Activation of MMP-2 was only found for DDR1a and not DDR1b, while elevated pro-MMP expression was reported for both isoforms, suggesting that the pathways regulating activation of MMPs are distinct from those regulating their expression, and more generally, that the pathways downstream of DDR1a and b are not identical.

Reciprocal regulation of collagen receptor activity by MMPs can also occur. At the most basic level, increasing MMP activity will result in degradation of collagen receptor substrates, and therefore antagonize receptor activation through a negative feedback mechanism. A further way by which MMP activity can antagonize receptor activation is through degradation of the receptors themselves. Both β1 integrin and DDR1 are substrates of MMPs, with MMP cleavage resulting in membrane shedding of extracellular fragments of these receptors (64-65). In the case of the integrins, shedding is thought to act as an additional means by which adhesion to the ECM is reduced, facilitating the detachment of cancer cells from the primary tumor site. In contrast, the biological significance of MMP-mediated DDR1 shedding has not been elucidated. However, collagen fibril organization *in-vitro* can be modulated by expression of the extracellular domains of both DDR1 and DDR2 (33). This could provide a mechanism whereby MMPs act to firstly release cells from the ECM though loss of integrin adhesion followed by subsequent assembly of newly synthesized matrix via DDR shedding.

5 Mechanical properties of collagen matrices as modulators of glioma invasion

In addition to providing biochemical signals, the mechanical properties of the collagen also play a role in modulating cancer cell behavior. Notably, the contribution of collagens to matrix stiffness is an area that has received considerable attention in recent years. In the following section we discuss matrix stiffness as a modulator of cancer cell behavior, and discuss the role of collagen receptors in influencing the response of tumor cells to the stiffness of their microenvironment.

The importance of matrix stiffness in the determination of cell behavior is exemplified by modulation of the malignant phenotype of transformed mammary epithelial cells by mechanical stimuli (66). In the case of glioma, the observations that brain tumors are stiffer than the surrounding brain parenchyma (67-69), and that cancer cell invasion often occurs along boundaries between structures of defined stiffness such as white matter tracts and blood vessels (70), has raised the idea that glioma cells may also respond to tissue stiffness in a similar manner to breast cancer. Some of the most compelling evidence that this is the case has come from studies performed in two-dimensional (2D) cell culture systems. That is systems where cells are plated on top of substrates of varying stiffness such as polyacrylamide or poly(dimethylsiloxane) gels functionalized with various extracellular matrix components. In particular, Ulrich et al have demonstrated that, *in vitro* modulation of bulk substrate stiffness affected the structure, proliferation, and motility of glioma cell lines in 2D culture (67).

While studies performed in 2D culture systems have gone some way to demonstrating that glioma cells are able to respond to the stiffness of their microenvironment, the extension of these studies into 3D model systems have shown that other physical properties of collagen matrices aside from stiffness can influence cancer cell invasion and proliferation. 3D cell culture models provide a basis for exploring physical interactions between the tumor cells and their environment, which are not amenable to 2D model systems. In particular, the presence of steric barriers to migration in 3D systems can have profound implications in the way cells invade, and such confounding factors have made it difficult to directly extrapolate results obtained in 2D culture to 3D model systems. Kauffman et al. have reported that invasion of glioma cells into 3D collagen matrices occurred at greater rates and with greater efficiency in gels formed from a higher concentration (and therefore stiffness) of collagen (49). However, since both ligand concentration and stiffness are varied concomitantly as collagen concentration is altered, it is not clear to what degree this effect on invasion can be attributed to either variable. One alternative system that has recently been proposed to circumvent this problem is to exploit edge effects inherent in 3D collagen gels to explore the contribution of collagen stiffness to cell migration. Rao et al used finite element analysis to demonstrate that collagen gels prepared on infinitely stiff substrates such as tissue culture plastic display greater stiffness close to the interface between the gel and the underlying substrate (71). GBM cells in the lower portion of the gels displayed more elongated morphology, more well-defined actin stress fibers, and greater migration rates than cells at positions closer to the top of the gels, suggesting that they were responding to the different mechanical properties of the gel present in these different locations.

