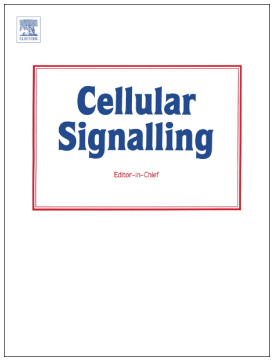

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Multifaceted Transforming Growth Factor-beta (TGFβ) Signalling in Glioblastoma

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Abstract

Glioblastoma (GBM) is an aggressive and devastating primary brain cancer which responds very poorly to treatment. The average survival time of patients is only 14–15 months from diagnosis [1, 2] so there is a clear and unmet need for the development of novel targeted therapies to improve patient outcomes. The multifunctional cytokine TGFβ plays fundamental roles in development, adult tissue homeostasis, tissue wound repair and immune responses. Dysfunction of TGFβ signalling has been implicated in both the development and progression of many tumour types including GBM, thereby potentially providing an actionable target for its treatment. This review will examine TGFβ signalling mechanisms and their role in the development and progression of GBM. The targeting of TGFβ signalling using a variety of approaches including the TGFβ binding protein Decorin will be highlighted as attractive therapeutic strategies.

Introduction

Glioblastoma (GBM)

A significant proportion of the human brain is made up of glial cells comprising four main subtypes: astrocytes, microglia, oligodendrocytes and their precursors NG2-glia [3]. Originally, glial cells were thought to function as a molecular scaffold responsible for neuronal structural integrity. However, more recent studies have identified wider functions of glial cells in neuronal guidance, survival, and myelination, as well as the formation and regulation of synapses (reviewed in [4]). Tumours that originate from glial tissue are termed glioma. Diagnosis and classification of subtypes of malignant gliomas are determined by histological features, which identify glioblastoma (GBM) as the most common glioma subtype (about 15% of all brain tumours), affecting about 3 individuals per 100000 people [5, 6].

While GBM can affect any age group, the highest prevalence is observed in individuals aged between 55 and 60 years old and in males. Clinical presentation of GBM is broadly subdivided into primary and secondary GBM, the majority (90%) being primary GBM which generally occurs in older patients (≥45 years old). Primary GBM develops rapidly without prior clinical evidence of a less malignant precursor lesion, while secondary GBMs typically develop through transformation of lower grade pre-existing astrocytomas. Secondary GBM, which account for the remaining 10% of cases, have better prognosis, and predominantly occur in younger patients (≤45 years). Despite the differences in age of onset and clinical histories, primary and secondary GBM are histologically indistinguishable, with common phenotypes being uncontrolled proliferation, high invasiveness and frequent resistance to both chemo and radiation therapy [7]. However, sequencing of GBM tumours has identified different molecular profiles of primary and secondary GBM tumours. Primary GBM are characterised by frequent amplification of the gene encoding the epidermal growth factor receptor (*EGFR*) (34%) and loss or mutation of phosphatase and tensin homolog gene, *PTEN* (24%), while secondary GBM frequently carry mutations in *TP53* (65%) and *IDH1* (at least 70%) [8]. Recent data indicate that IDH mutations are the most reliable biomarker of secondary GBM [9].

Currently, there is no conclusive evidence linking GBM to common environmental carcinogens such as smoking. The only recognised risk factor is prior exposure to ionising radiation [10], and although neurotropic viruses have been implicated, evidence to support a viral aetiology for GBM is also currently inconclusive[11, 12]. Given the lack of known predisposing factors, the vast majority of GBM cases are therefore considered to have arisen spontaneously. While the aetiology of the majority of GBM remains elusive, approximately 5% of all cases are the result of hereditary predisposition [13] caused by genetic disorders such as neurofibromatosis (types 1 and 2), tuberous sclerosis (TSC), von Hippel-Lindau disease (VHL), Cowden disease, Li-Fraumeni, and Turcot's, and Gorlin's syndromes. Within the remaining familial cases, the underlying hereditary cause, presumably combined with shared environmental influences, has not been delineated [14] although there is potential evidence for dominant inheritance of the disease [15]. However, genome-wide single nucleotide polymorphism linkage analysis looking at the predisposition to develop gliomas specifically identified a susceptibility locus at 17q12-21.32 [16] and

certain risk alleles (eg in *TERT* and *RTEL1*) have subsequently been associated with specific tumour molecular phenotypes [17]. Interestingly, meta-analysis has shown a marked reduction in glioma risk in those suffering from atopic and infectious diseases, suggesting that increased immune-surveillance might be protective against GBM [18, 19].

