Chapter 9
TGF Beta Signaling and Its Role in Glioma Pathogenesis

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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 cascades. Negative regulation of TGF-β/Smad signaling may occur through the inhibitory Smad6/7. Increased expression of TGF-β1-3 correlates with a degree of malignancy of human gliomas. TGF-β may contribute to tumor pathogenesis by direct support of tumor growth, self-renewal of glioma initiating stem cells and inhibiting of anti-tumor immunity. TGF-β1,2 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 promises as novel, potential anti-tumor therapeutics.

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

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Abbreviations

ADAMTS-1	Metalloproteinase and disintegrin-like domain
Akt	Protein kinase B/Akt kinase
ALKs	Activin-receptor-like kinases
AP-1	Activator protein
BMPs	Bone morphogenetic proteins
CTLs	Cytotoxic T lymphocytes
EGFR	Epidermal growth factor receptor
ECM	Extracellular matrix
ERK1/2	Extracellular signal-regulated kinases 1/2
GDFs	Growth and differentiation factors
JAK	Janus kinase
JNK c	Jun N-terminal kinases
LAP	Latency-associated peptide
LIF	Leukemia inhibitory factor
LTBP	Latent TGF-β binding protein
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
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TGF-β	Transforming growth factor β
TMZ	Temozolomide
TNFα	Tumor necrosis factor α
TRAF	TNF receptor associated factor
VEGF	Vascular endothelial growth factor.

9.1 Introduction

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that regulates cell proliferation, differentiation and extracellular matrix production (Jennings and Pietenpol 1998; Verrecchia and Mauviel 2002). The TGF-β belongs to a larger superfamily including: TGF-β, BMPs (bone morphogenetic proteins) and GDFs (growth and differentiation factors), sharing the characteristic fold of cystine-knot of the TGF superfamily. TGF-β and BMP/GDF 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 SMAD transcription factors (Rider and Mulloy 2010) (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 entire 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 has been implicated in the pathogenesis of a variety of diseases, including cancer and fibrosis. TGF-β binds and activates a membrane receptor serine/threonine kinase complex. Upon TGF-β binding, the receptor complex phosphorylates the transcription

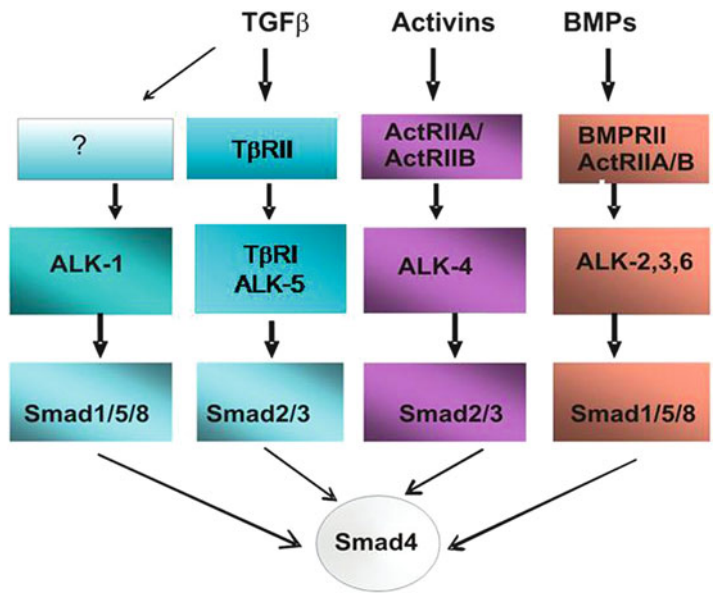

Fig. 9.1 The TGF-β superfamily signal transduction. Members of the TGF-β superfamily signal via a distinct type I and type II receptors. Ligand binding induces conformational changes and activation of kinase domains of the receptors. Smad 2/3 proteins are cytoplasmic transcription factors which are phosphorylated 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; TβRI, TGF- β type I receptor; TβRII, TGF- β type II receptor

factors Smad2 and Smad3 that bind to Smad4 and accumulate in the nucleus, where they regulate transcription (Massague et al. 2005; Schmierer and Hill 2007; ten Dijke and Hill 2004). TGF-β has a dual role in oncogenesis. It is 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 glioma, TGF-β becomes an oncogenic factor (Massague 2008; Seoane 2008).

