Differential regulation of TGF-β-induced, ALK-5-mediated VEGF release by SMAD2/3 versus SMAD1/5/8 signaling in glioblastoma.

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Abstract: BACKGROUND The transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) pathways have a major role in the pathogenesis of glioblastoma, notably immunosuppression, migration, and angiogenesis, but their interactions have remained poorly understood. METHODS We characterized TGF-β pathway activity in 9 long-term glioma cell lines (LTCs) and 4 glioma-initiating cell lines (GICs) in relation to constitutive and exogenous TGF-β-induced VEGF release. Results were validated using The Cancer Genome Atlas transcriptomics data. RESULTS Glioma cells exhibit heterogeneous patterns of constitutive TGF-β pathway activation reflected by phosphorylation not only of SMAD2 and SMAD3 but also of SMAD1/5/8. Constitutive TGF-β pathway activity depends on the type I TGF-β receptor, ALK-5, and accounts for up to 69% of constitutive VEGF release, which is positively regulated by SMAD2/3 and negatively regulated by SMAD1/5/8 signaling in a cell line-specific manner. Exogenous TGF-β induces VEGF release in most cell lines in a SMAD- and ALK-5-dependent manner. There is no correlation between the fold induction of VEGF secretion induced by TGF-β compared with hypoxia. The role of SMAD5 signaling is highly context and cell-line dependent with a VEGF inhibitory effect at low TGF-β and pSMAD2 levels and a stimulatory effect when TGF-β is abundant. CONCLUSIONS TGF-β regulates VEGF release by glioma cells in an ALK-5-dependent manner involving SMAD2, SMAD3, and SMAD1/5/8 signaling. This crosstalk between the TGF-β and VEGF pathways may open up new avenues of biomarker-driven exploratory clinical trials focusing on the microenvironment in glioblastoma.

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Differential regulation of TGF-β-induced, ALK-5-mediated VEGF release by SMAD2/3 versus SMAD1/5/8 signaling in glioblastoma

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ABSTRACT

Background: The transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) pathways have been attributed a major role in the pathogenesis in glioblastoma, notably immunosuppression, migration and angiogenesis, but their interactions have remained poorly understood.

Methods: We characterized TGF-β pathway activity in nine long-term glioma cell lines (LTC) and 4 glioma-initiating cell lines (GIC) in relation to constitutive and exogenous TGF-β-induced VEGF release. Results were validated using TCGA transcriptomics data.

Results: Glioma cells exhibit heterogeneous patterns of constitutive TGF-β pathway activation reflected by phosphorylation not only of SMAD2 and SMAD3, but also SMAD1/5/8. Constitutive TGF-β pathway activity depends on the type I TGF-β receptor, ALK-5, and accounts for up to 69% of constitutive VEGF release which is positively regulated by SMAD2/3 and negatively by SMAD1/5/8 signaling in a cell line-specific manner. Exogenous TGF-β induces VEGF release in most cell lines, in a SMAD- and ALK-5-dependent manner. There is no correlation between the fold induction of VEGF secretion induced by TGF-β compared to hypoxia. The role of SMAD5 signaling is highly context- and cell line dependent with a VEGF inhibitory effect at low TGF-β and pSMAD2 levels, and a stimulatory effect when TGF-β is abundant.

Conclusions: TGF-β regulates VEGF release by glioma cells in an ALK-5-dependent manner involving SMAD2, SMAD3 and SMAD1/5/8 signaling. This crosstalk between the TGF-β and VEGF pathways may open up new avenues of biomarker-driven exploratory clinical trials focusing on the microenvironment in glioblastoma.

Keywords: VEGF, TGF-β, glioblastoma, angiogenesis
INTRODUCTION

Transforming growth factor (TGF)-β, a cytokine with pleiotropic functions, plays a pivotal role in cancer biology and represents one of the key pathogenic factors in glioblastoma. Ligand binding leads to the phosphorylation of SMAD family proteins which are involved in the regulation of gene transcription. Activation of the TGF-β/SMAD pathway correlates with poor prognosis in glioma patients. Various anti-TGF-β strategies have been explored in rodent glioma models and clinical trials.