In contrast to the studies described above, several studies have either failed to show that glioma cells respond to matrix stiffness in 3D culture, or demonstrated effects that are inconsistent with results obtained in 2D systems. Yang et al used collagen gels nucleated at different temperatures, and with different concentrations of collagen to generate a panel of substrates of differing bulk stiffness and pore sizes (50). The authors then examined the invasion of C6 rat glioma spheroids into these gels. Somewhat surprisingly, only pore size

appeared to correlate with invasion distance, with gels of similar pore sizes, but differing bulk stiffness exhibiting similar migration rates. Another similarly intriguing result was the observation by Zaman et al that human prostate carcinoma cells invading 3D matrigel substrates showed increased migration in softer gels when integrin binding was attenuated (in contrast to 2D studies that suggest that increased migration should occur on stiffer substrates under similar perturbations) (72). One explanation proposed was that inhibiting integrin binding generated more rounded cells which were less able to move through the pores in the 3D gels.

Our current understanding of the functional relationship between matrix stiffness and glioma progression is at its infancy. While a framework for understanding the influence of the mechanical properties of the matrix on tumor growth and invasion exists, the specific details of how cells respond to these properties are still unknown. In particular, the precise role of collagen receptors requires further investigation. Despite the wealth of information available from various studies looking at integrin-mediated mechanosensing, the mechanisms by which integrins mediate assembly of specific signaling complex components in a spatial and temporal fashion in response to different mechanical stimuli is still lacking. In addition, while there is evidence DDRs play a role in mechanotransduction (73), the potential for this family of receptors to modulate the behavior of cells in response to different mechanical cues remains largely unexplored. Finally, glioma cells also exert forces on the surrounding collagen matrix and the nature of the traction forces exerted on the matrix by invading cells as well as the compressive forces derived from tumor growth also merit further investigation.

6. Summary and perspectives

In this review, we sought to summarize the important and often understated role of collagens in glioma progression. As illustrated in Figure 4, collagens are key components of the tumor microenvironment that influence tumor growth and behavior through a variety of pathways. Collagens provide both biochemical and mechanical cues that act through collagen receptors to modulate cell invasion, growth, and survival. At the same time, collagen provides both a substrate for, and a barrier to invasion, with interrelated biophysical properties such as ligand density and matrix pore-size influencing how cells are able to invade the surrounding tissue. MMPs are important modulators of this process, and activation of collagen receptors can up regulate MMP activity leading to increased tumor growth, survival and invasion. However, upregulation of MMP activity can also act in the opposite direction to antagonize collagen-receptor interactions, both through degradation of the collagen ligands, and the receptors themselves. Importantly, glioma cells are also able to extensively remodel their microenvironment, not only through the degradation and synthesis of collagens, but also by exerting physical forces on the surrounding collagen matrix in the form of both traction forces directed towards individual cells, and compressive forces directed away from the expanding tumor.

The biological complexity of collagen (subtypes, modifications and mechanical compliance) and its binding partners make this molecule a particularly challenging one to characterize using classical biochemical approaches. As highlighted in the preceding sections, there are still many aspects of collagen function and signaling that remain to be investigated. Despite our limited understanding of collagen function in brain tumors, there is emerging evidence that the functional interactions between collagens and their cognate receptors are potential candidates for targeted cancer therapy. For instance, β1 integrin blocking antibodies have shown efficacy in overcoming radioresistance in the breast cancer setting (74). Furthermore, inhibiting β1 integrin can sensitize breast cancer cells that have acquired resistance to tyrosine kinase inhibitors (75). Similarly, blocking antibodies against DDR1 are capable of

reducing glioma cell invasion (38). These preclinical studies highlight the importance of not only targeting the cancer cells directly but also disrupting tumor cell-matrix interactions. To that end, we envision that combination regimens based on standard-of-care or targeted agents (e.g., tyrosine kinase inhibitors) together with collagen receptor inhibitors would offer meaningful clinical benefit for glioblastoma patients.

Acknowledgments

The work in the author's laboratory is supported in part by the Institute of Cancer Research, The Wellcome Trust (WT089028) and BBSRC (BB/I014276/1).