With the advent of more advanced sequencing and transcriptional profiling technology, it has become apparent that GBM cells exhibit significant inter- and intra-tumour heterogeneity [20-24] and that the overarching general classification of primary and secondary GBM can be refined into several different subtypes [21-24]. Four molecular subtypes have been proposed, based on characteristic somatic alterations and gene expression signatures that are reminiscent of different tissue types [23]: neural progenitor cells (termed proneural (PN)), neurons (termed neural (NL)), mesenchymal tissues (mesenchymal (MES)) or proliferating cells and receptor tyrosine kinase activation (classical (CL)). Tumours in the PN subgroup, which has the highest percentage of occurrence in younger patients, exhibit an increased frequency of mutations in *IDH*1, *TP53* and *PIK3CA/PIK3R1* and amplifications and overexpression of *PDGFRA* [23]. Consistent with the younger age group three quarters of the PN subtype sequenced were secondary GBM. The N subtype is characterised by elevated levels of neural markers (such as *NEFL*, *GABRA1*, *SYT1* and *SLC12A5*). Despite having elevated mutation frequencies in the *EGFR*, *TP53*, and *PTEN* genes, the NL subtype displays no unique genetic alterations to distinguish it from other sub classes [23]. The MES subtype is associated with poor overall survival in patients and exhibits focal hemizygous deletion and mutations in *NF1*, and loss of *IDH1*, *PIK3R1* and *PDGFRA*. The MES subtype also expresses mesenchymal markers *MET* and *CHI3L1* [22], as well concurrent high level expression of components of the NFkB pathway such as RELB [23].Tumours of the CLA subtype display *EGFR* amplification and overexpression and may also express a constitutively active version of the gene (*EGFR* vIII) caused by deletion of exons 2–7. This subtype also exhibits homozygous deletion of *CDKN2A* [23]. Thus, despite the different GBM tumour subtypes being histologically very similar, it is now clear there is a substantial genetic variation between the different sub-classes. It should also be noted, however, that cells from different regions of a single tumour can exhibit genetic features of more than one, and sometimes all, of these proposed subtypes [25].

GBM cancer stem cells

Inter- and intra-tumour heterogeneity of GBM at the cellular, genomic and transcriptional levels is thought to be due, in part, to the presence of a subpopulation of cancer stem cells (CSC). The CSC model proposes that tumour cells possess some of the characteristics associated with untransformed, ‘normal tissue’ stem cells and consequently have the ability to self-renew and to give rise to all of the different cell types found within a tumour [26]. In GBM, glioma stem cells (GSC) [27-29] share many capacities associated with neural stem cells, including scope to self-renew, differentiate and the ability to form 3 dimensional neurosphere structures [30]. On their cell surface, GSC express a number of different neural stem cell antigen markers including CD133, Sox2 and nestin [31]. They also exhibit increased resistance to ionising radiation, which is thought to be a consequence of

upregulation of DNA-damage response proteins (ATM, ATR and CHK1) which presumably results in enhanced DNA damage repair [28, 32, 33]. Despite undergoing treatment, within a few months the vast majority of GBM patients experience tumour recurrence that is often localised at, or near, the site of initial treatment [34]. It has been postulated that GSCs are responsible for tumour recurrence [27-29] and are therefore a key target for potential new therapies.

Treatment of GBM

Current standard of care for GBM comprises maximal safe neurosurgical resection followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) chemotherapy [35]. Despite this multimodal therapeutic intervention, GBM responds very poorly to treatment, with patients surviving an average of only 12-18 months from diagnosis and 5 and 10 year survival rates of around 5.5% and 2.9% respectively [6]. Age and performance status are the most powerful predictors of survival, with older patients generally experiencing shorter survival [36, 37].

The ongoing failure to improve outcomes for GBM patients is multifactorial. Some areas of the brain are simply inoperable and this, combined with the highly infiltrative nature of GBM, inevitably results in incomplete resection and a significant burden of residual tumour cells [34]. Indeed, one of the main factors correlating with long term survival is the extent of tumour resection [38, 39]. Another major limiting factor in the effective treatment of GBM is the inability of many compounds to traverse the blood-brain barrier [40], which prevents the use of many cytotoxic agents that are effective against other solid cancers [41, 42]. TMZ, a DNA alkylating agent, is the only drug that has shown clinical efficacy and is a key component of standard of care for GBM. However, the majority of GBM tumours exhibit high expression of O^6-methylguanine methyltransferase (MGMT), which efficiently reverses the guanine methylation of DNA caused by TMZ treatment, thereby greatly reducing its efficacy [43]. Moreover, tumours that initially respond well to treatment with TMZ frequently become resistant, particularly when TMZ is administered as a monotherapy possibly through enhanced emergence of drug resistant subclones [34]. In support of this theory, acquired resistance to TMZ has been frequently observed in vitro in GBM derived cell lines [44].

Other potential avenues for the treatment of GBM include molecular targeted therapies such as gene therapy, antiangiogenic treatments, immune-based approaches like chimeric antigen receptor (CAR) T-cell immunotherapy and therapeutic vaccines [45, 46]. However, at the time of writing this review, many of these alternative therapies had either not made the transition from the laboratory to the clinic or have failed to show efficacy in clinical trials.

TGFβ Signalling in glioma

Dysregulation of cytokine production and release has long been known to play an essential role in glioma progression by modulating the local tumour microenvironment to promote tumour cell proliferation and invasion, angiogenesis, and immune evasion. Cytokines known to be upregulated in glioma include a number of interleukins (IL-6, IL-8, Il-10 and IL-12), TNFα, HIF-1 and VEGF while those downregulated include the interferons IFN-α, -β and -γ and interleukins IL-2

and IL12 [47, 48]. This key role of cytokine signalling cascades in glioma pathogenesis has made them an attractive target for potential new therapies, although none to date have proved effective in a clinical setting.

Work carried out in the 1990s suggests that the cytokine TGFβ plays a role in the progression of glioma [49]. In the central nervous system, production of TGFβ has been observed in several cell types, including neurons, astrocytes, and microglia [50]. Under normal physiological conditions TGFβ is expressed at low basal levels [51] undetectable by Immunohistochemical staining [52], however, upon brain injury a significant increase in expression of TGFβ is evident. The importance of this response is illustrated by observations that loss of TGFβ signalling results in increased neuronal death [51] [53]. Thus, it has been proposed that TGFβ has a protective role within the brain. Malignant gliomas like GBM express high levels of TGFβ [54-56] and these elevated cytokine levels correlate with poor prognosis. TGFβ can induce proliferation of gliomas [57] and addition of TGFβ to glioma cell lines can mediate an invasive glioma phenotype [58, 59]. Overall, there is increasing evidence to suggest that inhibition of TGFβ signalling might provide novel therapeutic options for GBM in tumours where TGFβ is acting to promote proliferation and survival.