TGF-β signaling is mediated via a heterodimeric receptor complex comprising type I and type II receptors. Canonical TGF-β signaling involves ligand binding to TGF-β receptor II (TGF-βRII) which associates with the type I receptor activin receptor-like kinase-5 (ALK-5), resulting in the phosphorylation of SMAD2 and SMAD3. Pharmacological ALK-5 inhibition is an effective treatment strategy in rodent glioma models. The involvement of another type I receptor in TGF-β signaling, the activin receptor-like kinase 1 (ALK-1), leading to the phosphorylation of SMAD1/5/8, has been characterized in endothelial cells. This alternative pathway may counteract the ALK-5/pSMAD2 pathway and thereby balance TGF-β signaling.

A role for TGF-β in modulating vascular endothelial growth factor (VEGF) release has been proposed in several cell types. Both negative and positive regulation of VEGF by TGF-β has been described in endothelial cells. In glioma cells, the effect of TGF-β on the regulation of VEGF release remains uncertain, too. For selected glioma cell lines, a time-dependent increase of VEGF release induced by exogenous TGF-β has been reported. Silencing of TGF-β1/2 or exposure to the ALK-5 inhibitor, SD-208, led to a reduced VEGF levels in the supernatant of LN-308 and LNT-229 glioma cells, indicating a role for TGF-β1/2 in the regulation of constitutive VEGF release. However, pharmacological inhibition of ALK-5 by SB431542 inhibited the TGF-β-evoked VEGF release, but had only minor effects on constitutive VEGF release in D270MG or D423MG cells. These observations raise the
possibility that VEGF is not only regulated via pSMAD2/3 signaling, but also involves other pathways in glioma cells.

MATERIALS AND METHODS

Cell culture

Nine long-term malignant glioma cell lines (LTC)\textsuperscript{17}, 4 glioma-initiating cell (GIC) lines\textsuperscript{18,19} and hCMEC/D3\textsuperscript{20} (supplementary material) were incubated under normoxia or in a hypoxia incubator (1% O\textsubscript{2}, 5% CO\textsubscript{2}, 37°C).

Reagents

Recombinant TGF-β\textsubscript{2} (R&D, Minneapolis, MN) was used for all stimulation studies. SD-208 (Scios Inc., Fremont, CA) inhibits ALK-5 at 0.048 µM in cell-free systems\textsuperscript{4}.

Immunoblot analyses

Whole cell lysates were subjected to SDS-PAGE under reducing conditions loading equal amounts of proteins (supplementary material). SiRNA-mediated knockdown was applied to identify the specific band. For quantitative correlation analyses of baseline expression of total and phosphorylated SMAD proteins, band intensity was analyzed via densitometry using ImageJ software (Open Source). To compare the relative induction of phosphorylation after stimulation, we scored the response into no (<20%), low (20%-50%), medium (>50%) or high induction of phosphorylation (>100% or no baseline phosphorylation).

Real-time PCR (RT-PCR)

Gene expression was determined via real-time PCR (RT-PCR, procedure and primer sequences see supplement) using glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene with the ΔC\textsubscript{TT}-method for relative quantification.

RNA interference
To silence gene expression, cells were transiently transfected using Metafectene® Pro (Biontex, Martinsried, Germany) for LTC and electroporation for GIC (Neon transfection system, Invitrogen, Basel, Switzerland) and siRNA pools (80-120 nM final concentration), containing four selected siRNA duplexes, each with a modification pattern addressing off-target effects caused by both strands (ON-TARGETplus, SMARTpool, ON-TARGETplus Non-Targeting Pool siRNA as a negative control (Dharmacon, Lafayette, CO, SA).

*Flow cytometry*

Signal intensity of flow cytometry analysis (supplementary material) was calculated as the ratio of mean fluorescence of specific versus isotype control antibody (specific fluorescence index, SFI).

*Enzyme-linked immunosorbent assay (ELISA)*

Supernatants (preparation as outlined in the supplement) were analyzed by ELISA for VEGF levels (ebioscience, Vienna, Austria) and TGF-β1/2 (R&D).