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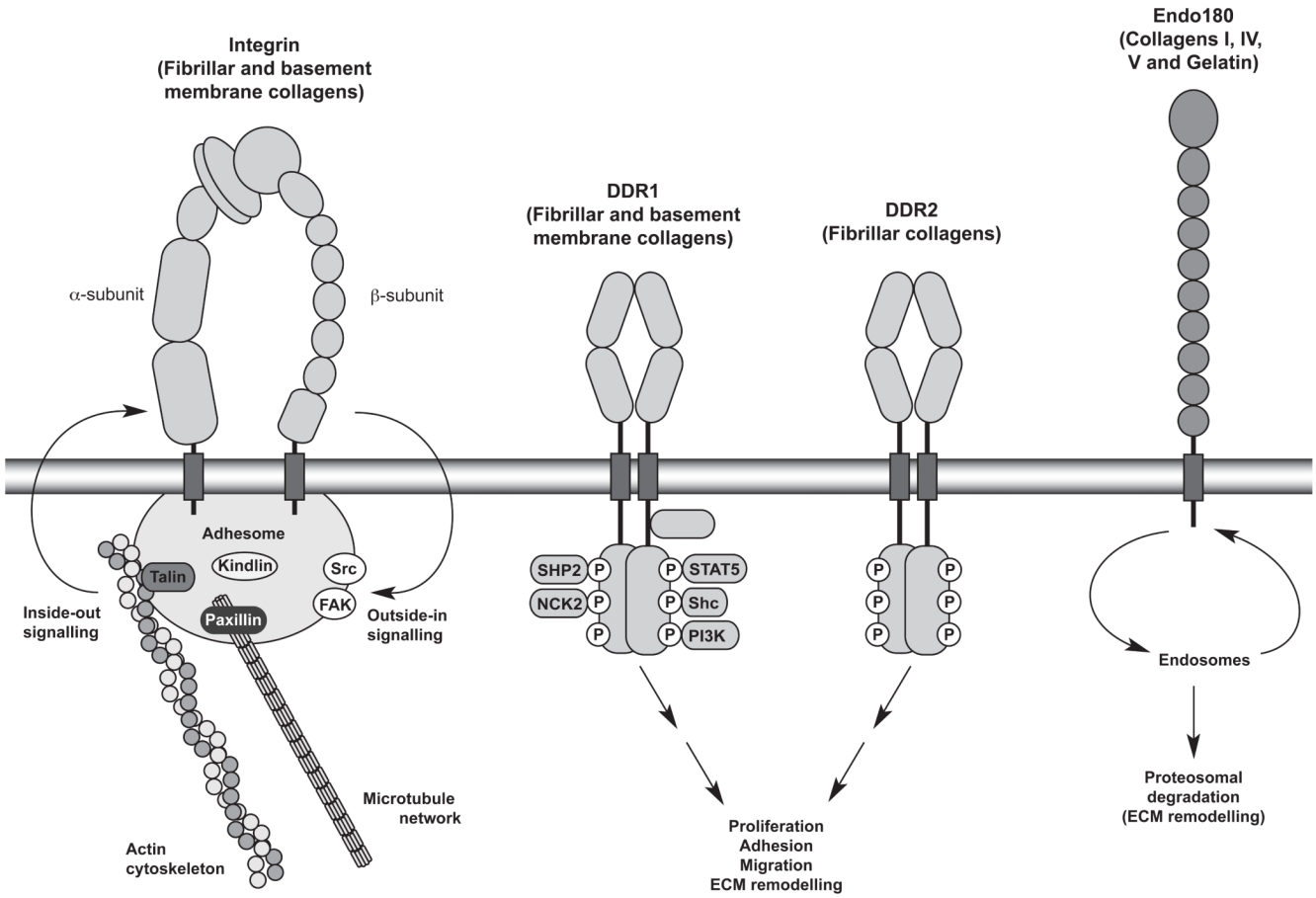

Figure 1. Integrin, discoidin domain, and Endo180 collagen receptors modulate distinct cellular processes in response to ligand binding

Integrins are heterodimers comprised of α and β subunits. Ligand binding results in assembly of adhesion complexes which interact with both the actin cytoskeleton and microtubule networks (outside-in signaling). Conversely, changes in the intracellular compartment affecting the adhesome or associated cytoskeletal components are able to modulate integrin affinity for its ECM ligand (inside-out signaling). Collagen binding to DDR1 and DDR2 leads to receptor phosphorylation, resulting in recruitment of adaptor proteins and activation of downstream signaling cascades. In contrast, collagen binding to Endo180 promotes cellular uptake and lysosomal degradation of the ligand.