Under physiological conditions TGF-β is expressed at a very low level in the brain but 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 and contributes to cell growth, invasion, and metastasis, and decreases host immune responses against tumor. TGF-β is highly active in high-grade glioma 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 with an unmethylated *PDGF-B* gene (Bruna et al. 2007). TGF-β1 produced by glioma infiltrating brain microglia/macrophages doubles glioma invasion *in vitro* and *in vivo* (Wesolowska et al. 2008). Recently, 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 induced the self-renewal capacity of those cells, but not of normal human neuroprogenitors, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway (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 role of TGF-β in pathogenesis of glioblastomas are still unknown. We summarized here data concerning molecular mechanisms of TGF activation, its signaling

and role in glioma pathogenesis. Furthermore, we discussed advances in 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 55-kDa polypeptides, which form 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 a latent complex. Such complexes are secreted and targeted to specific locations in the extracellular matrix by the appropriate LTBP (Hyytiainen et al. 2004). The latent state prevents the cytokine from eliciting a response until certain physiological conditions occur or until the target cell is reached. LTBPs are important for activation of the latent cytokine and as targeting molecules of the effects of TGF-β. The latency proteins also contribute to the cytokine stability: free TGF-β has a half life of about 2 min, whereas the latent form – 90 min. TGF-β is activated in an enzymatic process of LAP cleavage for example by plasmin (Grainger et al. 1995) or thrombospondin-1 which also cooperates with the plasmin- mediated process (Ribeiro et al. 1999). 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). Activation of TGF-β is a multi-step process and represents a regulatory stage responsible for a tight control of active TGF-β formation.

TGF-β signals through binding to the type II and type I serine/threonine kinase receptors (TβRII and TβRI, respectively), inducing their hetero-oligomerization that subsequently activates different, 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 TβRII and alters its conformation. Due to that TβRII can be recognized by TβRI. Constitutively active, intrinsic protein kinase of TβRII phosphorylates then the three serine and two threonine residues in the special domain of TβRI. Subsequently, the type I receptor directly phosphorylates Smad proteins on the C-terminal Ser-Ser-X-Ser motif. This phosphorylation is facilitated by other proteins: SARA (Smad anchor for receptor activation) and HRS/HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) (Miura et al. 2000; Tsukazaki et al. 1998) via the phospholipids-binding FYVE domain, acting as an adaptor to aid in recruitment of R-Smad2 to the TGF-β receptor complex, and by cPML (the cytoplasmic promyelocytic leukaemia), an adaptor that promotes ALK5 mediated receptor- Smad proteins (R-Smad) phosphorylation (Lin et al. 2004). R-Smad phosphorylation is coupled to TGF-β receptor internalization (Penheiter et al. 2002).

It is generally accepted that the TGF-β/Activin/Nodal subfamily activates Smad2 and 3 whereas the BMP/GDF/MIS subfamily functions 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, 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 (Attisano and Wrana 2002; 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: GTCT) or GC-rich sequences present in certain promoters (Fig. 9.2). Smad proteins have been shown to