*Luciferase reporter assay*

The pGL3 SBE-4-Luc plasmid (B. Vogelstein, Baltimore, MD) containing four copies of the Smad-binding element (SBE) GTCTAGAC²¹ was used for reporter gene assays (supplementary material).
RESULTS

*TGF-β pathway activity in human glioma cells*

We first analyzed the expression levels of TGF-β receptors, their ligands, constitutive levels of total SMAD2-5, of phosphorylated SMAD2, SMAD3, and SMAD1/5/8, and the TGF-β target gene plasminogen activator inhibitor (PAI)-1. Correlation analyses of these parameters were performed either for all cell lines pooled or separately for LTC (Table 1). A separate analysis for the GIC was omitted because of small sample size. We validated our results using Affymetrix gene expression data from the TCGA (Supplementary Table 1).

TGF-βRII, ALK-5 and ALK-1 mRNA were differentially expressed with a trend towards lower mRNA expression for all receptors in GIC. ALK-1 mRNA levels were lower than ALK-5 levels and more than 100-fold lower in glioma than in endothelial cells (Fig. 1A-C, left). The highest TGF-βRII protein levels were found for T98G cells while two of four GIC (T-269, S-24) had TGF-βRII levels at the detection limit. The SFI varied strongly for ALK-5 from 84 in LN-18 cells to 1.7 in LN-308 cells. ALK-1 protein was not detected at the cell surface in glioma cells, using hCMEC as a positive control (Fig. 1A-C, right). Overall, mRNA levels were not predictive of protein levels. TGF-β₁ and TGF-β₂ were heterogeneously expressed on mRNA and protein level (Fig. 1D,E). TGF-β₁ mRNA correlated with secreted TGF-β₁, but not with TGF-β₂ mRNA or protein. TGF-β₂ mRNA correlated with TGF-βRII mRNA in our as well as TCGA database (Table 1, supplementary Table 1).

pSMAD2 levels were highest in LN-428, LN-319, A172, LN-308, less in LN-18, D247 and T98G cells, below detection limit in the other cell lines, and correlated with pSMAD3 levels. pSMAD1/5/8 was low in 2 of 4 GIC (Fig. 1F). TGF-βRII and ALK-5 mRNA correlated with pSMAD2, and TGF-βRII surface expression with pSMAD1/5/8 levels. Phosphorylation of SMAD proteins as the proximate readout of TGF-β activity correlated with ligand expression as follows: pSMAD2 with TGF-β₁ and TGF-β₂ mRNA, pSMAD3 and pSMAD1/5/8 with TGF-β₂ protein and inversely SMAD5 protein with the sum of TGF-β₁ and TGF-β₂ protein.
PAI-1 mRNA levels did not correlate with TGF-β receptors, ligands (mRNA or protein) or downstream phosphorylation of SMAD (Fig. 1G, Table 1).

*Endogenous TGF-β promotes VEGF expression and release in a cell line-specific manner*

VEGF expression on mRNA or protein level was similar in LTC and GIC (Fig. 2A,B). TGF-β1 mRNA correlated with VEGF mRNA while total SMAD2 and total SMAD5 protein were inversely correlated with VEGF release. TGF-β1 and TGF-β2 mRNA correlated with VEGF mRNA and inversely also with SMAD2 mRNA in the TCGA database. However, neither TGF-β receptor mRNA nor protein in our cell line panel nor of gene expression data of the TCGA nor pSMAD2/pSMAD3 nor pSMAD1/5/8 levels correlated with VEGF mRNA. PAI-1 mRNA correlated with VEGF mRNA in the TCGA, but not in our dataset (Table 1, supplementary Table 1).

We chose LN-308 cells because of their high endogenous TGF-β expression and high constitutive SMAD2 and SMAD3 phosphorylation to study the regulation of constitutive VEGF release. We added selected analyses for U87MG and ZH-161 cells. In LN-308, TGF-βRII silencing, with an efficacy of 88% reduction assessed by RT-PCR (data not shown), reduced pSMAD2 and pSMAD3 levels, confirming the involvement of TGF-β and TGF-βRII in their constitutive regulation. Interestingly, pSMAD1/5/8 levels, too, were reduced upon silencing of TGF-βRII. Pharmacological inhibition of the kinase activity of ALK-5 by the small molecule inhibitor, SD-208 or silencing of ALK-5, led to reduced pSMAD2 and pSMAD3 levels, too, but did not affect constitutive pSMAD1/5/8 levels (Fig. 2C). To investigate the contribution of the different SMAD signaling pathways to constitutive VEGF release, we established gene silencing of SMAD2, SMAD3, SMAD2/3 in combination, or SMAD5 which led to specific reductions of the corresponding target proteins. As expected, pSMAD2, pSMAD3 and pSMAD1/5/8 were also reduced upon silencing of the respective SMAD proteins. In addition, silencing of SMAD proteins did not only affect their own
phosphorylation; instead, we observed reduced pSMAD3 upon silencing of SMAD2 in contrast to increased pSMAD2 upon silencing of SMAD3. pSMAD3 was increased upon silencing of SMAD5 while pSMAD2 was unaffected (Fig. 2D).