TGFβ Signalling Mechanisms

The TGFβ superfamily consists of a large number of multifunctional cytokines which play fundamental roles in development, adult tissue homeostasis, regulation of tissue, wound repair and immune responses. The superfamily includes activins, inhibins and bone morphogenetic proteins (BMP) [60] as well as the three isoforms of TGFβ (TGFβ1, β2, β3). *In vivo*, the vast majority of cells express at least one isoform of TGFβ. TGFβ is secreted as a Large Latent Complex (LLC) which can subsequently be targeted to the extracellular matrix (ECM) [61]. Following proteolytic and/or integrin mediated release from the LLC, TGFβ initiates signalling (illustrated in Figure 1) by binding a hetero-tetramer of two type I and type II TGFβ receptor serine/threonine kinases (TGFBRI and TGFBRII). The constitutively active TGFBRII in close proximity to TGFBR1 trans-phosphorylates TGFBRI in its regulatory GS domain [62, 63] hence activating the kinase domain of TGFBRI and initiating the canonical SMAD pathway to regulate gene expression [62, 63]. TGFβ also signals via non-canonical (SMAD-independent) pathways, in a cell type and context dependent manner, utilising a plethora of other cellular pathways including Nuclear Factor κB (NF-κB), Phosphatidylinositol-3-Kinase (PI3K)/AKT, Rho-like GTPase and Mitogen-Activated Protein Kinase (MAPK) pathways including MAPK1/3 (ERK2/1), MAPK8 (JNK1) and MAPK14 (p38)) [64, 65].

When dysregulated, TGFβ signalling plays a major role in the pathophysiology of many diseases, including several different types of cancer. Paradoxically, in cancer TGFβ can act as either a tumour suppressor or a tumour promoter dependent upon genetic and epigenetic changes present within the tumour cells [66, 67]. Generally, the tumour-suppressing effects associated with TGFβ are thought to occur within normal cells and early-stage tumours. Within this context, TGFβ negatively regulates both cell survival and proliferation. TGFβ inhibits cell cycle progression through induction of the cyclin dependent kinase inhibitors *CDKN2B* (p15) and *CDKN1A* (p21) while concurrently reducing expression of *MYC* (C-Myc) and *ID1-3*. Activation

of the TGFβ pathway also promotes genetic stability by enhancing expression of *TP*53 and *CHK*2, and may induce apoptosis via the up regulation of death-associated protein kinases (DAPK) and BCL2 family members such as PUMA [68-71].

Conversely, in the context of a more advanced tumour, TGFβ becomes an oncogenic factor promoting many of the hallmarks of cancer. These include excessive proliferation, increased cell survival, a stem cell like phenotype, immunosuppression, angiogenesis, epithelial to mesenchymal transition (EMT) (reviewed in [72]), tumour invasion and ultimately metastasis [73, 74].

The apparent paradox associated with the roles of TGFβ in cancer development and progression makes targeting the pathway for therapeutic use challenging. TGFβ's specific role in a tumour needs to be ascertained prior to commencement of treatment. In tumours that are addicted to TGFβ signalling for growth and/or require it for dissemination, inhibition of the pathway may prove beneficial. However, where TGFβ acts in a tumour suppressor role, inhibition of the pathway could be detrimental. Therefore, in a therapeutic setting it is clearly important that the precise nature of TGFβ signalling within a specific tumour should be delineated to inform the potential use of TGFβ inhibitors.

The Role of TGFβ Signalling in GBM

Tumour cell autonomous TGFβ signalling

Malignant glioma cells reportedly express autocrine TGFβ1 and TGFβ2 [75, 76]. Elevated levels of TGFβ2 and TGFβ3 have also been observed within tumour tissue, with hyper-activation (measured by phosphorylated-SMAD2 of the canonical TGFβ/SMAD pathway) correlating with poor patient prognosis [57, 77]. *In vitro* however, consistent with this notion of a TGFβ paradox, the functional outcome of TGFβ signalling may vary. Thus, TGFβ has been demonstrated to both positively and negatively regulate growth both in glioma cell lines [78-82] and in primary tumour cells derived from patient biopsies [57]. Additionally, loss of the TGFβ growth inhibitory response has been observed in glioma cells of a higher grade [83, 84]. Glioma cell lines can be broadly classified in 3 groups based on whether TGFβ inhibits (U87 MG), promotes (U373 MG) or has no major effect on proliferation [57, 80-82, 85]. In cell lines that are either growth promoted or inhibited by TGFβ, inhibition of TGFβ signalling markedly reduces or increase cellular proliferation, respectively [57]. Whether elevated levels of TGFβ can promote proliferation may, at least in part, be due to epigenetic changes within the tumour. For example, TGFβ can induce expression of pro-proliferative PDGF-B, but only in gliomas with an unmethlyated *PDGFB* gene promoter [57]. The methylation status of the *PDGFB* promoter, may be partially predictive in determining the response of a GBM tumour to TGFβ, and hence the aggressiveness of the tumour.