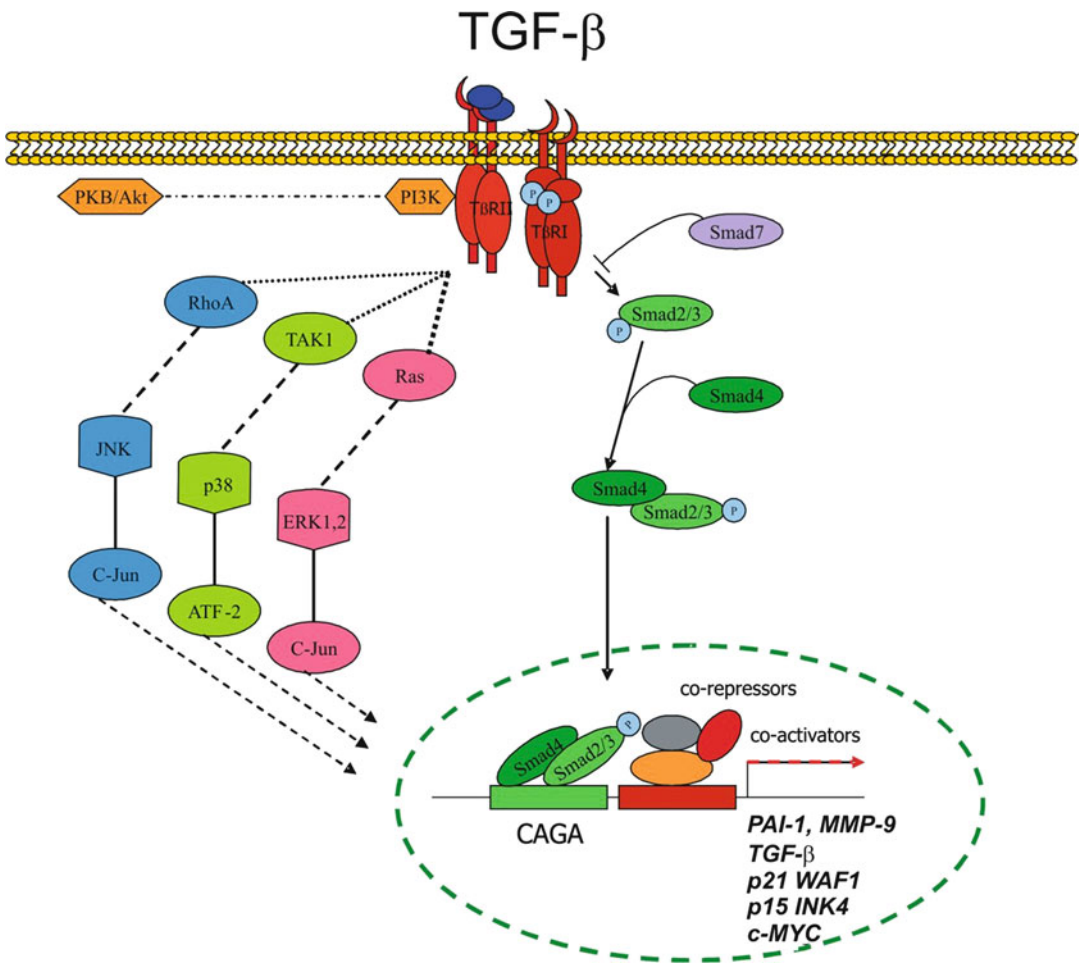

Fig. 9.2 Activation of TGF-β type I and type II receptors (TβRI, TβRII) leads to activation of receptor kinases, phoshorylation of receptor-Smad proteins (R-Smads), that form a complex with Smad4, which translocates to the nucleus. The pathway is regulated by the activity of the inhibitory Smad7. TGF-β 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 kinase B/Akt (PKB/Akt). The interaction with these MAP kinases may positively or negatively regulate Smad transcriptional activity

cooperate with a wide variety of transcription factors to regulate gene expression (Massague et al. 2005; Moustakas et al. 2001).

Besides the canonical TGF-β/Smad pathway, TGF-β can directly activate non-Smad signaling pathways (Moustakas and Heldin 2005; Zhang 2009), including 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). ERK could be phosphorylated via TβRI-induced phosphorylation of ShcA protein which associates with adaptor Grb2 and Sos proteins, thereby initiating the well-characterised pathway linking receptor tyrosine kinases with ERK MAP kinases (Lee et al. 2007). In addition, small GTPases such as Ras, Rho, Rac and CDC42, have been implicated in non-Smad TGF-β signaling in epithelial cells (Bhowmick et al. 2001; Edlund et al. 2002; Wilkes et al. 2003). For example, activation of Ras, extracellular signal-regulated kinases 1/2 (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), whereas activation of protein kinase A contributes to TGF-β signaling in murine mesangial cells (Wang et al. 1998). We demonstrated that TGF-β1 which did not affect 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 reported for C2C12, Mv1Lu, and HaCaT cells (Hanafusa et al. 1999; Karsdal et al. 2003).