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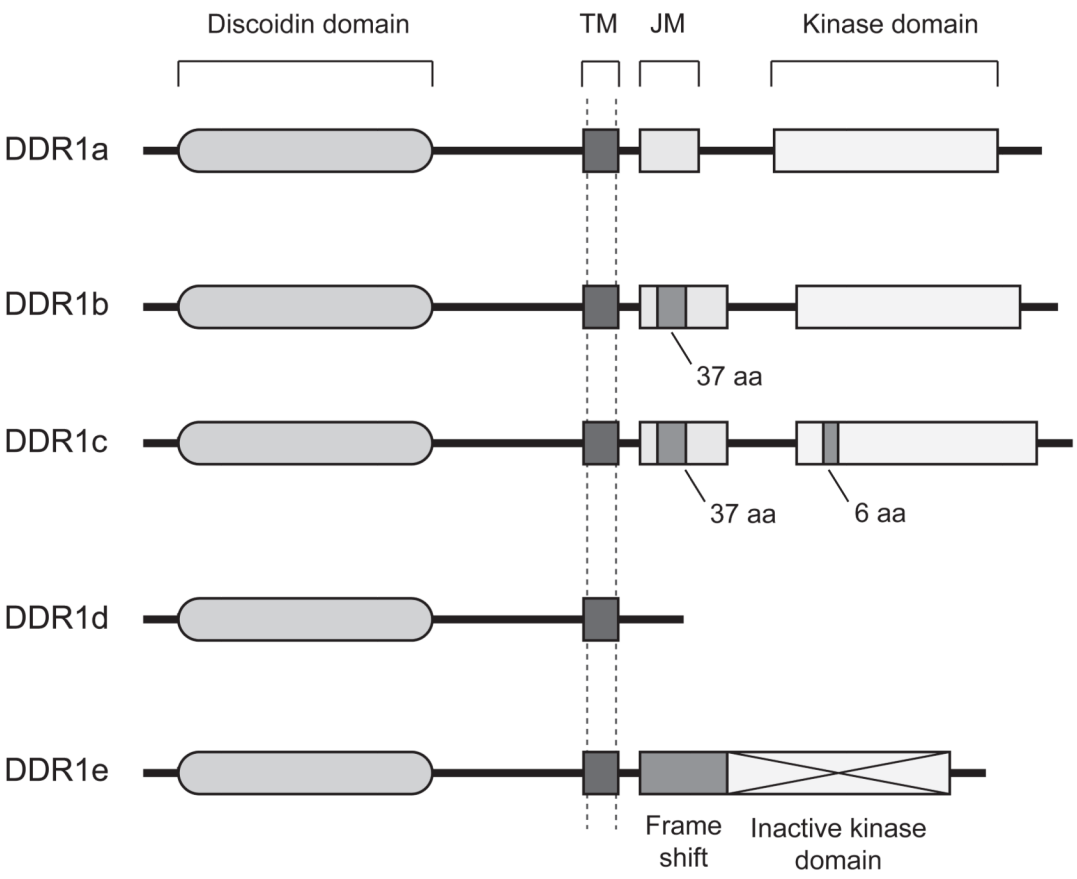

Figure 2. Splice variants of DDR1

DDR1c is the longest form. DDR1a and b both lack 6 amino acids in the kinase domain, while DDR1a also lacks an additional 37 amino acids in the juxtamembrane domain. DDR1d is truncated such that the entire kinase domain is absent. DDR1e contains a frame-shift resulting in inactivation of the kinase domain.

