

Chapter 9

Recent Advances in Understanding Mechanisms of TGF Beta Signaling and Its Role in Glioma Pathogenesis



Bozena Kaminska and Salvador Cyranowski

Abstract Transforming growth factor beta (TGF- β) signaling is involved in the regulation of proliferation, differentiation and survival/or apoptosis of many cells, including glioma cells. TGF- β acts via specific receptors activating multiple intracellular pathways resulting in phosphorylation of receptor-regulated Smad2/3 proteins that associate with the common mediator, Smad4. Such complex translocates to the nucleus, binds to DNA and regulates transcription of many genes. Furthermore, TGF- β -activated kinase-1 (TAK1) is a component of TGF- β signaling and activates mitogen-activated protein kinase (MAPK) cascades. Negative regulation of TGF- β /Smad signaling may occur through the inhibitory Smad6/7. While genetic alterations in genes related to TGF- β signaling are relatively rare in gliomas, the altered expression of those genes is a frequent event. The increased expression of TGF- β 1–3 correlates with a degree of malignancy of human gliomas. TGF- β may contribute to tumor pathogenesis in many ways: by direct support of tumor growth, by maintaining self-renewal of glioma initiating stem cells and inhibiting anti-tumor immunity. Glioma initiating cells are dedifferentiated cells that retain many stem cell-like properties, play a role in tumor initiation and contribute to its recurrence. TGF- β 1,2 stimulate expression of the vascular endothelial growth factor as well as the plasminogen activator inhibitor and some metalloproteinases that are involved in vascular remodeling, angiogenesis and degradation of the extracellular matrix. Inhibitors of TGF- β signaling reduce viability and invasion of gliomas in animal models and show a great promise as novel, potential anti-tumor therapeutics.

Keywords TGF- β signaling · TGF- β receptors · Activin-receptor-like kinases · MAP kinases · Smad proteins · Gliomas

Abbreviations

ADAMTS-1 metalloproteinase and disintegrin-like domain
Akt protein kinase B/Akt kinase
ALKs activin-receptor-like kinases

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AP-1	activator protein 1
BMPs	bone morphogenetic proteins
CTLs	cytotoxic T lymphocytes;
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal-regulated kinases 1/2
GDFs	growth and differentiation factors
Id	inhibitor of DNA binding
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LAP	latency-associated peptide
LIF	leukemia inhibitory factor
LTBP	latent TGF- β binding protein
MMP	metalloproteinase
MT1-MMP	membrane-type 1 matrix metalloproteinase
p38 MAPK	P38 mitogen-activated protein kinases
R-Smads	receptor-Smad proteins
SARA	Smad anchor for receptor activation
STAT	signal transducer and activator of transcription
TAK1	TGF- β -activated kinase-1
TGF- β	transforming growth factor β
TMZ	temozolomide
TNF α	tumor necrosis factor α
TRAF	TNF receptor associated factor
T β RI	TGF- β type I receptor
T β RII	TGF- β type II receptor
VEGF	vascular endothelial growth factor

9.1 Introduction

Transforming growth factor-beta (TGF- β) is a multifunctional cytokine which regulates cell proliferation, differentiation and extracellular matrix production (Ferrer et al. 2018; Verrecchia and Mauviel 2002). Additional members of TGF- β superfamily include: bone morphogenetic proteins (BMPs), activins (ACV) and Nodal proteins. The BMP family consists of 20 ligands, subdivided based on sequence similarity, and affinities for specific receptors into four subgroups: the BMP2/4, the BMP5–7, the BMP9/10 and the GDF5–7 (growth and differentiation factor). BMP signaling can be modulated by co-receptors such as endoglin and agonistic or antagonistic extracellular proteins such as BMPER (BMP-binding endothelial cell precursor-derived regulator). Nodal and Activins are secreted proteins, expressed during embryonic development and implicated in mesoderm formation and left-right axis specification (Shen 2007).

All TGF- β family members are synthesized as longer precursors with an N-terminal signal peptide followed by a large pro-domain (serving as a chaperone required for folding and secretion of the mature protein), a furin protease cleavage site, and a C-terminal mature polypeptide. The mature polypeptides are converted into disulfide bonds-linked dimers, which can interact with receptors and initiate the downstream signaling. In contrast to TGF- β , the activity of BMPs and activins depends on spatio-temporal regulation of their expression during development (Budi et al. 2017).

TGF- β and BMPs/GDFs form homo- and hetero-dimers that interact with combinations of type I and type II receptor dimers to produce multiple signaling complexes, leading to the activation of

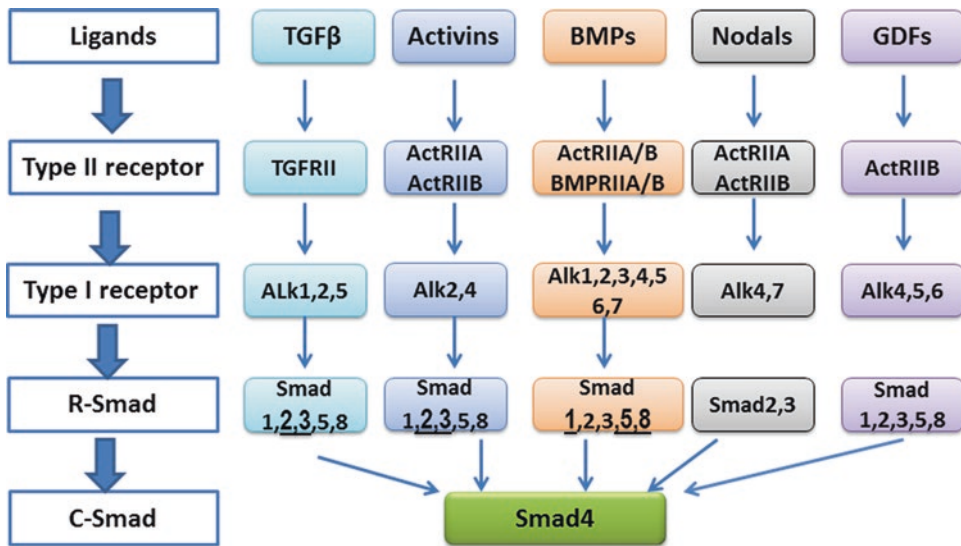


Fig. 9.1 The TGF- β superfamily signal transduction. Members of the TGF- β superfamily signal via distinct type I and type II receptors, which induces conformational changes and activation of kinase domains of the receptors. Smad 2/3 proteins are cytoplasmic transcription factors which are phosphorylated by a serine-threonine kinase associated with the receptor. Hetero-oligomeric complex of the R-Smad (receptor Smad proteins) associates with Smad 4, translocates to the nucleus and binds to specific DNA sequence in the promoters of target genes to regulate transcription. Four distinct type II, seven type I receptors and five R-Smads have been identified. ActR, activin receptor; ALK, activin-receptor-like-kinase; BMPR, BMP receptor; T β RI, TGF- β type I receptor; T β RII, TGF- β type II receptor

SMAD transcription factors and gene expression (Massague et al. 2005; Rider and Mulloy 2010; Schmierer and Hill 2007; ten Dijke and Hill 2004) (Fig. 9.1). The effects of distinct TGF- β isoforms depend on the type, differentiation state and physiological conditions of target cells (Bottner et al. 2000). Functional analysis of genes in the TGF- β signaling pathway in mice, either lacking specific genes or expressing dominant negative forms of particular proteins, provided new insights into the signaling cascades, their interaction and specificity (Goumans and Mummery 2000).