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 TβRI 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). A recent study provided evidence that TGF-β type I receptor (TβRI) can by-pass canonical signaling pathways. It has been shown that TβRI undergoes cleavage by TACE (TNF-α converting enzyme) in cancer cells and that the liberated intracellular domain (ICD) of TβRI associates with the transcriptional regulator p300 to activate genes involved in tumor cell invasiveness, such as *Snail* and *MMP-2*. The TβRI 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

A broad array of Smad interacting partners and diverse post-translational modifications of Smads have been identified (Itoh and ten Dijke 2007). The Smad signaling pathway may be negatively regulated by the inhibitory Smad6 and Smad7 (Nakao et al. 1997). Inhibitory Smad7 acts to oppose the signal mediated by R-Smads by forming stable associations with activated type I receptors, thus preventing phosphorylation of R-Smads (Shi and Massague 2003).

Other mechanisms by which I-Smads antagonize signaling include: interactions with Smad4, preventing R-Smad–Smad4 complex formation; recruitment of E3-ubiquitin ligases Smurf1 and 2 to induce type I receptor ubiquitination and subsequent receptor degradation; direct repression of Smad-induced transcriptional responses. 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

The Smad binding element – the CAGAC sequence is calculated to be present on average every 1,024 bp in the genome, or about once in the regulatory region of any average size gene. Also 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: Fast1, Mixer, Jun/Fos, Runx, ATF3, E2F4/5 are highly responsive to different inputs (reviewed in Massague and Wotton 2000). 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 that have histone acetyltransferase activity and may increase transcription of target genes by inducing chromatin remodeling (Massague and Wotton 2000).

TGF-β1 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-β1 induction and antisense against AP-1 components *c-jun* and *c-fos* blocked autocrine TGF-β1-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 (Alexandrow and Moses 1995; Siegel et al. 2003a, b). 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).

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. Recent studies show that the overall cancer risk is increased by 70 and 19 % among TGFBR1*6A homozygotes and heterozygotes, respectively (Bian et al. 2003). The expression of downstream components of TGF-β signaling has been evaluated in glioma cell lines and glioma speciments and those analyses showed alterations in many components of this pathway.

Early studies indicated that TGF-β1 induces endogenous PAI-1 protein synthesis, Smad binding element/(CAGA)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, but are generally resistant to its growth inhibitory effects. Analysis of TGF-β effects on 12 human glioma cell lines showed that the cytokine mildly inhibited or had no effect on the cell growth and stimulated growth of two lines. The majority of glioma lines had homozygous deletions of the *p15(INK4B)* gene, only in three lines TGF-β slightly induced $p21^{WAF1/CIP}$ expression.

Other studies have shown that expression of the SMAD2, SMAD3 and SMAD4 proteins was lower in the 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 ten malignant glioma cell lines. Seven of the ten 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. All glioma cell lines expressed the SnoN protein and its expression was not modulated by a treatment with TGF-β1; 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-β1 treatment in a majority of glioma cell lines suggesting dysfunction of TGF-β-induced growth inhibitory signals (Zhang et al. 2006).