Additionally, TGFβ also promotes the upregulation of microRNA182 (MIR182), which subsequently inhibits the activity of the deubiquitinase enzyme ubiquitin carboxyl-terminal hydrolase (CYLD). Under normal cellular conditions active CYLD helps to dampen down NFκB signalling. The NFκB pathway plays an important role in the

development of cancer (reviewed in [86]). Briefly, increased NFκB signalling promotes the ability of cells to proliferate and undergo EMT, whilst also suppressing cellular apoptosis and stimulating angiogenesis. The reduction of CYLD activity caused by TGFβ-mediated induction of MIR182, therefore, results in sustained constitutive activation of NFκB in human glioma cells [87-89]. NFκB-mediated EMT is thought to contribute to the radiation resistance observed in tumour cells [90].

Interestingly, standard treatment of GBM (surgery and chemo-radiotherapy) may also result in increased TGFβ signalling. TGFβ's role in modulating a wound response has been well described in detail elsewhere [91]. It is highly likely that these pathways are also activated by tumour resection. In support of this notion, enhanced expression of TGFβ1 was observed after localised brain injury in rats [92]. In patient derived GBM cell lines, treatment with TMZ resulted in a dose-dependent increase in gene expression and cellular levels of TGFβ [93]. Exposure of cells to ionising radiation (IR) is also known to activate TGFβ [94] and in GBM this has the potential to enhance invasiveness via activation of MMP-2 [95, 96]. Additionally, after IR treatment, TGFβ seems to confer a protective effect against radiation induced damage [97]. Conversely, inhibition of TGFBR1 has been shown to enhance radiosensitivity [98] in a variety of cancer cell lines including GBM [82, 99]. It would be of interest to examine if cellular levels of TGFβ ligands and downstream biomarkers of pathway activation also increase in a dose dependent manner in GBM patients treated with radiotherapy.

Hence, conventional therapy may increase TGFβ levels at the site of treatment. Given that local recurrence of GBM generally occurs at, or adjacent to, the treatment volume, it is tempting to speculate that the treatment induced increases in TGFβ signalling may contribute to tumour recurrence. In certain contexts, TGFβ has well-established pro-oncogenic effects, and therefore may play a fundamental role in the development of GBM's highly malignant phenotype.

Pro-invasive TGFβ signalling

One of the characteristics of, and major challenges in treating, GBM is the highly invasive nature of the tumours. Invasion of malignant cells requires digestion of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs). TGFβ modulates the cellular levels of several MMP proteins including MMP-2 and MMP-9 [100, 101]. In parallel, TGFβ also decreases the level of tissue inhibitors of MMPs (TIMPs) consequently promoting ECM degradation and facilitating cellular invasion [102, 103]. All three TGFβ isoforms also have the capacity to induce EMT in epithelial cells [104-106] where cells exhibit decreased expression of epithelial markers such as E-cadherin, and enhanced expression of mesenchymal markers such fibronectin and vimentin [104]. The junctions connecting adjacent cells are removed and cells lose their polarity cues. TGFβ has been reported to induce features of the EMT-associated MES subtype in GBM cells through upregulation of transcriptional factors ZEB1, TWIST1 and SNAIL1 [107-109]. Additionally, TGFβ also has the potential to induce MIR10a/b and MIR182, which can enhance invasion of GBM cells via PTEN suppression and TGFβ/MIR182/NFκB crosstalk respectively [95, 110]. RNAi-mediated knock down of TGFBR2 severely impairs glioma invasion and tumourgenicity [76], thus, TGFβ signalling via its receptors can contribute to the migration and invasion of GBM tumour cells.

TGFβ in the maintenance of Glioma Stem Cells (GSC)

GSCs express a higher level of TGFβ2 than differentiated glioma cells [111] and autocrine TGFβ signalling is crucial in the maintenance of GSC tumorigenicity [112]. The TGFβ/SMAD pathway can induce the transcription of leukaemia inhibitory factor (LIF) which, in turn, activates JAK-STAT signalling, promotes GSC self-renewal and ultimately prevents differentiation [113]. Canonical SMAD-dependant TGFβ signalling also directly induces the expression of SYR box 4 (SOX4) which increases expression of the transcription factor SOX2 [112] and thus promotes a stem cell like phenotype in embryonic and neural stem cells [114]. Through these signalling pathways, TGFβ can significantly increase the self-renewing capacity while limiting the differentiation of GSCs.

TGFβ signalling within the tumour microenvironment

TGFβ signalling and angiogenesis in GBM

New blood vessel formation, or angiogenesis, is critical for growth beyond a tumour mass of approximately $2mm^3$ [115]. At larger sizes, a lack of new blood vessels limits availability of oxygen and nutrients and hinders tumour growth. *In vivo,* TGFβ1 can induce angiogenesis both directly [116, 117] and also indirectly via enhanced expression and activity of various pro-angiogenic proteins such as vascular endothelial growth factor (VEGF). VEGF is essential for increasing the permeability and proliferation of endothelial cells in vessel walls. Studies indicate a synergistic relationship between TGFβ signalling and hypoxia in VEGF transcriptional regulation [118]. In GBM the transcription of VEGF is controlled by TGFβ [119, 120]. GBM cells also exhibit elevated HIF levels [121] which enhance the transcription of *VEGF* [122], thus, the control of *VEGF* transcription could be modulated via cross-talk between HIF and TGFβ. TGFβ secreted by GBM tumour cells can also promote angiogenesis by increasing the expression of insulin like growth factor-binding protein 7 (IGFBP7) [123]. In a human GBM cell zebrafish xenograft model, TGFβ1 enhanced the induction of angiogenesis which could be repressed by the addition of a JNK (c-Jun N-terminal kinase) inhibitor [124]. Therefore, TGFβ probably contributes to modulation of angiogenesis resulting in the highly vascularised tumours that are characteristic of GBM.