Deregulation of TGF- β expression or signaling plays key roles in the pathogenesis of a variety of diseases, including cancer and fibrosis. During oncogenesis, the TGF- β signaling has a dual function. On the one hand, TGF- β acts as a strong inhibitor of proliferation of normal epithelial cells and astrocytes, and is considered a tumor suppressor factor. On the other hand, in some tumor types, and specifically in high-grade gliomas, TGF- β becomes an oncogenic factor (Massague 2008). Under physiological conditions, TGF- β is expressed at a very low level in the brain, and its expression strongly increases after injury (Lindholm et al. 1992). TGF- β inhibits proliferation of normal astrocytes, but loses its growth-inhibitory potential towards gliomas, due to alterations in the expression of cell cycle inhibitors. There is growing evidence that TGF- β is actively secreted by tumor cells in the later stages of cancer progression where it stimulates cell growth, invasion, and metastasis, while decreasing host immune responses against tumor. TGF- β is highly active in high-grade gliomas, and elevated TGF- β activity confers poor prognosis in glioma patients (Rich 2003; Penuelas et al. 2009). TGF- β induces cell proliferation and tumor progression through the induction of platelet derived growth factor-B (PDGF-B) in human gliomas (Bruna et al. 2007). TGF- β 1 produced by glioma infiltrating brain microglia/macrophages doubles glioma invasion *in vitro* and *in vivo* (Wesolowska et al. 2008). TGF- β has been shown to cooperate with leukemia inhibitory factor (LIF) in inducing the self-renewal and preventing the differentiation of glioma initiating stem cells (Ikushima et al. 2009; Penuelas et al. 2009). TGF- β leads to the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway, specifically increasing the self-renewal capacity of glioma initiating

stem cells but not normal human neural progenitors (Penuelas et al. 2009). Despite a large number of data regarding TGF- β expression in different brain tumors, the molecular mechanisms underlying the expression, signaling and specific roles of TGF- β in the pathogenesis of gliomas are not fully understood. We summarize the data concerning molecular mechanisms of TGF activation, its signaling and role in glioma pathogenesis. Furthermore, we discuss advances in the development of molecular and pharmacological inhibitors of TGF- β signaling as cancer therapeutics.

9.2 A Brief Summary of Mechanisms of TGF- β Signaling in Normal and Malignant Cells

9.2.1 Components and Mechanisms of TGF- β Signaling

TGF- β is synthesized as a 55-kDa polypeptide, which forms a dimer shortly after production and is further cleaved in the Golgi apparatus by furine-like proteases to form a small latent TGF- β (Dubois et al. 1995). The mature 25-kDa TGF- β protein is non-covalently bound to the latency-associated peptide (LAP) and the latent TGF- β binding protein (LTBP). TGF- β is secreted as an inactive complex and the appropriate LTBP targets the protein to specific locations in the extracellular matrix – ECM (Hyytiainen et al. 2004). The latent state prevents the cytokine from eliciting a premature response or until a target cell is reached. LTBPs are important for the final activation of the cytokine. The latency proteins also contribute to the cytokine stability: free TGF- β has a half-life of about 2 minutes, whereas the latent form – 90 minutes. In higher grade gliomas, the levels of the LTBP-1 are increased and the proteins secreted by malignant cells associate with ECM. The LTBP-1 release can be decreased by the inhibition of furine-like protease activity. Interestingly, overexpression of LTBP-1 in glioma cells increases TGF- β activity and Smad2 phosphorylation, while the LTBP-1 gene silencing has the opposite effect (Tritschler et al. 2009).

TGF- β can be activated in an enzymatic process of LAP cleavage by plasmin (Grainger et al. 1995) or by thrombospondin-1, which also cooperates with the plasmin-mediated cleavage (Ribeiro et al. 1999). In some cells, a latent TGF- β 1 is found in association with the transmembrane protein GARP (glycoprotein A repetitions predominant) which anchors a latent TGF- β to the cell membrane (Budi et al. 2017). Other proteins participating in TGF- β activation *in vivo* include integrins (Annes et al. 2004) and matrix metalloproteinases (membrane-type 1 matrix metalloproteinase, MT1-MMP) (Tatti et al. 2008). Therefore, activation of TGF- β is a multi-step, tightly controlled process necessary for the subsequent downstream signaling.

TGF- β signals through binding to the type II and type I serine/threonine kinase receptors (T β RII and T β RI, respectively), inducing their hetero-oligomerization and subsequent activation of specific intracellular signaling pathways (Groppe et al. 2008). Five type II and seven type I receptors, also termed activin-receptor-like kinases (ALKs), have been identified (Shi and Massague 2003) (Fig. 9.1). TGF- β first binds to T β RII and alters its conformation. T β RII phosphorylates the cytoplasmic domain of T β RI. Subsequently, phosphorylated type I receptors such as ALK1 and ALK5 directly phosphorylate the receptor-regulated Smad (R-Smad) proteins on the C-terminal Ser-Ser-X-Ser motif. The phosphorylation is facilitated by adaptor proteins, including SARA (Smad anchor for receptor activation) and HRS/HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) via the phospholipid-binding FYVE domain, acting as an adaptor during recruitment of R-Smad2 to the TGF- β receptor complex (Miura et al. 2000; Tsukazaki et al. 1998). Another adaptor protein is cPML (the cytoplasmic promyelocytic leukaemia), which promotes phosphorylation of receptor- Smad proteins (R-Smad) by ALK5 (Lin et al. 2004). Nodal and Activin bind to their common receptors, the Activin-*like* (Alk) type I receptors Alk4 and 7, with Cripto-1 as a co-receptor for Nodal signaling (Shen 2007). BMPs bind to type 1 (ALK1/3/6) receptors and type 2 (BMPRII, ActRIIA, ActRIIB) transmembrane receptors with

serine/threonine kinase (Pauklin and Vallier 2015). R-Smad phosphorylation is coupled to TGF- β receptor internalization (Penheiter et al. 2002).

The TGF- β /Activin/Nodal subfamily activates Smad2 and 3, whereas the BMP/GDF subfamily functions mostly through Smad1, 5, and 8 (Goumans and Mummery 2000, Nakao et al. 1997) (Fig. 9.1). The MH2 (MAD-homology-2) domain is highly conserved among all Smad proteins and is responsible for receptor interaction, the formation of homo- and heteromeric Smad complexes, and direct contact with the nuclear pore complex for shuttling to the nucleus. Phosphorylation of the two serine residues in the SXS motif of the MH2 domain activates R-Smad (Piek et al. 1999; Shi and Massague 2003). Once phosphorylated, R-Smads dissociate from the receptor/SARA complex and form an oligomeric complex with the common mediator, Smad4, whereupon they translocate to the nucleus and interact with Smad binding elements (SBE). The SBE is composed of the consensus palindromic sequence GTCTAGAC for Smad3 and Smad4 or GC-rich sequences present in certain promoters (Fig. 9.2). Smad proteins have poor affinity to