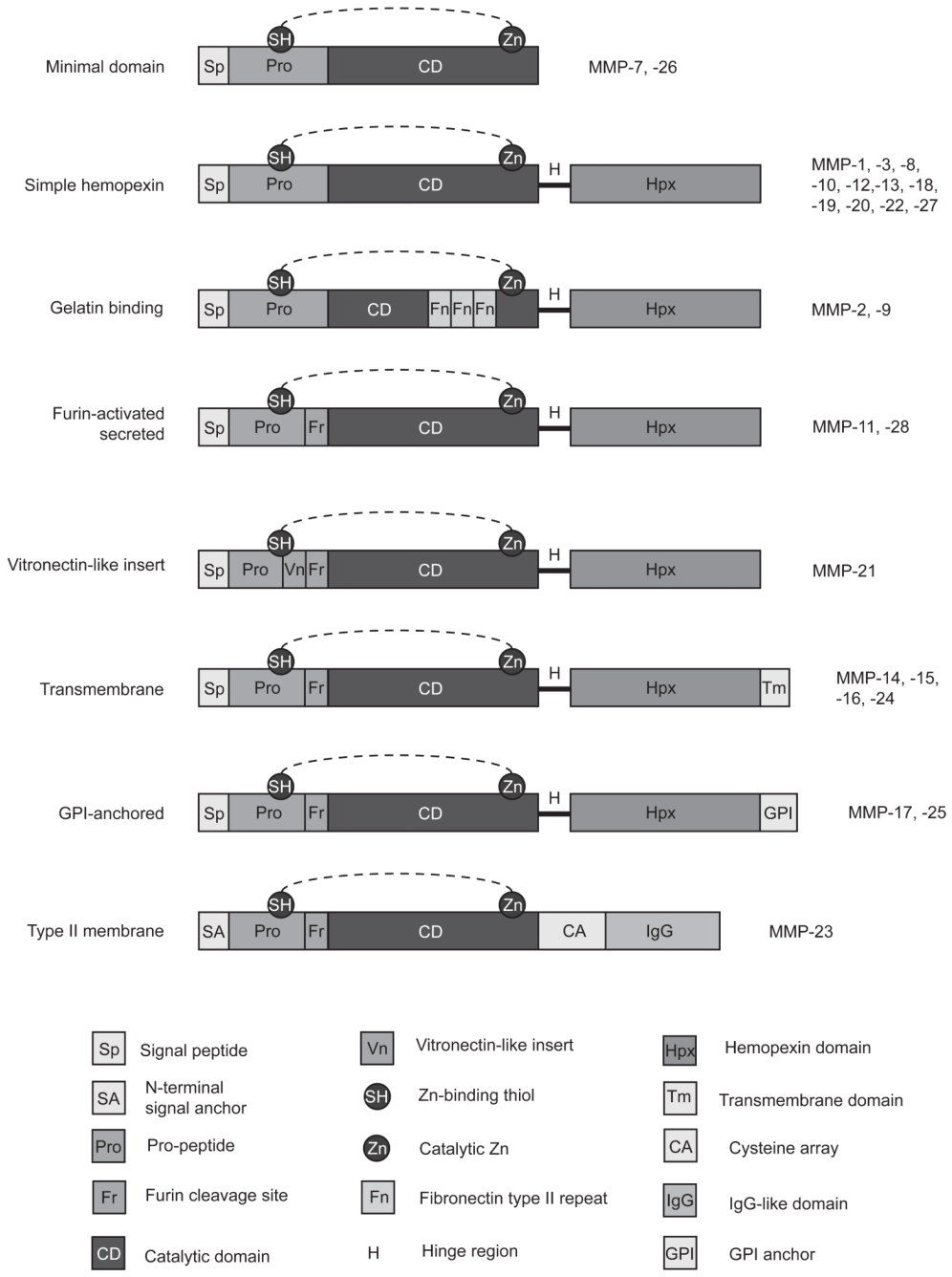

Figure 3. The domain structure of the MMPs includes

Common elements include a secretory signal peptide (SP); a regulatory pro-peptide (Pro); a catalytic domain (CD); and a C-terminal hemopexin domain (Hpx) linked to the catalytic domain *via* a hinge region (H). Five of the structural groups are secreted, while three are associated with the cell membrane (transmembrane, GPI-anchored, and type II membrane groups). MMPs are first synthesized in an inactive pro-form in which interactions between a cysteine thiol in the pro-peptide domain (SH) and the catalytic zinc ion (Zn) prevent substrate cleavage.