Modulation of Immune responses by TGFβ in GBM

Recent studies indicate that TGFβ signalling can influence the tumour microenvironment and contribute to cytotoxic T cell exclusion and immune evasion in metastatic urothelial cancer and experimental models of breast and colon cancer [125, 126]. Similar mechanisms may also operate in GBM and this clearly warrants further investigation. As mentioned previously, activation of immunosurveillance has been implicated as a protective mechanism against GBM [18, 19] however, local and systemic suppression of the immune system has been well documented in GBM patients [127] [128]. The elevated levels of TGFβ ligands observed within the GBM tumour microenvironment are thought to contribute to this immunosuppression. TGFβ specifically prevents production of granzyme A/B, interferon gamma and perforin, molecules that are directly involved in T and Natural Killer (NK) cell-mediated tumour cytotoxicity [129]. Additionally, TGFβ1 downregulates the NK group 2D activating receptor on NK cells as well as the corresponding ligand on GBM

tumour cells, which aids evasion from T and NK cells. TGFβ2 reduces expression of the human leukocyte antigen (D related) on malignant GBM cells [130-132],promotes the production of FoxP3+ T regulatory cells and stimulates macrophages of the M2 phenotype to produce IL-10, all of which have an immunosuppressive effect and are associated with poor prognosis in GBM [133, 134]. To summarise, TGFβ has the capacity to influence a number of factors that contribute to a microenvironment that supports tumour progression and growth, ultimately leading to treatment failure [68].

Inhibition of TGFβ as a therapeutic treatment in GBM

Given the multiple lines of evidence indicating that TGFβ can act in a pro-tumourigenic manner in GBM (summarised in Figure 2) it follows that TGFβ signalling may be a promising therapeutic target for its treatment. In support of this strategy, genetic approaches to knock down expression of TGFβ or TGFβ receptors in glioma cells have been shown to limit migration, invasion and tumourigenicity [76, 135].

Several different inhibitors modulating TGFβ signalling at different points in the pathway have been developed (Figure 3). The first class regulates TGFβ signalling by limiting the availability of free ligand, thereby preventing receptor binding and activation and inhibiting subsequent downstream pathways. These include antisense oligonucleotides targeting TGFβ mRNAs, blocking monoclonal antibodies and receptor ectodomain-based ligand traps that sequester the TGFβ ligand. A second class of inhibitors do not prevent binding of TGFβ to its receptor but inhibit receptor kinase activity, halting the downstream signal transduction pathway [85, 136]. Building on the abundance of clinical and experimental data suggesting a role for TGFβ signalling in the progression of GBM, a variety of these inhibitors have been tested both *in vitro* and *in vivo* in preclinical models of glioma [82, 85, 99, 136-139].

The first class of inhibitors, which limits ligand availability, includes trabedersen (or AP12009), an antisense oligonucleotide complementary to the human TGFβ2 mRNA sequence. In patient-derived glioma cells, treatment with trabedersen significantly reduces TGFβ2 protein secretion compared to the controls [139]. A phase II clinical trial examined the effectiveness of two different doses of trabedersen (10 and 80 μM) compared to standard chemotherapy in the treatment of recurrent GBM or anaplastic astrocytoma (AA) [140]. In GBM, both doses of trabedersen used within the study, were equivalent to standard chemotherapy. Interestingly in AA patients, trabedersen did show sufficient promise to justify a follow-up phase III trial (NCT00761280). However, this trial was halted due to insufficient recruitment of patients [140]. In mouse models of glioma, use of a pan-TGFβ neutralising antibody (1D11) yielded contradictory results depending on the immune context, with complete remission only observed in immunocompetent mice [141]. A human monoclonal antibody neutralising all TGFβ isoforms GC1008A (also known as fresolimumab) was evaluated in a small Phase II study in recurrent high-grade glioma patients which included 9 patients with primary and 1 with secondary GBM. While fresolimumab was able to efficiently pass the blood brain barrier and penetrate the tumour mass, the trial was halted after 12 participants due to lack of any discernible clinical benefit [142]. P144 is a peptide encompassing amino acids 730-743 from the membrane-proximal ligand-binding domain of betaglycan and acts as ligand trap for TGFβ [143]. *In vitro* treatment of GBM cell lines with P144 results in decreased proliferation, migration, invasiveness, and tumorigenicity and, *in vivo*, P144 was reported to impair

tumour growth resulting in a concurrent increase in survival [144]. Similarly, the expression of a soluble version of TGFBR2 in glioma cells, results in a reduction of the phosphorylation of SMAD2 and ultimately led to a delay in growth [145].

The kinase inhibitor class, targeting TGFβ receptors, includes LY2109761 which inhibits both TGFβ receptors. *In vitro*, LY2109761 reduced the survival of glioma cell lines and increased their sensitivity to radiation. *In vivo*, it significantly reduced tumour growth and invasion resulting in prolonged survival [99] [138]. SB-431542 is another small molecule kinase inhibitor with high specificity for TGFBR1 [146]. In glioma cell lines, treatment with SB-431542 results in inhibition of both phosphorylation and subsequent nuclear translocation of the SMAD 2/3/4 complex [85]. Expression of VEGF and PAI-1, both downstream targets for TGFβ signalling are also reduced [85]. The TGFBR1 kinase inhibitor galunisertib (LY2157299 monohydrate) has exhibited an acceptable safety profile in clinical trials and, as such, is considered to be one of the most promising TGFβ inhibitors for clinical use. A phase I trial that included GBM patients indicated promising activity in this setting [147] but unfortunately the follow-up phase II trial indicated that galunisertib did not improve overall survival, either as a single agent or in combination with lomustine [148]. Recurrent GBM is notoriously challenging to treat however, with tumour cells exhibiting high levels of resistance to therapy. Indeed, the lack of any clinical benefit observed in clinical trials of the other TGFβ inhibitors described above, may also be a consequence of their use in recurrent GBM as opposed to use as an adjuvant or in combination with standard first line therapies. Galunisertib in combination with TMZ and radio/chemotherapy is currently being trialled in patients with newly diagnosed malignant glioma (NCT01220271), however, at the time of preparing this review, no clinical outcome information was available.