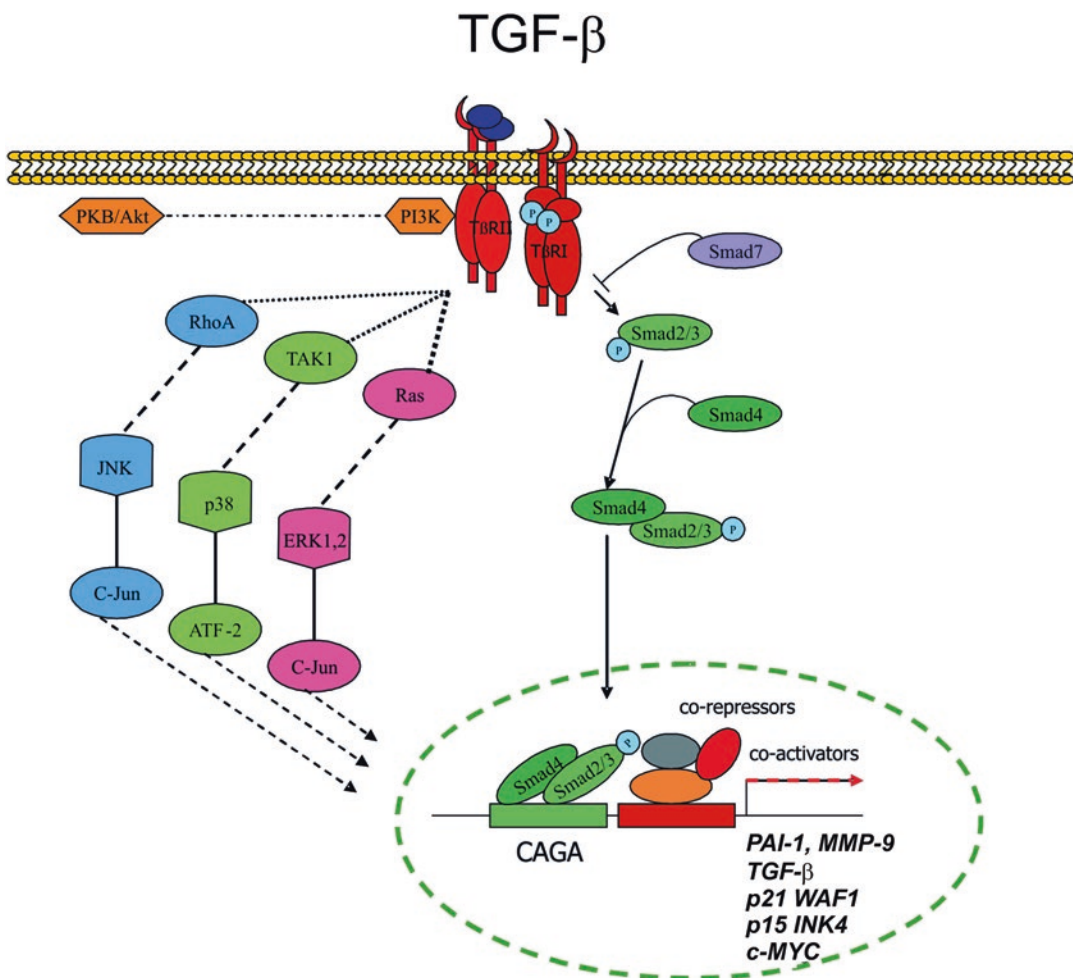


Fig. 9.2 Activation of TGF- β type I and type II receptors (T β RI, T β RII) leads to activation of receptor kinases, phosphorylation of receptor-Smad proteins (R-Smads), forming a complex with Smad4, which translocates to the nucleus. The pathway is regulated by the activity of the inhibitory Smad7. TGF- β can activate several mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), p38 MAPK, phosphatidylinositol 3-kinase (PI3K) and protein kinaseB/Akt (PKB/Akt). The interaction with these MAP kinases may positively or negatively regulate Smad transcriptional activity

DNA and have been shown to cooperate with a wide variety of high-affinity DNA-binding transcription factors to regulate gene expression (Massague et al. 2005; Moustakas et al. 2001). Nodal ligands signal via the type I receptor ALK4/7 and the type II receptors ActRIIA and ActRIIB, as well as through Smad2/3 (Shen 2007). TGF- β -induced Smad signaling cooperates with different factors and signaling pathways, which offers versatile means to elicit various cellular responses (Budi et al. 2017).

Besides the canonical TGF- β /Smad pathway, TGF- β can directly activate non-Smad signaling pathways (Moustakas and Heldin 2005; Zhang 2009), including induction of PI3K–Akt–mTOR signaling, activation of small GTPases such as Ras, Rho, Rac and CDC42 (Edlund et al. 2002; Budi et al. 2017; Wilkes et al. 2003), and the mitogen activated protein (MAP) kinases. TGF- β activated kinase-1 (TAK1), a member of the MEKK family and activator of JNK and p38 MAPK, is activated by TGF- β (Yamaguchi et al. 1995). T β RI-induced phosphorylation of ShcA protein, which associates with adaptor Grb2 and Sos proteins, leads to the phosphorylation of extracellular signal-regulated kinases (ERK), thereby initiating the well-characterized pathway and linking receptor tyrosine kinases with ERK MAP kinases (Lee et al. 2007). Activation of Ras, ERK1/2, and c-Jun N-terminal kinase (JNK) by TGF- β signaling have been reported in primary intestinal epithelial cells and some breast cancer cell lines (Mulder 2000). TAK1 and TAB1 have been demonstrated as upstream signal transducers activating the MKK3–p38 MAPK signaling cascade that leads to the induction of type I collagen expression by TGF- β 1 in mouse glomerular mesangial cells (Kim et al. 2007). We reported that TGF- β 1, which did not affect the viability or proliferation of human glioblastoma T98G cells, increased transcriptional responses exemplified by the induction of *MMP-9* expression. TGF- β -induced nuclear translocation of the SMAD3/SMAD4 complex and activation of SMAD-dependent promoter was paralleled by the selective activation of p38 MAPK, and phosphorylation of its substrates: ATF2 and c-Jun proteins, leading to transient activation of AP-1 transcription factor (Dziembowska et al. 2007; Kaminska 2009). Selective activation of p38 MAPK, with no apparent activation of JNK after TGF- β stimulation, was detected in C2C12, Mv1Lu, and HaCaT cells (Hanafusa et al. 1999; Karsdal et al. 2003).

There are reports of interactions between Smad dependent and independent pathways. TGF- β 1 or Smad7 overexpression induce apoptosis of human prostate cancer PC-3U cells by activation of the TAK1–MKK3–p38 MAPK pathway. Forced expression of dominant negative p38, dominant negative MKK3, or incubation with the p38 selective inhibitor prevented TGF- β 1-induced apoptosis. The expression of Smad7 was required for TGF- β -induced activation of MKK3 and p38 kinases. Endogenous Smad7 could interact with TAK1 and MKK3 and phosphorylated p38 in a ligand-dependent manner, suggesting that Smad7 may act as a scaffolding protein, and facilitate TAK1- and MKK3-mediated activation of p38 (Edlund et al. 2003).

TGF- β 1 may also influence cancer metabolism. TGF- β 1 up-regulates *PFKFB3* mRNA and protein expression (PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases), which results in an increase in fructose 2,6-bisphosphate concentration, glucose uptake, glycolysis and lactate production in T98G glioma cells. Increases in *PFKFB3* mRNA and protein expression and Fru-2,6-P₂ concentration are mediated by Smad3, p38 MAPK, and PI3K/Akt signaling (Rodríguez-García et al. 2017).

Mechanisms underlying non-canonical TGF- β signaling may involve the adaptor protein TRAF6, the ubiquitin ligase (E3), which interacts with a consensus motif present in T β RI and forms a complex required for TGF- β -induced autoubiquitylation of TRAF6 and subsequent activation of the TAK1–p38/JNK pathway in a receptor kinase-independent manner. Activation of Smad2 is not dependent on TRAF6 (Sorrentino et al. 2008). Furthermore, T β RI can be cleaved by TACE (TNF- α converting enzyme) in cancer cells and the liberated intracellular domain (ICD) of T β RI associates with the transcriptional regulator p300 to activate genes involved in tumor cell invasiveness, such as *Snail* and *MMP-2*. The T β RI ICD was localized in the nuclei of different types of tumor cells in tissue sections, but not in normal epithelial cells (Mu et al. 2011).

9.2.2 *Negative Regulators of TGF- β Signaling*

The Smad signaling pathway may be negatively regulated by the inhibitory Smad6 and Smad7 – (I-Smads) (Nakao et al. 1997; Itoh and ten Dijke 2007). While R-Smads form via the MH2 domain stable associations with activated type I receptors, inhibitory Smads act mainly to oppose this association, thus preventing phosphorylation of R-Smads (Shi and Massague 2003; Miyazawa and Miyazono 2017). R-Smads have a Ser-Ser-X-Ser (SSXS) motif at their C-terminus, which is phosphorylated by the type I receptors, whereas I-Smads and the co-Smad lack such a motif. Smad6 preferentially inhibits Smad signaling initiated by BMP type I receptors ALK-3 and ALK-6, whereas Smad7 inhibits both TGF- β - and BMP-induced Smad signaling (Shi and Massague 2003; Miyazawa and Miyazono 2017). A Smad7 mutant that fails to interact with T β RI retains its ability to inhibit TGF- β signaling (Kamiya et al. 2010). Other mechanisms by which I-Smads antagonize signaling include mechanisms such as: down-regulation of cell surface type I receptors, prevention of complex formation between R-Smads and co-Smads. I-Smads also regulate non-Smad signaling pathways induced by TGF- β family proteins. I-Smad may block interactions with Smad4, preventing R-Smad–Smad4 complex formation; recruit E3-ubiquitin ligases Smurf1 and 2 to induce type I receptor ubiquitination and subsequent receptor degradation; directly repress Smad-induced transcriptional responses (Miyazawa and Miyazono 2017). Another important way of inhibition of the R-Smad function is through the recruitment of transcriptional co-repressors such as c-Ski and SnoN. Co-repressors Ski and SnoN can be recruited to Smad binding elements in a Smad4-dependent manner and inhibit the basal expression of TGF- β -responsive genes such as *Smad7* (ten Dijke and Hill 2004).