Up-regulation of *TGF-β2* mRNA expression was observed in EGFRvIII-positive GBM patients and in astrocytoma U87MG cells overexpressing EGFRvIII as compared with U87MG cells (Zhang et al. 2011c). This suggests that the loss of p15INK4B, a cell cycle inhibitor, may explain, in part, the selective loss of growth inhibition by TGF-β 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 (Alexandrow and Moses 1995; Siegel et al. 2003b). Cooperatively, these gene responses mediate cell cycle arrest in the G_0/G_1 phase of the cell cycle. Mechanisms for Smad-mediated repression of c-*myc* and *Id* have been elucidated. A TGFβ inhibitory element (TIE) located between positions −92 and −63 relative to the c-*myc* P2 transcription start site mediates repression in response to TGFβ in human skin keratinocytes and mammary epithelial cells (Chen et al. 2002; Siegel et al. 2003a, b). 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 also 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. 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

The invasion of neoplastic cells into brain tissue is a pathologic hallmark of gliomas and contributes to the failure of current therapies (surgery, radiation and chemotherapy). Gliomas are highly invasive, partly due to a unique composition of the brain parenchyma, composed mainly of hyaluronan and devoid of rigid protein barriers made up of collagen, fibronectin and laminin. Proteases secreted during glioma progression degrade extracellular matrix (ECM) allowing tumor cells to spread and diffusely infiltrate the brain parenchyma (Rao 2003).

TGF-β1 is an important mediator of invasion in malignant gliomas. The effects of TGF-β1 on mobility and migration are associated with changes in ECM components, including Tenascin C, fibronectin, laminin, vitronectin, MMP-2 and MMP-9 (Hau et al. 2006; Platten et al. 2001). Exogenous TGF-β1 directly increased the motility of glioma cells by enhancing expression of subunits of $\alpha_{2,5}$, β_3 integrin (Platten et al. 2000) as well as by up-regulating the activity of metalloproteinases: MMP-2, 9 and MT1-MMP (Wick et al. 2001). Increased enzymatic degradation of extracellular matrix proteins may facilitate tumor spread (Platten et al. 2001; Wick et al. 2001). TGF-*β*2-specific phosphorothioate antisense molecules 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 the degradation of versican V1. In T98G glioblastoma cells TGF-β1-induced migration and invasiveness were blocked by exposure to an ADAM17 inhibitor, TAPI-2. TGF-β1 can up-regulate *ADAM17* mRNA and protein expression and the ADAMTS-1 (metalloproteinase and disintegrin-like domain) proteases are known to cleave versican (Lu et al. 2011). A different study demonstrated that cleavage of brevican, another member

of the lectican family by ADAMTS-5 is functionally 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 expression and their activity that leads to degradation of extracellular matrix proteoglycans (e.g. aggrecan, brevican and versican).

9.4.3 TGF-β1 as Pro-Angiogenic Factor

Genetic studies have revealed a role for TGF-β1 and its receptors in embryonic vascular assembly, the establishment and maintenance of vessel wall integrity (Pepper 1997). In gliomas TGF-β1 may act as an angiogenic factor promoting neovascularization. TGF-β signaling stimulates 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 maturation of blood vessels (Goumans et al. 2002). Some studies demonstrated that hypoxia and TGF-β signaling pathways can synergize in the regulation of *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).

Furthermore, integrin-mediated activation of TGF-β by astrocytes may influence endothelial cell function. The integrin α(v)β8 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 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-β1 is a tumor-secreted factor capable of IGFBP7 induction in endothelial cells (Pen et al. 2008).

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

Autocrine TGF-β signaling has be shown to play an essential role in maintenance of stem-like phenotype of glioma-initiating cells (GICs). TGF-β induced expression of *Sox2*, a stemness gene, and this induction was mediated by *Sox4*, a direct TGF-β target gene. Inhibitors of TGF-β signaling promoted GIC differentiation, and reduced their tumorigenicity in intracranial transplantation assay (Ikushima et al. 2009). 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 (Ikushima et al. 2009; Penuelas et al. 2009). TGF-β induced the self-renewal capacity of those cells, but not of normal human neuroprogenitors, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway (Penuelas et al. 2009).