Despite promising *in vitro* data demonstrating that TGFβ inhibition could be an amenable target for treatment of GBM, no TGFβ pathway inhibitors tested so far have proven of clinical benefit to GBM patients. The reasons for this are likely manifold. Firstly, many preclinical *in vitro* and *in vivo* models used in the past fail to recapitulate the complexity of human disease and treatment, potentially giving rise to results that lack biological or clinical relevance. This is perhaps evident from the number of promising therapies identified in pre-clinical studies that have failed to translate into the clinic. Indeed, there has been no successful addition of a chemotherapeutic to GBM standard of care since TMZ. However, there is increasing development and use of more sophisticated and biologically relevant model systems, such as using 3D cell culture and patient derived cell lines in place of commercial lines [149]. The use of pre-clinical imaging modalities and small animal targeted radiotherapy platforms is also becoming more widespread, allowing for improved treatment planning and delivery in *in vivo* studies. Applying these strategies to TGFβ research may lead ultimately to more clinically translatable results. For instance, radiotherapy (RT) is standard of care for all GBM patients. However, radiation has been widely demonstrated to modulate cell signalling to produce a more aggressive and invasive phenotype in GBM cells and, importantly, to stimulate TGFβ secretion [150-155], potentially augmenting therapeutic response. The incorporation of state-of-the-art pre-clinical RT technologies that allow image guided, targeted delivery of multiple, clinically relevant fractions in combination with orthotopic models using primary cell lines in pre-clinical TGFβ studies may aid the identification and selection of promising therapies and predictive biomarkers in the future.

Poor patient response may also be in part due to insufficient delivery of therapeutic agents to the tumour bulk and invasive margins across the blood brain barrier, one of the major challenges in glioblastoma research. Although systemic administration of chemotherapy has been traditionally the preferred route, the combination of poor delivery, dose limiting side effects and short serum half-life has restricted progress and lead to researchers searching for alternative delivery methods. Recent studies have indicated that local delivery of chemotherapeutic agents through application of hydrogels or polymeric wafers into the resection cavity following surgery have the potential to enhance patient survival [156]. This approach allows maximum delivery and continuous slow release of therapeutic reagent to the remaining tumour cells while avoiding unwanted toxicity. Glioma cells are highly infiltrative however and this local application may not be sufficient to penetrate the brain parenchyma to reach the invading cells, thus the need for systemic administration may be unavoidable. The recent development of targeted delivery of chemotherapeutics across the BBB using receptor- mediated nanoparticle technology have so far produced promising results in the pre-clinical setting [157-159].

Despite the current challenges, there is clear scope for the development and application novel strategies for the targeting of TGFβ signalling. One potential new therapeutic avenue is the use of the glycoprotein decorin (DCN).

Decorin as a Potential Anti-Cancer Therapy in Glioblastoma

Mammalian DCN is the prototypical protein of the small leucine-rich repeat proteoglycan (SLRP) family. These proteins comprise a vital constituent of the ECM. The *DCN* gene resides on chromosome 12q21.33 and produces a primary translation product that is 329 amino acids in length, with a predicted native size of 42 kDa. As a glycoprotein DCN undergoes extensive post-translational modification, with specific residues of the protein covalently linked to a glycosaminoglycan chain of either chondroitin or dermatan sulphate. Consequently, the apparent molecular weight of the protein can vary considerably from the predicted size. The specific type of modification seems to be contingent on tissue type [160]. DCN is thought to have four functional domains [161, 162] and consists of 12 repeats of a globular protein core which is made up of tandem leucine-rich repeats (LRRs) flanked by cysteine-rich disulphide domains [163]. The LRR repeats form a curved solenoid structure [160] allowing interaction with a number of different substrates, including TGFβ, fibronectin, collagen, EGFR and VEGFR [164] (reviewed in [165]). DCN is predominantly synthesised and secreted by fibroblasts and mesenchymal stromal cells and is also found in the cytoplasm of epithelial cells (including neurons and astrocytes of the brain) [166, 167]. The first work characterising the function of DCN, suggested that it acted exclusively as a matrix proteoglycan, binding to type I, II and IV collagens to regulate fibrillogenesis [161]. In mouse models, DCN-null mice exhibit abnormal collagen morphology and skin fragility confirming a role linked to fibrillogenesis [168].

With respect to TGFβ signalling, at development stage E12, DCN null mice exhibit an increase in levels of TGFβ, SMAD2, SMAD3, and phosphorylated SMAD2/3 indicative of elevated activation of the canonical TGFβ signalling pathway. At E18, TGFβ is no longer elevated, while levels of SMAD2/3 are significantly reduced in DCN null mice compared to control mice [169]. A separate study also observed that

knockout mice exhibit increased expression of TGFβ1 and a concurrent increase in phosphorylation of ERK1/2 and SMAD3 but, interestingly, not SMAD2 [170]. This work suggests that within the context of foetal development, DCN can modulate the expression of TGFβ, SMAD2 and SMAD3, acting as both a negative and positive regulator of signalling.