9.2.3 *Transcriptional Responses Induced by TGF- β Signaling*

All R-Smads (except Smad2) and Smad 4 bind DNA directly. The preferred binding site of the Smad3 and Smad4 MH1 domains was defined as the SBE (Zawel et al. 1998). Smad binding to the SBE lacks selectivity, as Smads 1, 3 and 4 have a similar affinity for the SBE. Therefore, additional DNA contacts are necessary for specific, high-affinity binding of a Smad complex to a target gene. Smads can achieve high-affinity, selective interactions with DNA by associating with DNA-binding partners, forming complexes of specific composition and geometry. Most of the Smad transcriptional partners identified to date, including Fast1, Mixer, Jun/Fos, Runx, ATF3, E2F4/5, are highly responsive to different stimuli (reviewed in Massague and Wotton 2000). Smad3 binds directly to the promoter region of *c-myc* through TGF- β inhibitory element and represses its transcription (Yagi et al. 2002). Global expression profiling and chromatin immunoprecipitation followed by sequencing (ChIP-seq) have defined Smad target genes and binding sites in various cell types and demonstrated that those sites vary between cell types and differentiation stages (Mullen et al. 2011). Studies comparing Smad2 and Smad3 knockout mouse phenotypes and activation of TGF- β target genes, suggest that Smad2 and Smad3 have distinct roles and sets of transcriptional targets (Goumans and Mummery 2000). Smad2 plays important roles in embryogenesis, whereas Smad3 regulates cell growth and cell migration after birth (Brown et al. 2007).

Smad-mediated transcription is affected by the presence of OCT4, SOX2 and NANOG transcription factors in embryonic stem cells, interaction with other signal-induced factors, and interplay with transcriptional repressors and the polycomb group proteins (Gaarenstroom and Hill 2014). FAST-1 is a member of the winged-helix family of DNA-binding proteins and interacts with Smad2–Smad4 or Smad3–Smad4 complexes, but not with BMP-activated Smad complexes (Chen et al. 1998). Smad3–Smad4 complex and an AP-1 (activator protein-1) complex synergize in the transcriptional activation from the *c-Jun* promoter by binding to separate sites located 120 bp apart from each other (Wong et al.

1999). Smads recruit the general co-activators p300 and CBP (Creb binding protein) that have histone acetyltransferase activity and may increase transcription of target genes by inducing chromatin remodeling (Massague and Wotton 2000; Gaarenstroom and Hill 2014). The requirement of BRG1/SMARCA4, a component of the SWI/SNF nucleosome remodeling complex, to be recruited by Smad2/3 in response to TGF- β signaling has been demonstrated (Ross et al. 2006; Xi et al. 2008;).

TGF- β 1 can activate its mRNA expression and thereby its own secretion in Smad- and AP-1-dependent manner in many cell types, including glioma cells (Jachimczak et al. 1996; Jennings et al. 1991; Kim et al. 1990; Van Obberghen-Schilling et al. 1988). There are three AP-1-binding elements contributing to TGF- β 1 induction and antisense oligodeoxynucleotides against the AP-1 components *c-jun* and *c-fos* blocked autocrine TGF- β 1-induced expression. The TGF- β cytostatic program involves transcriptional activation of the cyclin-dependent kinase inhibitors p21WAF1/Cip1 and p15Ink4b, and repression of transcription of genes encoding transcription factors *c-myc* and Id1-Id3 (inhibitor of DNA-binding 1–3) (Siegel et al. 2003). Negative regulators of TGF- β signaling such as *I-Smads* and *SnoN* are direct target genes of TGF- β and participate in negative feedback loops (Derynck and Zhang 2003; Massague and Wotton 2000). The canonical model has been challenged in some studies employing global gene expression profiling and showing that a subset of TGF- β induced genes does not require Smad4 (Ijichi et al. 2004; Levy and Hill 2005).

The target genes controlled by the same Smad-cofactor complexes represent a synexpression group. An Id-like helix-loop-helix protein, a human homologue of Maid (HHM), is a synexpression group-restricted regulator of TGF- β signaling. HHM suppressed TGF- β -induced growth inhibition and cell migration, but not epithelial-mesenchymal transition. In addition, HHM inhibited TGF- β -induced expression of plasminogen activator inhibitor-type 1 (*PAI-1*), *PDGF-B*, and *p21WAF*, but not *Snail*. Olig1 is one of the Smad-binding transcription factors affected by HHM. Olig1 interacts with Smad2/3 in response to TGF- β stimulation, and is involved in transcriptional activation of *PAI-1* and *PDGF-B*. HHM, but not Id proteins, inhibited TGF- β signaling-dependent association of Olig1 with Smad2/3 through physical interaction with Olig1. Thus, HHM appears to regulate a subset of TGF- β target genes, including the Olig1-Smad synexpression group (Ikushima et al. 2008).

The SKI-related proto-oncogene *SNON* (*SKIL*), that encodes a potent negative regulator of TGF- β -related signaling, physically interacts with SMAD2, SMAD3, and SMAD4, disrupts their assembly and prevents SMAD2 and SMAD3 from associating with the transcriptional co-activator p300/CBP. It recruits a nuclear receptor co-repressor (N-CoR) and histone deacetylase (HDAC). It keeps TGF- β /Nodal/Activin target genes repressed. Upon activation of TGF- β /Nodal/Activin signaling, which occurs during mesendodermal differentiation, *SNON* and *SKI* are rapidly degraded by the proteasome, exposing the SBEs and allowing for the binding of the activated Smad3–Smad4 complexes to gene promoters (Tsuneyoshi et al. 2012).

9.3 Deregulation of TGF- β Signaling in Gliomas

There is growing evidence that alterations in TGF- β signaling pathway components modify cancer risk. Approximately 14% of the general population carry *TGFBR1**6A, a variant of the *TGFBR1* gene that results in decreased TGF- β -mediated growth inhibition. Subsequent studies showed that the overall cancer risk is increased by 70% and 19% among *TGFBR1**6A homozygotes and heterozygotes, respectively (Wang et al. 2012). The recent comprehensive study of TGF- β signaling network across 33 different cancer types reported a list of 43 genes that encode proteins from the canonical TGF- β pathway: 3 TGF- β proteins, 8 bone morphogenetic proteins (BMPs) and 9 activin (ACV); 3 TGF- β receptors and 1 interacting protein (TGFBRAP1), 3 BMP receptors and 6 ACV receptors; 8 Smads; and 2 adaptor proteins (SPTBN1 and ZFYVE9) (Korkut et al. 2018). The frequency of mutations was generally low, but 39% of the tumors contained an alteration in at least one of the 43 core genes.

SMAD4 (4%) and *SPTBN1* (4%) were the most frequently altered. Collectively, BMP ligands had an alteration frequency of 13% and SMAD-encoding genes had heterozygous deletion frequencies around 20%. Tumors rarely had more than one altered gene within a category (Korkut et al. 2018). Interestingly, there were no mutations in the canonical TGF- β pathway in glioblastomas.

Deregulation of the TGF β signaling pathway could be due to altered expression of ligands, receptors or intracellular mediators of the pathway. Up-regulation of *TGF- β 2* mRNA expression was observed in EGFRvIII-positive GBM patients and in astrocytoma U87MG cells overexpressing EGFRvIII as compared with wild type U87MG cells (Zhang et al. 2011c). The expression of downstream components of TGF- β signaling has been evaluated in glioma cell lines and glioma specimens and those analyses showed alterations in many components of this pathway. Early studies indicated that TGF- β 1 induces endogenous PAI-1 protein synthesis, Smad binding element-(SBE)12-luciferase-reporter activity, as well as mRNA expression of *SMAD6* and *SMAD7* in all tested human glioma cell lines that suggested unaffected TGF- β signaling (Piek et al. 1999). On the other hand, high-grade human gliomas secrete TGF- β 1, but are generally resistant to its growth inhibitory effects. Analysis of TGF- β 1 effects on 12 human glioma cell lines showed that the cytokine mildly inhibited or had no effect on the cell growth, with exception of two cell lines, where it stimulated the growth. The majority of glioma lines had homozygous deletions of the *p15(INK4B)* gene, only in three lines TGF- β 1 slightly induced *p21^{WAF1/CIP}* expression.