Silencing of TGF-βRII reduced VEGF release in LN-308 cells (Fig. 2E). Inhibition of ALK-5 by RNA interference or SD-208 reduced VEGF release in U87MG and LN-308 cells, too, although the reduction was not significant in ZH-161 (Fig. 2F). SMAD silencing had differential effects on VEGF release: SMAD2 or SMAD3 gene silencing reduced constitutive VEGF levels in U87MG and LN-308, but not ZH-161 (Fig. 2G). Even co-silencing of SMAD2 and SMAD3 failed to affect VEGF release in ZH-161, but further reduced VEGF levels in LN-308 exceeding the effect of silencing SMAD2 or SMAD3 alone. In contrast, silencing of SMAD5 increased the levels of constitutive VEGF release in U87MG cells, but had no effects in LN-308 or ZH-161 (Fig. 2H).

*Exogenous TGF-β promotes VEGF release in human malignant glioma cells*

We next assessed glioma cell responsiveness to exogenous TGF-β. All cell lines showed increased pSMAD2 and - except for S-24 - also pSMAD3, albeit to a differential extent. Relative pSMAD2 induction correlated inversely with the endogenous pSMAD2 ($r=-0.66$, $p=0.01$). pSMAD1/5/8 was increased in LN-319, A172 and U87MG LTC and T-325, ZH-161, S-24 GIC (Fig. 3A). We next monitored the increase of TGF-β-dependent transcriptional activity using a SMAD-binding element (SBE) reporter plasmid\(^\text{21}\). TGF-β induced SBE reporter activity in all cell lines except in LN-428, A172, T-269 and S-24 cells. The highest response was observed in U87MG (26-fold) (Fig. 3B). Sufficient TGF-βRII surface expression was necessary for the induction of SBE reporter activity, given the poor response in T-269 and S-24 cells which exhibit TGF-βRII levels at the detection limit (Fig. 1A). In addition, high responsiveness correlated with pSMAD3 induction by TGF-β ($r=0.62$, $p=0.02$).
Surprisingly, the reporter-non-responsive cell lines LN-428 and A172 expressed high levels of ALK-5, in contrast to the highly responsive cell line U87MG (Fig. 1B).

VEGF levels increased after TGF-β treatment in all cell lines except LN-428, A172 and S-24. The most pronounced increase was seen in U87MG cells (9-fold) (Fig. 3C). The response in U87MG was maintained but reduced in extent when these cells were switched to GIC culture conditions. In ZH-161, the response was further increased when switched to serum-containing medium for 2 passages. Under GIC conditions, basal VEGF synthesis was increased in U87MG both on mRNA and protein level and in ZH-161 on protein level (Supplementary Figure 1). Cell lines not exhibiting TGF-β2-dependent VEGF induction showed only minor induction of pSMAD2 and pSMAD3 by TGF-β2 (Fig. 3A). The induction of VEGF by TGF-β correlated with the induction of pSMAD3 (r=0.59, p=0.04) and reporter responsiveness (r=0.71, p=0.006). To assess whether cell lines not increasing VEGF release in response to TGF-β were generally less responsive to transcriptional activation of the VEGF gene, we exposed the same cell lines to hypoxia (1% O2, 24 h) as a major driver of VEGF gene transcription. Hypoxia led to increased VEGF levels in almost all cell lines including LN-428, A-172 and S-24 which did not increase VEGF release upon stimulation with TGF-β.