TGF-β inhibitors can target the glioma initiating cell population in human glioblastoma patients. A GIC-enriched cell population expresses high levels of CD44 and Id1 (inhibitor of DNA-binding protein), and tend to be located in a perivascular niche. The inhibition of the TGF-β pathway (blockade of TβIR) decreased the $CD44^{high}/Id1^{high}$ GIC pop-ulation through reduction of Id1 and Id3 transcription factors levels, inhibiting capacity of those cells to initiate tumors. High CD44 and Id1 levels confer poor prognosis in GBM patients (Anido et al. 2010). A recent study showed that temozolomide (TMZ), an alkylating agent with anti-tumor efficacy for malignant gliomas, inhibits the invasion of

glioma-initiating cells. TMZ reduced the TGF-β2-mediated invasion, and down-regulated TGF-β2 expression at the mRNA and protein levels (Zhang et al. 2011a, b, c).

9.4.5 TGF-β Signaling in Tumor-Mediated Immunosuppression

TGF-β plays a crucial role in the escape of gliomas from host immunity. The anti–tumor response in patients with glioma may be ineffective because of loss of a specific tumor antigen and professional antigen presenting cells in the brain. TGF-β1 enhances this effect by inhibition of MHC class II expression on glioma cells, macrophages and microglia (Zagzag et al. 2005). TGF-β1 exerts an immunosuppressive effect on all cells of the immune system (Beck et al. 2001; Chen et al. 2005; Jachimczak et al. 1993) by blocking differentiation into cytotoxic T lymphocytes (CTLs) and $CD4^+$ cells, and their maturation to Th1 or Th2 phenotype (Gorelik and Flavell 2001). TGF-β1 inhibits 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).

TGF-β1 can also suppress activation of macrophages by down-regulation of TNFα, H_2O_2 and NO production, enhancing at the same time the production of the immunosuppressive cytokine IL-10 by macrophages (Maeda et al. 1995). TGF-β1 decreased the activating receptor NKG2D on the surface of NK cells and $CD8^+$ T cells in glioma patients, rendering them less efficient at tumor cell killing (Crane et al. 2010). Interference with TGF-β1 orTGF-β2 expression by a small RNA interference (RNAi) technology 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. NK cells isolated from mice bearing LNT-229 glioma cells deficient in TGF-β1 showed an activated phenotype (Friese et al. 2004).

Blocking of TGF-β1 signaling in the immune system cells led to enhanced anti-tumor response. Both thymoma and melanoma tumors were eradicated in animals expressing a dominant negative TβRII under the control of a T- specific promoter (Gorelik and Flavell 2001). CTLs transduced with a vector expressing dominant negative TβRII were resistant to the anti-proliferative and anti-cytotoxic effects of exogenous TGF-β1 in EBV-positive Hodgkin disease (Bollard et al. 2002). C6 glioma cells depleted of TβRII by expression of the specific TβRII shRNA formed significantly smaller subcutanous tumors in mice than control glioma cells (Wesolowska et al. 2008). Taken together, efforts to bypass TGF-β-mediated immunosuppression represent an attractive therapeutic strategy for the treatment of gliomas.

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

TGF-β signaling pathway is emerging as an attractive target in cancer and inhibitors of this pathway are tested in cancer clinical trials, showing reduction of tumor progression and improvement in overall survival. Table 9.1 summarizes recent 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 alpha 2-macroglobulin plasma protein that bind TGF-β (Won et al. 1999). Two humanized monoclonal antibodies: CAT-192 specific to TGF-β1 and CAT-152 against TGF-β2 were promising in preclinical 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), but 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). A systemic inhibition of TGF-β signaling with a