Other studies have shown that expression of the SMAD2, SMAD3 and SMAD4 proteins was lower in tested glioma cell lines, while expression of the SMAD7 protein was similar to that in normal astrocytes. In particular, SMAD3 expression was low or very low in nine out of the 10 malignant glioma cell lines. Seven of the 10 glioma cell lines exhibited lower levels of nuclear translocation of SMAD2 and SMAD3, and two cell lines expressing very low levels of SMAD3 showed no nuclear translocation. SnoN and Ski proteins are inhibitors of TGF- β signaling. All glioma cell lines expressed the inhibitory SnoN protein and its expression was not modulated by treatment with TGF- β 1; three glioma cell lines expressed high levels of the Ski protein. The expression of the *p21^{WAF1/CIP}*, *p15(INK4B)*, cyclin-dependent kinase-4 (CDK4), and cyclin D1 proteins was not altered by TGF- β 1 treatment in a majority of glioma cell lines suggesting dysfunction of TGF- β -induced growth inhibitory signals (Zhang et al. 2006). The loss of *p15INK4B*, a cell cycle inhibitor, may explain, in part, the selective loss of TGF- β –induced growth inhibition in gliomas (Held-Feindt et al. 2003; Rich et al. 1999). Most glioma lines retained other TGF- β -mediated responses, such as extracellular matrix protein and angiogenic factor secretion, which may contribute to increased malignant behavior.

9.4 Functions of TGF- β Signaling in Glioma Biology

9.4.1 TGF- β Signaling in Controlling Cell Proliferation

The TGF- β cytostatic program involves transcriptional activation of genes coding for the cyclin-dependent kinase inhibitors *p21WAF1/Cip1* and *p15Ink4b*, and repression of *c-myc* and *Id1-Id3* genes (Siegel et al. 2003, Seoane et al. 2004). Cooperatively, these gene responses mediate cell cycle arrest in the G₀/G₁ phase of the cell cycle. Mechanisms for Smad-mediated repression of *c-myc* and *Id* have been elucidated. A TGF β inhibitory element (TIE) located between positions –92 and –63 relative to the *c-myc* P2 transcription start site mediates repression in response to TGF β in human skin keratinocytes and mammary epithelial cells (Chen et al. 2002; Siegel et al. 2003). *c-Myc*, which binds to the *p21Cip1* and *p15Ink4b* promoters in mitogen-stimulated cells, must be down-regulated before the *p21Cip1* and *p15Ink4b* genes could be activated by TGF- β . *c-Myc* down-regulation by TGF- β renders those promoters competent for activation that requires another transactivation complex (Seoane et al. 2001). FoxO proteins (Fox - members of the Forkhead box) have been identified as key partners of

SMAD3 and SMAD4 in the TGF- β -dependent generation of the *p21Cip1* activation complex. FoxO-Smad complexes are inhibited by FoxG1, a distinct Forkhead family member, and the combined actions of FoxG1 and phosphatidylinositol 3-kinase (PI3K) signaling (negatively regulating Forkhead proteins) mediate resistance of human glioblastoma cells to TGF- β -induced growth inhibition. These three pathways — the SMAD, PI3K, and FoxG1 - converge on FoxO factors in the control of glioblastoma cell proliferation (Seoane et al. 2004).

9.4.2 TGF- β Signaling in the Regulation of Invasion

Gliomas are highly invasive, partly due to a unique structure of the brain parenchyma, composed mainly of hyaluronan and devoid of rigid protein barriers made up of collagen, fibronectin and laminin. Proteases induced and secreted during glioma progression such as matrix metalloproteinases (MMPs), ADAMTS proteases (ADAMs with thrombospondin motifs), plasminogen activators (PAs), lysosomal cysteine peptidases called cathepsins cleave ECM components allowing tumor cells to spread and diffusely infiltrate the brain parenchyma. MMPs and ADAMTS cleave ECM components such as proteoglycans aggrecan, versican, neurocan and brevican with selective preferences. Cell surface proteases of the ADAM family, but also serine proteases, regulate the activity of growth factors and chemokines that subsequently act as stimulants in glioma microenvironment (Ferrer et al. 2018). The invasion of glioma cells into a brain tissue renders surgical resections difficult and contributes to the failure of current therapies (surgery, radiation and chemotherapy).

TGF- β 1 is an important mediator of invasion in malignant gliomas. The effects of TGF- β 1 on mobility and migration are associated with changes in the expression of ECM components, including Tenascin C, fibronectin, laminin, vitronectin, MMP-2 and MMP-9 (Hau et al. 2006; Platten et al. 2001). Exogenous TGF- β 1 directly increased the motility of glioma cells by enhancing expression of integrin α_2 , α_5 and β_3 subunits (Platten et al. 2000), as well as by up-regulating the activity of MMP-2, 9 and MT1-MMP (Wick et al. 2001). Downregulation of TGF- β 2 expression with specific antisense oligodeoxynucleotides (ODNs) inhibited glioma migration and down-regulated *VERSICAN* expression as determined by gene arrays (Arslan et al. 2007). Versican, a large multi-domain chondroitin sulfate proteoglycan, is a major component of the extracellular matrix. The largest splice variants of Versican, V0 and V1, are the predominant forms present in most glioma cell lines. TGF- β 2 has been shown to up-regulate expression the Versican V0/V1 isoforms and to modulate glioma migration (Arslan et al. 2007). TGF- β 2 was capable of increasing MMP-2 activity and induced degradation of Versican V1. TGF- β 1-induced migration and invasiveness of T98G glioblastoma cells were blocked by exposure to an ADAM17 inhibitor - TAPI-2. TGF- β 1 can up-regulate *ADAM17* mRNA, and protein expression and the ADAMTS-1 proteases are known to cleave Versican (Lu et al. 2011). Cleavage of Brevican, another member of the lectican family by ADAMTS-5 is involved in glioma invasion *in vivo* (Nakada et al. 2005). It is likely that TGF- β induces migration and invasiveness via up-regulation of ADAMs and MMPs expression and their activity leads to degradation of extracellular matrix proteoglycans (e.g. Aggrecan, Brevican and Versican).

Lactate dehydrogenase type A (LDH-A) is an important enzyme catalyzing pyruvate into lactate and is excessively expressed by tumor cells in response to a decrease of the extracellular pH, which leads to apoptosis of non-tumor cells and invasion of malignant cells. It has been found that lactate regulates TGF- β 2 expression and glioma cell migration via induction of Thrombospondin-1. Moreover, TGF- β 2 enhanced expression, secretion, and activation of MMP-2 and augmented the cell surface expression of integrin $\alpha v\beta$ receptors (Baumann et al. 2009).

9.4.3 TGF- β 1 as Pro-angiogenic Factor

Genetic studies have revealed a role for TGF- β 1 and its receptors in embryonic angiogenesis, the establishment and maintenance of vessel wall integrity (Pepper 1997). In gliomas, TGF- β 1 acts as an angiogenic factor promoting neovascularization. TGF- β signaling stimulates the production of vascular endothelial growth factor (VEGF), which is a major stimulus in the promotion of angiogenesis, as well as plasminogen activator inhibitor (PAI-I) involved in the maturation of blood vessels (Goumans et al. 2002). Some studies demonstrated that hypoxia and TGF- β signaling pathways synergize in the regulation of the *VEGF* gene expression at the transcriptional level. This cooperation has been mapped on the human *VEGF* gene promoter within a region at -1006 to -954 that contains functional DNA-binding sequences for HIF-1 (hypoxia -inducible factor) and SMADs (Sanchez-Elsner et al. 2001). Gene expression profiling of GBM and low grade glioma vessels revealed increased *VEGF-A* and *TGF β 2* signaling in the tumor microenvironment associated with many changes in gene expression noted in GBM vessels. An enrichment of Smad target genes within the distinct gene signature of GBM vessels was validated by detection of a significant increase of Smad complexes in the vasculature of GBMs (Dieterich et al. 2012). A systematic analysis of TGF- β pathway activity and TGF- β -induced VEGF release in 9 long-term glioma cell lines and 4 GSCs cultures revealed interesting associations. Glioma cells displayed heterogeneous patterns of endogenous TGF- β pathway activation, having phosphorylated SMAD2 and SMAD3, but also SMAD1/5/8. Basal activation depended on the type I TGF- β receptor, ALK-5, accounted for up to 69% of constitutive VEGF release, which was regulated positively by SMAD2/3 and negatively by SMAD1/5/8 in a cell line-specific manner. Exogenous TGF- β induced the VEGF release in most cell lines in a SMAD- and ALK-5-dependent manner (Seystahl et al. 2015).