Conversely, LNT-229 cells were unaffected, and D247MG showed even reduced VEGF release under hypoxia (Fig. 3D). Of note, hypoxia-induced VEGF release was overall higher in GIC, ranging from 1.9-fold (ZH-161) to 32-fold (T-269) than in LTC, ranging from 1.3-fold (LN-18) to 2.1-fold (U87MG). Accordingly, we found only a minor induction of HIF-1α protein by hypoxia in LNT-229 which increased VEGF release in response to TGF-β, but not to hypoxia. Indeed, under normoxic conditions, we detected higher HIF-1α protein levels in ZH-161 than in LNT-229 cells. Of note, treatment with TGF-β left HIF-1α protein unaffected and there was no correlation between the magnitude of the VEGF response to TGF-β versus hypoxia (data not shown). We selected U87MG and ZH-161 cells to examine the time and concentration dependence of VEGF induction by TGF-β. On mRNA level, the highest
increase of VEGF (14-fold for U87MG and 1.4-fold for ZH-161) was observed at 4 h for U87MG and at 24-48 h for ZH-161 h (Fig. 3E). The induction of VEGF release into the supernatant was with 9-fold highest at 24 h for U87MG and 2-fold at 24-48 h in ZH-161 cells (Fig. 3F). Further, the induction of VEGF was concentration-dependent in U87MG and ZH-161 cells on mRNA (Fig. 3G) and protein level (Fig. 3H).

**Molecular pathways mediating TGF-β-evoked VEGF release in glioma cells**

To explore the pathways mediating the stimulatory effect of exogenous TGF-β on VEGF release, we combined the inhibition of ALK-5 by SD-208 or siRNA, or siRNA-mediated gene silencing of SMAD2, SMAD3 or SMAD5, with the addition of exogenous TGF-β₂. As expected, inhibition of ALK-5 by SD-208 or RNA interference reduced TGF-β₂-evoked SMAD2 and SMAD3 phosphorylation in U87MG and ZH-161 cells. Interestingly, the induction of SMAD1/5/8 phosphorylation was abrogated by inhibition or silencing of ALK-5, too, suggesting a hitherto unrecognized TGF-β-dependent signal transduction pathway from ALK-5 to SMAD1/5/8 in glioma cells. Both pharmacological inhibition and silencing of ALK-5 interfered with the TGF-β₂-mediated increase of VEGF (Fig. 4A, right panel). Silencing of SMAD2, SMAD3 or SMAD5 in U87MG or ZH-161 cells led to specific reductions of the target protein and reduced pSMAD2, pSMAD3 and pSMAD1/5/8 levels, respectively (Fig. 4B). Interestingly, TGF-β₂-induced SMAD1/5/8 phosphorylation was further increased in case of SMAD2 or SMAD3 gene silencing in U87MG, but not in ZH-161, consistent with substrate competition of SMAD proteins for ALK-5 (Fig. 4B). TGF-β₂-evoked transcriptional activity was reduced upon silencing of either SMAD2, SMAD3 or SMAD5 in U87MG. A similar trend was observed in ZH-161, however, the reduction was only significant upon silencing of SMAD3 alone or combined with SMAD2 (Fig. 4C). In both cell lines, the TGF-β₂-evoked VEGF release was reduced upon silencing of SMAD2 or SMAD3. Co-silencing of SMAD2 and SMAD3 had no superior effect over single silencing in
ZH-161. Further, silencing of SMAD5 reduced TGF-β2-evoked VEGF release in U87MG while the reduction was not significant in ZH-161 cells (Fig. 4D).
DISCUSSION

Targeting angiogenesis is one of the major current strategies of glioblastoma treatment, but enthusiasm for this approach has declined after the failure of several anti-angiogenic agents in phase III trials\textsuperscript{23-25}. Although the anti-VEGF antibody bevacizumab showed activity in recurrent glioblastoma in phase II trials\textsuperscript{26,27}, no survival gain was achieved in two phase III trials in newly diagnosed glioblastoma\textsuperscript{28,29}. Further, various attempts to improve anti-angiogenic therapy by combination with cytotoxics were unsuccessful\textsuperscript{30}. Nevertheless, VEGF is still one of the most promising targets to interfere with the malignant phenotype of glioblastoma. While hypoxia is considered the key driver of VEGF release in glioblastoma, hypoxia-independent control of VEGF release has received little attention.