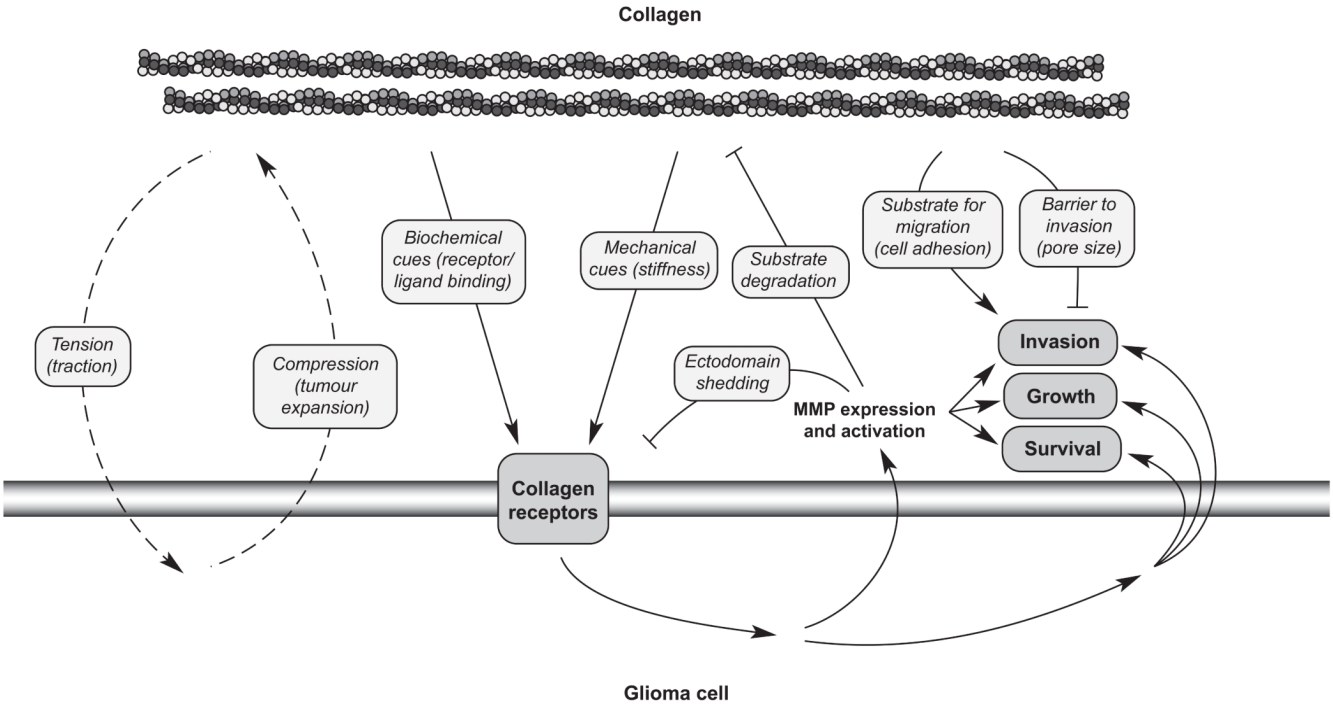

Figure 4. Interactions between glioma cells and the collagen ECM

Collagens provide both biochemical and mechanical cues that act through collagen receptors to modulate cell invasion, growth, and survival. At the same time, collagen provides both a substrate for, and a barrier to invasion. Activation of collagen receptors can upregulate MMP activity leading to increased tumor growth, survival and invasion. However, upregulation of MMP activity can also act in a negative feedback loop to antagonize collagen-receptor interactions, both through degradation of the collagen ligands, and the receptors themselves. Collagen organization is also affected by both traction forces exerted on the matrix by individual cells, and compressive forces arising from expansion of the tumor (dashed arrows).

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Table 1

Collagen-degrading MMPs and their substrates (adapted from (Hagemann et al., 2012)).

MMP	Alternative names	Collagen substrates
MMP-1		Collagen I,II, III, VII, VIII, X, XI
MMP-2		Collagen I, III, IV, V, VII, X, XI,
MMP-3		Collagen III, IV, V, VII, IX, X, XI
MMP-7		Collagen I, IV
MMP-8		Collagen I,II, III, IX, X
MMP-9		Collagen IV, V, VII, X, XI, XIV
MMP-10		Collagen III, IV, V
MMP-12		Collagen I, IV,
MMP-13		Collagen I,II, III, VI, IX, X, XIV
MMP-14	MT-MMP1	Collagen I,II, III,
MMP-16	MT-MMP3	Collagen III
MMP-19	MMP-18	Collagen I, IV
MMP-25	MT-MMP6	Collagen IV
MMP-26		Collagen IV