Furthermore, integrin-mediated activation of TGF- β by astrocytes may influence endothelial cell function. The integrin α v β 8 on human astrocytes is a major cell surface receptor for a latent TGF- β and acts as a central regulator of brain vessel differentiation and stabilization through regulation of TGF- β activation and expression of TGF- β -responsive genes, most notably *PAI-1* and *THROMBOSPONDIN-1* (Cambier et al. 2005; Tchaicha et al. 2011). Insulin-like growth factor-binding protein 7 (IGFBP7) is a selective biomarker of glioblastoma (GBM) vessels, strongly expressed in tumor endothelial cells and a vascular basement membrane. Conditioned media from human U87MG glioma cells up-regulated expression of *IGFBP7* mRNA and protein in human brain endothelial cells. Addition of pan-TGF-beta-neutralizing antibody or the ALK5 antagonist, SB431542, blocked *IGFBP7* expression, indicating that TGF- β 1 is a tumor-secreted factor inducing IGFBP7 in endothelial cells (Pen et al. 2008).

9.4.4 A Role of TGF- β Signaling in Glioma Cancer Initiating Cells

Several studies have identified a rare subpopulation of cells with stem cell-like properties in gliomas (reviewed in Lathia et al. 2015). Glioma stem cells (GSCs), known also as glioma initiating cells, are considered to be cells responsible for the initiation, propagation, maintenance and recurrence of these tumors, indicating that therapies that target the GSCs might significantly improve the poor prognosis associated with gliomas (Chen et al. 2012). GSCs are characterized by the expression of neural stem cell (NSC) antigens such as Oct4, Sox2/4, Nanog and CD133, and possess the capacity of self-renewal, multi-lineage differentiation and the ability to generate spheres when cultured in a serum-free medium in a presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). GSCs overexpress certain ATP-binding cassette transporters such as ABCG2 that can excrete

chemotherapeutic drugs such as temozolomide (TMZ) out of GSCs and cause drug resistance. The origin of GSCs is still debatable but data from animal glioma models suggest that GSCs could originate from the malignant transformation of NSCs or more differentiated glial/neuronal progenitors (Alcantara Llaguno et al. 2009), while others advocate for the notion that differentiated cells reverse to the less differentiated or stem cell state (Friedmann-Morvinski and Verma 2014). Elimination of GSCs from a bulk tumors is regarded as a prerequisite for developing successful therapeutic strategies against gliomas. In animal models, the eradication of GSCs effectively blocked tumor growth, and remaining tumor cells were non-invasive and lacked tumor growth potential (Chen et al. 2012).

Ikushima et al. demonstrated that autocrine TGF- β signaling plays an important role in the maintenance of stemness in GSCs (Ikushima et al. 2009). Treatment of GSCs with inhibitors of the TGF- β pathway reduced their potency to grow tumors as intracranial xenografts in immune-compromised mice. TGF- β preserved the stemness of these cells by inducing the expression of the transcription factor SOX4, which in turn induced a *SOX2* gene. Further studies demonstrated that TGF- β has to cooperate with leukemia inhibitory factor (LIF) in inducing the self-renewal and preventing the differentiation of glioma initiating stem cells (Penuelas et al. 2009). TGF- β induced the self-renewal capacity of those cells, but not of normal human neural progenitors, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway (Penuelas et al. 2009). A subset of human gliomas expresses high levels of LIF, which correlates with high expression of TGF- β 2 and neuroprogenitor cell markers (Penuelas et al. 2009).

Inhibition of TGF- β signaling in the glioma initiating cell population in human glioblastoma patients reduced the numbers and potential of GSCs. A population enriched in GSCs expressing high levels of CD44 and Id1 was found in a perivascular niche. The inhibition of the TGF- β pathway (blockade of T β IR) decreased the CD44^{high}/Id1^{high} cell population through reduction of Id1 and Id3 transcription factors levels, inhibiting a capacity of those cells to initiate tumors. High CD44 and Id1 levels were found to correlate with a poor prognosis in GBM patients (Anido et al. 2010). TMZ, an alkylating agent with anti-tumor efficacy for malignant gliomas, inhibited the invasion of glioma-initiating cells, down-regulated TGF- β 2 expression at the mRNA and protein levels and reduced the TGF- β 2-mediated invasion (Zhang et al. 2011a).

Glioblastoma-infiltrating microglia (brain resident macrophages) and peripheral macrophages (collectively known as GAMs), when exposed to a tumor, release TGF- β 1 (Wesolowska et al. 2008). Ye et al. (2012) demonstrated that in primary human gliomas and orthotopic transplanted syngeneic gliomas, the number of GAMs at the invasive front correlates with the presence of CD133+ GSCs, and GAMs produce high levels of TGF- β 1. Down-regulation of the TGF- β receptor II (TGFBR2) with short hairpin RNA inhibited the invasiveness of GSCs and mechanistically it was a consequence of the reduced expression of the metalloproteinase MMP-9 in GSCs depleted of TGFBR2 (Ye et al. 2012).

Interestingly, the *ex vivo* exposure of human GSCs to BMP4 eliminated the capacity of transplanted cells to form intracerebral tumors. Also, *in vivo* delivery of BMP4 effectively reduced the CD133+ population, blocked the growth of the human GBM cells and cured the animals. BMP4 activated their receptors (BMPRs) and triggered the Smad signaling in cells isolated from human GBMs, which reduced proliferation and increased expression of markers of neural differentiation without affecting cell viability (Piccirillo et al. 2006). BMP4 enhanced differentiation and reduced the pool of GSCs (Ikushima et al. 2009). BMP7 variant (BMP7v) reduced the proliferation of cultured GSCs, and stem cell marker expression, while enhancing expression of neuronal and astrocyte differentiation markers. BMP7v decreased tumor growth and stem cell marker expression in subcutaneous and orthotopic GSC xenografts. In the orthotopic model, BMP7v reduced invasion of cells to the brain parenchyma, impaired angiogenesis, and reduced mortality (Tate et al. 2012). While BMPs bind to type I and type II receptor homodimers and activate the Smad signaling, they phosphorylate distinct Smads1, 5, and 8, therefore downstream transcriptional responses are different. Altogether, those studies show an essential role of autocrine TGF- β signaling in the glioma initiating cell population in human GBMs, and indicate that inhibition of this signaling pathway or switch to BMP signaling pathway could provide new strategies to eradicate GBMs.

9.4.5 *TGF- β Signaling in Tumor-Mediated Immunosuppression*

TGF- β plays a crucial role in the escape of gliomas from the host immunity. The anti-tumor response in patients with glioma could be ineffective because of the loss of specific tumor antigens and shutting down activities of professional antigen presenting cells. TGF- β 1 enhances this effect by inhibition of MHC class II expression on glioma cells, macrophages and microglia (Zagzag et al. 2005). TGF- β 1 exerts an immunosuppressive effect on all cells of the immune system by blocking differentiation into cytotoxic T lymphocytes (CTLs) and CD4⁺ cells, and their maturation to Th1 or Th2 phenotype (Beck et al. 2001; Chen et al. 2005; Jachimczak et al. 1993). TGF- β 1 inhibits the generation of cytotoxic CD8⁺ T cell subpopulation and directly suppresses the expression of cytotoxic molecules such as Granzyme B and Perforin that are crucial for the cytolytic action of lymphocytes (Smyth et al. 1991). In CTLs TGF- β inhibits the expression of five genes coding for Perforin, Granzyme A, Granzyme B, Fas ligand, and Interferon γ that are crucial for CTL-mediated tumor cytotoxicity. Repression of Granzyme B and Interferon- γ involved binding of activated Smad and ATF1 transcription factors to gene promoter regions. Application of TGF- β neutralizing antibody in mice enabled tumor clearance and restored cytotoxic gene expression in antigen-specific CTLs (Thomas and Massagué 2005).