TGF-\(\beta\), an important molecule shaping the microenvironment in glioblastoma, was characterized here as a positive regulator of the VEGF pathway. We provide a comprehensive analysis of the TGF-\(\beta\) pathway and its control of VEGF release in LTC and GIC models. Specifically, we analyzed the role of endogenous TGF-\(\beta\) as a model of autocrine signaling versus that of exogenous TGF-\(\beta\), mimicking paracrine signals from the microenvironment. As a precondition for autocrine signaling, TGF-\(\beta\)RII and ALK-5 were widely expressed whereas ALK-1 was not (Fig. 1), indicating that an ALK-1/pSMAD1/5/8 signaling axis of VEGF release characterized in endothelial cells\textsuperscript{12} is not operating in glioma cells. All LTC released TGF-\(\beta_1\) and TGF-\(\beta_2\), while TGF-\(\beta_2\) was not detected in 2 of 4 GIC, confirming that therapeutic approaches targeting only one TGF-\(\beta\) isoform are insufficient. Most cell lines exhibited phosphorylated SMAD2, SMAD3 or SMAD5, suggesting constitutive TGF-\(\beta\) pathway activation, confirmed by reduced pSMAD levels when TGF-\(\beta\)RII or ALK-5 expression were suppressed, or ALK-5 inhibited pharmacologically (Fig. 2C). In non-endothelial cells, BMP are considered as major drivers of SMAD1/5/8 phosphorylation, however, recently TGF-\(\beta\)-dependent phosphorylation of SMAD1/5/8 has been described,
although not yet for glioma cells. We found that pSMAD1/5/8 levels in cellular lysates correlate with TGF-βRII levels at the surface and that TGF-βRII gene silencing reduced pSMAD1/5/8 levels (Fig. 2C). That inhibition of ALK-5 via SD-208 or ALK-5 gene silencing left pSMAD1/5/8 unaffected, suggests that a type-I receptor other than ALK-5 mediates SMAD1/5/8 phosphorylation in response to TGF-β. Still, we cannot rule out that these effects are mediated via minor ALK-1 levels escaping detection on protein level.

To study the effect of endogenous TGF-β signaling on constitutive VEGF release, we interfered with different components of the TGF-β pathway. TGF-βRII gene silencing reduced VEGF release in LN-308 cells, confirming a role for TGF-β in the regulation of VEGF release in this cell line which releases high levels of active TGF-β.

Furthermore, VEGF release required ALK-5 activity in LN-308 and U87MG cells, although not ZH-161 cells (Fig. 2F). This might suggest a robust TGF-β-independent VEGF secretion in GIC potentially due to constitutive HIF expression at normoxic conditions which we confirmed for ZH-161. SMAD2 and SMAD3 are necessary for TGF-β-dependent regulation of VEGF in LN-308 and U87MG cells, but again ZH-161 was unaffected even by combined SMAD2/3 silencing which was most effective in reducing VEGF in LN-308 cells (Fig. 2G).

The role of SMAD5 in the regulation of constitutive VEGF release is more complex. LN-308 and ZH-161 did not show major changes upon SMAD5 gene silencing whereas U87MG showed increased VEGF secretion (Fig. 2H). This can be explained by an indirect effect: BMP-2 being expressed by U87MG destabilizes HIF-1α in glioma cells. Thus, depletion of SMAD5 resulting in reduced BMP signaling might increase the stability of HIF-1α, promoting VEGF release. Further, in endothelial cells a direct inhibitory effect of BMP-9 on VEGF via ALK-1/pSMAD1/5/8 signaling has been described. Thus, TGF-β contributes to constitutive VEGF release in glioma cells via a TGF-βRII/ALK-5/SMAD2/3 signaling pathway. The pSMAD1/5/8 signaling axis driven by both TGF-β and BMP might be indirectly involved in selected cell lines, possibly as a negative feedback mechanism.
TGF-β is secreted not only by glioma cells but also by the tumor microenvironment, eg, endothelial, immune or microglial cells. Thus, we studied the effect of exogenous TGF-β on VEGF release. We observed a differential pattern of responsiveness to TGF-β as determined by changes in SMAD phosphorylation and reporter activity (Fig. 3A,B). We found that SMAD1/5/8 is also phosphorylated via TGF-β and ALK-5 in some glioma cell lines and may contribute to enhanced VEGF release (Fig. 3A, 4A,D). Sufficient TGF-βRII cell surface expression and phosphorylation of SMAD2/3 are necessary, but not sufficient, for SBE activation (Fig. 3B) and VEGF release (Fig. 3C), suggesting the involvement of additional downstream effectors. That high constitutive levels of pSMAD2 negatively correlated with the inducibility of pSMAD2 by exogenous TGF-β, suggests a saturation of the signaling pathway by autocrine TGF-β, leading to a decreased sensitivity towards exogenous TGF-β. ALK-5 expression was not predictive for sensitivity to exogenous TGF-β since cell lines with poor activation of the SBE reporter expressed rather high levels of ALK-5, and vice versa. Probably, post-transcriptional processes, e.g., involving deubiquitinating enzymes regulating TGF-β receptor stability modulate sensitivity towards TGF-β.