Subsequently, DCN has been implicated in a number of other important biological processes including cell proliferation, differentiation, migration, metastasis, autophagy, inflammation, immunomodulation, and wound repair [162, 171]. Of interest, DCN can act as a tumour suppressor through inhibition of tumour growth and angiogenesis [172]. In metastatic breast cancer, loss of DCN correlates with an increased incidence of progressive disease and is associated with poor prognosis [173].

Decorin and TGFβ

The core region of DCN allows interaction with a number of different proteins. Through this domain, DCN has the capacity to bind to all 3 active isoforms of TGFβ [164, 174]. In CHO cells, exogenous DCN was sufficient to arrest TGFβ-induced proliferation [175]. The affinity DCN has for TGFβ may suppress signalling by competing for binding to the ligand with its appropriate receptor [176].

DCN may also modulate TGFβ signalling via an alternative pathway that does not directly prevent the binding of active isoforms of TGFβ to the appropriate receptor complex. This alternative regulatory pathway involves the Ca2+/calmodium-dependent kinase II phosphorylation of a negative regulatory site on SMAD2 (serine-240) [177]. In the absence of additional TGFβ, DCN induced the formation and nuclear translocation of the SMAD2/SMAD4 complex. The authors speculated that DCN could regulate canonical TGFβ signalling via sequestration of the cytoplasmic pool of co-SMAD4 to the nucleus, thereby limiting its availability for TGFβ receptor initiated SMAD signalling [177].

Additionally, given that DCN is an ECM protein and that DCN-TGFβ-binding is reversible, DCN has been proposed to act as a reservoir for TGFβ [174]. TGFβ stimulates the upregulation of DCN which may, in turn, directly inhibit the expression of TGFβ mRNA, acting as part of a negative feedback loop [178, 179]. Therefore, DCN has the potential to modulate TGFβ signalling at multiple different levels and is considered to be a naturally-occurring antagonist of TGFβ which may have potential therapeutic uses in TGFβ-driven disease.

Regulation of TGFβ signalling by Decorin

The therapeutic potential of DCN to regulate TGFβ signalling was first examined in a rat model of kidney inflammation. The fibrosis observed in the kidney is a consequence of elevated levels of TGFβ1, and this process is markedly reduced by addition of DCN [179]. TGFβ signalling is also involved in the wound healing response. In this context, the addition of recombinant DCN downregulates TGFβ1 and TGFβ signalling in models of hypertrophic scarring in skin [180, 181]. Acute and chronic adult lung disease is also associated with excessive TGFβ signalling levels [182] and expression of exogenous DCN within the airway epithelium is sufficient to

dampen down elevated signalling [183]. In DCN null mice, abnormal regulation of TGFβ signalling is rescued by addition of exogenous DCN [169].

Given the significant body of evidence implicating elevated TGFβ signalling in glioma progression, the effect of DCN on GBM has also been investigated. Ectopic expression of DCN in human and rat glioma cell lines inhibits mRNA transcription and bioactivity of TGFβ1 and TGFβ2 and also results in significant tumour regression of C6 glioma cells *in vivo* [167]. DCN treatment is also associated with decreased cell migration and infiltration [184, 185]. Animals receiving an intracranial injection of DCN expressing cells survive significantly longer than those injected with control tumour cells [186]. These studies also suggest that DCN contributes to reduced TGFβ pathway activity by preventing the synthesis and release of TGFβ1 and TGFβ2 [167, 186]. Tumours treated with DCN, regress after an initial period of growth. The regression is marked by increased infiltration of T and B immune cells into the tumour, which can be reversed by suppression of the immune system. This suggests that DCN abrogates the TGFβ-induced immunosuppressive state [167], suppresses TGFβ synthesis and promotes GBM tumour regression.

While this review has focused on using DCN to regulate TGFβ signalling, it should be noted that the biological effects of DCN are not solely limited to modulation of TGFβ function [165]. As shown in Figure 4, DCN also interacts with a myriad of different signalling molecules via direct binding to receptor tyrosine kinases such as EGFR, IGFR and VEGFR. DCN therefore has the potential to regulate a number of disparate pathways involved in inflammation, angiogenesis, autophagy and mitophagy [172, 187-189]. One of the major challenges in the chemotherapeutic treatment of malignant tumours is the ability of tumour cells to adapt and develop resistance to the drugs used, which is acutely problematic when chemotherapy options are limited. However, in mouse models, resistance of tumours to cisplatin or cyclophosphamide can be restored by intravenous injection of DCN [190, 191]. Therefore, the ability of DCN to restrict tumour cell adaption by simultaneously targeting a number of pathways may be its greatest asset as an adjuvant therapy.

However, as a note of caution, although several independent studies have demonstrated that DCN inhibits TGFβ's bioactivity [192, 193], DCN can also increase TGFβ-TGFβRII binding, with a consequential increase in TGFβ signalling [194]. In GBM, floating GSC neurospheres exhibit increased expression of DCN alongside increased resistance to TMZ [195]. More intriguingly, in GBM patients, high levels of DCN in tumours seem to correlate with shorter survival, which contradicts DCN's perceived role as a tumour suppressor [196]. Therefore, modulation of cell signalling may not be simply a function of DCN's role as a ligand trap but may be more reminiscent of TGFβ itself with its ability to either positively or negatively affect pathways being context dependent.