T regulatory cells (Treg), characterized by the expression of the forkhead box P 3 (Foxp3), are potent modulators of the anti-tumour responses. Tregs producing a cytokine IL-17 (named the IL-17⁺ Tregs) were found in high-grade glioma tissues. Those cells produced TGF- β . The proliferation of CD8⁺ T cells was suppressed by the IL-17⁺(Tregs, and this inhibitory effect was partially abrogated by neutralizing antibodies to TGF- β or IL-17 alone, and completely abrogated by neutralizing antibodies against both cytokines (Liang et al. 2014).

TGF- β 1 can also suppress activation of macrophages by down-regulation of TNF α , H₂O₂ and NO production, enhancing at the same time the production of the immunosuppressive cytokine IL-10 by macrophages (Maeda et al. 1995). TGF- β 1 decreased the activating receptor NKG2D on the surface of natural killer (NK) cells and CD8⁺ T cells in glioma patients, rendering them less efficient at tumor cell killing (Crane et al. 2010). Interference with TGF- β 1 or TGF- β 2 expression by siRNAs prevented down-regulation of NKG2D on immune cells mediated by LNT-229 glioma-conditioned medium and strongly promoted their recognition and lysis by CD8⁺ T and NK cells. Mice bearing LNT-229 glioma cells deficient in TGF- β 1 had NK cells with an activated phenotype (Friese et al. 2004).

Taken together, efforts to bypass TGF- β -mediated immunosuppression represent an attractive therapeutic strategy for the treatment of gliomas. Due to multiple modes of TGF- β action, it is difficult to distinguish which anti-tumor effects of the inhibitors of TGF- β signaling are mediated via modulation of host immunity.

9.5 Molecular and Pharmacological Strategies to Interfere with TGF- β Signaling for Potential Therapeutic Intervention in Gliomas

9.5.1 *Antibodies Inhibiting TGF- β Signaling*

TGF- β signaling pathway has emerged as an attractive target in cancer and inhibitors of this pathway demonstrated promising activities in various preclinical and clinical cancer trials, showing a reduction of tumor growth and improvement in overall survival. Table 9.1 summarizes therapeutic approaches targeting the TGF- β pathway. Some anti-TGF- β strategies are based on blocking the interaction between the cytokine and its receptor, e.g. by an application of a soluble TGFIIR or human α 2-macroglobulin plasma protein that binds TGF- β (Won et al. 1999). Two humanized monoclonal antibodies, CAT-192 specific to TGF- β 1 and CAT-152 against TGF- β 2, were promising in preclinical

Table 9.1 Selected inhibitors of TGF- β signaling tested for glioma therapy

Agent	Type	Development status	Company	References
Fresolimumab GC-1008	TGF- β 1,2 & 3 mAb	Phase II	Sanofi S.A.	den Hollander et al. (2015)
Trabedersen AP-12009	Oligonucleotide anti TGF- β 2	Phase III	Isarna Therapeutics	Hau et al. (2007))
LY550410 LY580276	Small molecule T β RI inhibitor	Preclinical	Eli Lilly & Co.	Sawyer et al. (2004)
LY2109761	T β R I/II dual inhibitor	Preclinical	Eli Lilly & Co.	Melisi et al. (2008)
SB-505124	Small molecule T β RI inhibitor	Preclinical	GlaxoSmithKline	DaCosta et al. (2004)
SB-431542	Small molecule T β RI inhibitor	Preclinical	GlaxoSmithKline	Hjelmeland et al. (2004)
Galunisertib LY2157299	Small molecule T β RI inhibitor	Phase II	Eli Lilly & Co.	Rodon et al. (2015)

trials for a treatment of fibrosis associated with nephropathy (Benigni et al. 2003) or as anti-scarring agents in glaucoma surgery (Mead et al. 2003). However, the CAT-152 mAb was not effective in preventing the progression of fibrosis in patients undergoing glaucoma surgery in a phase III study (Khaw et al. 2007). Further development of CAT-192 by Cambridge Antibody Technology was abandoned in favor of a newly developed drug candidate – an anti-panTGF- β (α -TGF- β 1, 2 & 3) monoclonal antibody GC-1008 (fresolimumab, Sanofi S.A.), which completed phase I and/or II in clinical trials involving patients with idiopathic pulmonary fibrosis (NCT00125385), and focal segmental glomerulosclerosis (NCT00464321; Trachtman et al. 2011; CT01665391; Vincenti et al. 2017). In patients with renal cell carcinoma and malignant melanoma, fresolimumab did not show toxicity and exerted anti-tumor activity in a phase I study (NCT00356460; Morris et al. 2014). In 2015, a PET imaging study revealed high penetrance of isotope-conjugated fresolimumab (^{89}Zr -fresolimumab) into brains of patients with recurrent high-grade gliomas, but there was no clinically significant outcomes in disease progression (NCT01472731, den Hollander et al. 2015). In a recently completed radiotherapy-based phase II trial, GC-1008 in higher doses yielded positive outcomes for patients with metastatic breast cancer (NCT01401062; Formenti et al. 2018). Currently, fresolimumab is to be assessed in two phase I and/or II trials, one involving combination therapy with stereotactic radiotherapy (SARC-ATAC, Stereotactic Ablative Radiotherapy and anti-TGF β Antibody Combination.) against non-small cell lung carcinoma (NCT02581787); and the other against osteogenesis imperfecta (NCT03064074).

A systemic inhibition of TGF- β signaling with another panTGF- β -neutralizing monoclonal antibody, 1D11, improved the therapeutic efficacy of glioma-associated antigen peptide vaccines in mice bearing orthotopic GL261 gliomas (Ueda et al. 2009). The antibody was subsequently tested in a couple of bone disease murine models but never entered clinical investigation. A TGF- β neutralizing antibody targeting exclusively TGF- β 1 and TGF- β 2, XOMA-089 (XPA089), is being developed by XOMA Corporation/Novartis and is currently in a pre-clinical phase. The antibody has been used in a combination study with checkpoint inhibitor PD-1 in mice and improved the outcome of immunotherapy (Terabe et al. 2017). In the same study, researchers pointed the putative advantage of XPA089 as a TGF- β 1/2 inhibitor over pan-TGF- β inhibitors (e.g. fresolimumab) as a third isoform TGF- β 3 was reported to have had positive outcomes in breast cancer (Laverty et al. 2009), and its inhibition might not be desired. However, more recent reports suggest that TGF- β 3 might be a gate-keeper of downstream signaling to other TGF- β isoforms and its inhibition may be a promising strategy in targeting dysfunctional TGF- β signaling in GBM (Seystahl et al. 2017).

Another approach utilizing monoclonal antibodies has been developed by Eli Lilly & Co. The company has designed an inhibitor targeting a receptor TGFRII. LY3022869, also known as TR1 and previously as IMC-TR1, has yielded exceptional responses in murine models (Zhong et al. 2010),

which prompted its introduction to the clinical investigation two years later. The drug candidate has completed phase I clinical trial in patients with solid tumors that failed standard therapies in 2014 (NCT01646203); however, the main goal of the study was never achieved as infusions of the drug formulation evoked a strong cytokine release syndrome and forestalled maximum tolerated dose (MTD) determination (Tolcher et al. 2017).