Table 9.1 Development of inhibitors of TGF-β signaling for glioma therapy

Agent	Type	Development status	Company	References
Lerdelimumb CAT-152	TGF-β2 mAb	Phase III	Cambridge Antibody Technology	Mead et al. (2003)
Metelimumab CAT-192	TGF-β1 mAb	Phase II	Cambridge Antibody Technology	Benigni et al. (2003)
AP-12009	Oligonucleotide anti TGF-β2	Phase II	Antisense Pharma	Hau et al. (2007)
AP-11014	Oligonucleotide anti TGF-β2	Preclinical	Antisense Pharma	Schlingensiepen et al. (2004)
LY550410 LY580276	Small molecule TβRI inhibitor	Preclinical	Eli Lilly Pharmaceuticals	Sawyer et al. (2004)
LY2109761	TβR I/II dual inhibitor	Preclinical	Eli Lilly Pharmaceuticals	Melisi et al. (2008)
SB505124	Small molecule TβRI inhibitor	Preclinical	GlaxoSmithKline	DaCosta Byfield et al. (2004)
SB-431542	Small molecule TβRI inhibitor	Preclinical	GlaxoSmithKline	Hjelmeland et al. (2004)
SD-208	Small molecule TßRI inhibitor	Preclinical	Academic Institution	Uhl et al. (2004)

TGF-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 phosphorothioate antisense oligodeoxynucleotides specific to TGF-β2 were effective in blocking TGF-β2 expression in malignant glioma and reversing cytokine effects on lymphocyte proliferation and autologous tumor cytotoxicity (Jachimczak et al. 1993). The antisense oligonucleotide trabedersen (AP 12009), specifically blocking *TGF-β2* mRNA was tested in I/II studies and a randomized, active-controlled dose-finding phase II study, and led to long-lasting tumor responses and promising survival data in high-grade glioma patients with recurrent or refractory tumor disease (Hau et al. 2007, 2011; Schlingensiepen et al. 2006, 2008). Survival of modified C6 glioma cells transfected with the TGF-β2 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 glioma (Fakhrai et al. 2006). Intracranial administration of antisense TGF-β2 ODNs with a systemic tumor vaccine improved survival of 9L glioma-bearing Fisher 344 rats (Liu et al. 2007).

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 a mouse model of glioma (Friese et al. 2004). We have developed vectors coding for small hairpin RNA (shRNA) that effectively silenced the expression of TGF-β receptor Type II gene expression, abolished TGF-activated SMAD signaling and reduced activation of the PAI-1 promoter in rat and human glioblastoma cells (Wesolowska et al. 2008).

Many small molecules inhibiting 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 and SB-505124 has been designed. Such compounds consist of a domain with a hydrogen-bond acceptor (which may be imidazole core, pyrozole ring or quinoline scaffold) essential for blocking of kinase activity. Several studies demonstrated physiological efficacy of such molecules, as well as their kinase inhibitory activity (DaCosta Byfield et al. 2004; Sawyer et al. 2004). A small-molecule inhibitor SD-208 (an antagonist of TGF-β receptor) significantly prolonged survival of glioma-bearing mice, enhancing the immunogenicity of glioma cells, while their migratory and invasion properties were diminished (Uhl et al. 2004).

A small molecule inhibitor of the type I TGF-β receptor – 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). SX-007, an orally active, small-molecule TGF-β RI kinase inhibitor reduced TGF-β-mediated invasion in cell culture and reversed immune suppression, and improved the median survival in an in vivo SMA-560 glioma model (Tran et al. 2007). LY2109761, a novel TGF-β receptor type I and type II dual inhibitor, has shown a SMAD2-selective inhibitory profile with antitumor 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 and in combination with fractionated radiation and temozolomide delayed tumor growth in human xenograft tumors growing subcutaneously on BALB/c nu/nu mice (Zhang et al. 2011a, b, c).

A recent study evaluated a panel of small molecule inhibitors of type 1 receptor serine threonine kinases (ALKs1-7): inhibitors of the TGF-β pathway (SB-431542, SB-505124, LY-364947 and A-83-01) and the BMP pathway (Dorsomorphin and LDN-193189) against 123 protein kinases. 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).

A large–molecule antagonist of TGF-β signaling are considered to be more selective and having 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 miRNA specifically and efficiently into tumor cells *in vivo* remains a great challenge (Tiemann and Rossi 2009). Lentiviral or adeno-associated vectors expressing shRNA in cultured mammalian cells and in whole animals may be a promising approach for a specific, efficient, and stable knockdown of various genes.

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