Concluding Remarks

GBM is the most predominant and aggressive primary brain cancer and currently remains incurable with a devastating prognosis of 14–15 months survival after diagnosis. The current treatment regime involves surgical resection followed by adjuvant radiotherapy and temozolomide chemotherapy, however in many cases, treatment only extends patient survival by a few months. Treatment failure can be

attributed to the invasive nature of GBM, substantial tumour heterogeneity and the presence of GSC populations. In the last decade, the search for new treatments for GBM has been a major focus of cancer research. Oncogenic TGFβ is known to drive GBM carcinogenesis and because of this growth factor addiction, TGFβ inhibitors could be ideal targeted therapies. Current understanding identifies DCN, a naturally-occurring TGFβ antagonist, as having the capacity to prevent GBM growth and brain infiltration. However conflicting research has also implicated DCN in enhancing TGFβ bioactivity and consequently promoting carcinogenesis. Given the uncertainty about the specific biological effects of TGFβ and DCN in the context of GBM, further study and the development of appropriate biomarkers to help identify which patients may benefit from treatment with TGFβ inhibitors (such as DCN) would aid a precision medicine approach. Additionally, future clinical trials of TGFβ inhibitors should be designed to test their efficacy in the first line settings and in combination with immune checkpoint blockade regimens.

Figure legends

Figure 1. TGFβ SMAD canonical and non-SMAD signalling pathways. TGFβ is released from its extracellular ligand trap and activated by either: proteolysis by matrix metalloproteinases or plasmin, integrins like αvβ6 or αvβ8, reactive oxygen species or Thrombospondin. Free TGF-β then binds to a heterotetrameric receptor complex composed of TGF□R1 and TGFBR2, enabling TGFBR2 to phosphorylate TGFBR1. The activation of TGFBR1 allows for the recruitment and subsequent phosphorylation of SMAD2 or SMAD3 (pSMAD). pSMAD2/3 then associates with SMAD4 in a heterotrimeric complex which translocates to the nucleus where, along with other co-factors and transcription factors, it binds to target gene promoters to positively or negatively regulate their expression. In addition, less well characterised non-canonical SMAD-independent pathways via PI3K, MAPK, SHC, TRAF, NFκB and RHO may be activated to regulate a number of cellular pathways.
Abbreviations: Transforming Growth Factor Beta Receptor 1 (TGFBR1), Transforming Growth Factor Beta Receptor 2 (TGFBR2), Small Mothers Against Decapentaplegic (SMAD), Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), Mechanistic target of rapamycin kinase (mTOR), ribosomal protein S6 kinase B1 (S6K), growth factor receptor bound protein 2 (GRB2), Son of Sevenless (SOS), TNF receptor-associated factor (TRAF), Transforming growth factor β-activated kinase 1 (TAK1), Mitogen-activated protein kinase kinase (MKK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38), par-6 family cell polarity regulator alpha (PAR6), Smad-Ubiquitin Regulatory Factors (SMURFs), Rho-associated protein kinase (ROCK), LIM domain kinase (LIMK), IκB Kinase (IKK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).

Figure 2. In GBM, pro-tumourigenic TGFβ signalling promotes tumour aggressiveness. TGFβ can promote: angiogenesis, cancer stem cell phenotypes, tumour cell migration and invasion, and immune-suppression thus contributing a

tumour micro environment that is supportive of chemo- and radio-therapy resistant cells.

Figure 3. Summary of TGFβ inhibitors. Trabedersen (or AP12009) an antisense oligonucleotide complementary to the human TGFβ2 mRNA sequence, helps prevent translation of TGFβ2. Fresolimumab (GC1008A) is a TGFβ neutralising antibody, while P144 is a short peptide that has a strong affinity for TGFβ and acts as ligand trap. These three different compounds limit the bio-availability of free TGFβ ligand that is able to bind to the receptor, preventing ligand dependent activation of the TGFβ signalling pathway. The second class of inhibitors are small molecules inhibit the kinase activity of the receptor, therefore halting the downstream signal transduction pathway.

Figure 4. Modulation of other signalling pathways by Decorin. A. Decorin (DCN) is a proteoglycan named after its ability to 'decorate' extracellular matrix proteins such as collagen. DCN is heavily modified by glycosaminoglycans allowing the protein to bind to a wide variety of cellular targets. Decorin has four domains. I: signal and propeptide. II: amino terminus (of mature protein). III: core protein. IV: carboxyl terminus.Decorin can act as a ligand for a number of receptor tyrosine kinases, modulating the associated downstream pathways. Abbreviations: Decorin (DCN), Epidermal growth factor receptor (EGFR), Insulin-like growth factor (IGFR), Vascular endothelial growth factor receptor 2 (VEGFR2), Hepatocyte growth factor receptor (Met), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), Trichoplein keratin filament binding (TCHP)/Mitostatin, TIMP metallopeptidase inhibitor 3 (TIMP3), catenin beta 1 (CTNNB1)/β-Catenin, hypoxia inducible factor 1 subunit alpha (HIF1α), vascular endothelial growth factor A (VEGFA), protein kinase AMP-activated catalytic subunit alpha 2 (PRKAA2)/AMPK, mTOR (mechanistic target of rapamycin kinase), MAPK (Mitogen-activated protein kinase), p21 (cyclin dependent kinase inhibitor 1A), RHOA (ras homolog family member A), ROCK1 (Rho associated coiled-coil containing protein kinase 1) and THBS1 (thrombospondin 1).

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This review highlights the complex roles of TGFβ signaling in glioblastoma.