9.5.2 Antisense Oligodeoxynucleotides and Short Interfering RNAs Inhibiting TGF- β Signaling

The phosphorothioate antisense oligodeoxynucleotides (ODN) specific to TGF- β 2 were effective in blocking TGF- β 2 expression in malignant gliomas, and reversing cytokine effects on lymphocyte proliferation and autologous tumor cytotoxicity (Jachimczak et al. 1993). The antisense oligonucleotide, trabedersen (AP 12009), specifically blocking *TGF- β 2* mRNA was tested in three phase I/II studies (NCT00844064) and a randomized, active-controlled dose-finding phase II study (NCT00431561). Trabedersen led to long-lasting tumor responses and promising survival data in high-grade glioma patients with recurrent tumor (Hau et al. 2007, 2011; Schlingensiepen et al. 2006, 2008). Survival of modified C6 glioma cells transfected with the TGF- β 2 antisense vector was improved (Liau et al. 1998) leading to phase I clinical trial of a TGF-beta antisense-modified tumor cell vaccine in patients with advanced gliomas (Fakhrai et al. 2006). Intracranial administration of antisense TGF- β 2 ODNs with a systemic tumor vaccine improved the survival of 9 L glioma-bearing Fisher 344 rats (Liu et al. 2007). In late 2008 trabedersen entered a phase III clinical study with patients with anaplastic astrocytoma (AA) or GBM (NCT00761280), with the aim to determine survival rate in comparison to a standard of care therapy with temozolomide. The study was terminated in 2012 due to insufficient patient numbers recruited for the trial (27 patients in total), leaving results of the study solely descriptive. The survival rate at 12 months for trabedersen was 57.1% compared to 53.8% for TMZ, at 18 months 42.9% and 38.5%, respectively, and at 21 months 35.7% and 23.1%, respectively.

Strategies based on RNA interference are used to block TGF- β signaling in glioma cells. Blockade of cytokine expression using siRNA against TGF- β inhibited tumor cell migration, invasiveness and restored anti-tumor immune response in mouse gliomas (Friese et al. 2004). Vectors coding for short hairpin RNA (shRNA) that effectively silenced the expression of human TGF- β receptor Type II, abolished the TGF- β -activated SMAD signaling and reduced activation of the *PAI-1* promoter in rat and human glioblastoma cells (Wesolowska et al. 2008). As of 2019, none clinical trials involving RNAi-based TGF- β pathway inhibitors have been registered.

9.5.3 Small Molecules Inhibitors of the Catalytic Activity of TGF- β Receptor Kinase

Many small molecule inhibitors of the catalytic activity of TGF- β receptor kinase have been developed. A group of competitive inhibitors of the ATP binding site of TGF receptor Type I kinase, such as LY550410, LY580276 (Eli Lilly & Co.) and SB-505124 (GlaxoSmithKline) has been designed. Such compounds consist of a domain with a hydrogen-bond acceptor (e.g. imidazole core, pyroazole ring or quinoline scaffold), which is essential for blocking kinase activity. Several studies demonstrated the physiological efficacy of such molecules, as well as their kinase inhibitory activity (DaCosta et al. 2004, Sawyer et al. 2004). In 2016 Jiang et al. indicated an advantage of SB-505124 as a drug candidate owing to oral administration route due to high bio-compatibility in a pharmacokinetics

study in a rat model (Jiang et al. 2016). A small-molecule inhibitor SD-208 (an antagonist of TGF- β receptor I) significantly prolonged survival of glioma-bearing mice, enhancing the immunogenicity of glioma cells, while diminishing their migratory and invasion properties (Uhl et al. 2004). An anti-tumor efficacy of SD-208 was well documented in a metastatic melanoma mouse model, where it reduced melanoma bone metastases and prolonged survival of animals in the treated group compared to controls (Mohammad et al. 2011). Another small molecule inhibitor of the type I TGF- β receptor developed by GlaxoSmithKline - SB-431542 - blocked phosphorylation and nuclear translocation of SMAD proteins, abolished TGF- β -mediated up-regulation of critical genes, inhibited proliferation and motility of human glioma cells (Hjelmeland et al. 2004). Interestingly, SB-431524 promoted murine and human dendritic cell maturation and cytokine production *in vitro* and, more importantly, significantly induced CTL activity against implanted intraperitoneally colon cancer tumor *in vivo* (Tanaka et al. 2010). SX-007, an orally active small-molecule TGF- β RI kinase inhibitor reduced the TGF- β -mediated invasion in cell cultures, reversed immune suppression, and improved the median survival in an *in vivo* SMA-560 glioma model (Tran et al. 2007). These findings are particularly interesting in the context of enhancing the immunogenicity of tumors such as GBMs that are known to be poorly infiltrated by immune cells.

LY2109761, a TGF- β receptor type I and type II dual inhibitor, selectively inhibited SMAD2 signaling and showed anti-tumor activity in various tumor models (Melisi et al. 2008). LY2109761 reduced clonogenic survival of U87 and T98 glioma cells, had anti-migratory and anti-angiogenic effects in Matrigel migration and tube formation assays. Furthermore, LY2109761 alone or in combination with fractionated radiation and TMZ delayed tumor growth in human xenografts growing subcutaneously in BALB/c nu/nu mice (Zhang et al. 2011b).

A panel of small molecule inhibitors of type 1 receptor serine threonine kinases (ALKs1–7), including inhibitors of the TGF- β pathway (SB-431542, SB-505124, LY-364947 and A-83-01) and the BMP pathway (Dorsomorphin and LDN-193189), has been developed and tested. The inhibitors of the TGF- β pathway were found to be more selective than the inhibitors of the BMP pathway, and SB-505124 was recommended as the best inhibitor of ALKs 4, 5 and 7 (Vogt et al. 2011).

Many of these small molecule blockers of TGF- β receptor kinase remain in the preclinical phase and serve as good and frequently very specific tools in elucidating outcomes of TGF- β pathway inhibition *in vitro* and *in vivo*. However, a novel small molecule TGF β RI kinase blocker galunisertib (LY2157299, Eli Lilly & Co.) has recently emerged as a promising candidate for clinical testing and is still under active clinical investigation. Galunisertib has been registered for 23 clinical trials so far (mainly phase 1 and 2), targeting numerous cancers, including metastatic colorectal, metastatic pancreatic, recurrent NSCLC, triple negative breast, hepatocellular carcinoma, glioma and more. In a first-in-human dose (FHD) study initiated in 2005 for glioma patients (NCT01682187), galunisertib exhibited clinical response in 21.4% of enrolled patients, showing no cardiotoxicity, no secondary malignancies due to TGF- β inhibition and a generally favorable safety profile (Rodon et al. 2015). Interestingly, the same study reported a possible link between TGF- β signaling and an *IDH1* mutation status as, similar to trabedersen, galunisertib evoked more profound clinical responses in patients with lower WHO grade gliomas, i.e. gliomas associated with *IDH1* mutations. Safety and dosing of galunisertib for glioma patients has been further investigated in two circumstantially initiated studies: one involving patients with recurrent glioma as a second-line treatment along lomustine (NCT01220271), the other involving patients with newly diagnosed gliomas as a first-line therapy combined with TMZ and radiotherapy (NCT01582269). The interim data released for the first study revealed no added side effects or toxicity for galunisertib and lomustine compared to lomustine alone (Carpentier et al. 2013). The interim data for the latter pointed preserved or elevated CTL levels in patients after galunisertib administration compared to patients treated with TMZ only (Wick et al. 2013), pointing towards galunisertib-dependent alleviation of immunosuppression mediated by TGF- β pathway. Most recent reports indicate that galunisertib can modulate anti-tumor T cell activity alone and in combination with PD-1/PD-L1 checkpoint inhibitors (Holmgaard et al. 2018).

Summarizing, the studies of antibodies, large or small molecules interfering with TGF β signaling brought a plethora of promising results in preclinical studies, but did not fulfill its promise in more advanced clinical trials. Large-molecule antagonists of TGF- β signaling are considered to be more selective and have a broader action than small-molecule inhibitors. Despite earlier predictions of severe toxicity, neutralizing antibodies to TGF- β are well tolerated and have potent anti-metastatic activity. Si/shRNAs are recognized as a new class of potential therapeutics against a wide range of diseases, however delivering siRNA or shRNA specifically and efficiently into tumor cells *in vivo* remains a great challenge (Tiemann and Rossi 2009). Lentiviral or adenoviral-associated vectors expressing shRNA in cultured mammalian cells and in the whole animals may be a promising approach for a specific, efficient, and stable knockdown of various genes.

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