Exogenous TGF-β induced VEGF release in most cell lines (Fig. 3C). Cells unresponsive to TGF-β did were still responsive to hypoxia with regard to VEGF induction, placing differential regulation upstream of the VEGF promotor (Fig. 3D). TGF-β-dependent SMAD3 phosphorylation as well as transcriptional SBE activation correlated with TGF-β-evoked VEGF release, although no such correlation was seen for pSMAD2 or the TGF-β target gene, PAI-1. A differential activation of the transcription factor Sp1, a major driver of VEGF gene expression in non-hypoxic conditions, via competing SMAD signaling pathways might play a role in this context. Of note, the VEGF promotor itself contains SBE sites and promotor activity is increased by SMADs. That depletion of SMAD5 led to increased constitutive VEGF release (Fig. 2H), but reduced TGF-β-evoked VEGF release (Fig. 4D) in U87MG cells might be explained best via the dual activation of pSMAD1/5/8 by TGF-β and BMP. While
endogenous BMP signaling might inhibit constitutive VEGF expression either indirectly via destabilization of HIF\textsuperscript{37} or directly via BMP-9-ALK1 signaling as described for endothelial cells\textsuperscript{38}, a relative predominance of TGF-β signaling might occur in the pharmacological context of exogenous stimulation by TGF-β inducing VEGF both via pSMAD2/3 and pSMAD1/5/8 signaling (Fig. 5).

In summary, we show that TGF-β regulation of VEGF release in glioma cells depends, in an ALK-5-dependent manner, not only on SMAD2/3-dependent pathways, but also pSMAD1/5/8 signaling, suggesting that anti-TGF-β strategies may indirectly inhibit VEGF pathway activation in glioblastoma and this mechanism accounts for some of the angiogenic activity attributed to TGF-β. Moreover, since we have recently illustrated how integrin inhibition down-regulates TGF-β pathway activation in glioblastoma\textsuperscript{42}, combinatorial therapeutic approaches targeting sequentially or in parallel integrins, TGF-β and VEGF based on preferential pathway activation may allow for better treatment options for glioblastoma.

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References


Figure legends

Figure 1. **TGF-β receptor expression and TGF-β pathway activity in human glioma cells.**

A-C. Expression levels of TGF-β-RII (A), ALK-5 (B) and ALK-1 (C) on mRNA level assessed by RT-PCR (left) and protein level (SFI) assessed by flow cytometry (middle) (A,B). Representative flow cytometry profiles are shown on the right. Data are expressed as mean and SEM of 2-4 independent experiments. D. Expression of TGF-β₁ (squares) and TGF-β₂ (circles) mRNA assessed by RT-PCR. E. LTC were serum-starved for 24 h and then exposed to serum-free medium for 24 h, GIC were seeded, 24 h later changed to fresh medium, and supernatants harvested 24 h thereafter. TGF-β₁ (open bars) or TGF-β₂ (striped bars) released into the supernatant were analyzed by ELISA (mean and SEM, n=2, in duplicates, “n.d.” below detection limit). F. Whole cell lysates of LTC (24 h serum-starved) or GIC were subjected to immunoblotting. Representative loading controls for GAPDH or β-actin are included. G. PAI-1 mRNA expression assessed by RT-PCR.

Figure 2. **Control of VEGF release by constitutive TGF-β pathway activity.** A. VEGF mRNA (A) analyzed by RT-PCR and VEGF release into the supernatant (B) prepared as in Fig. 1D and analyzed by ELISA (mean and SEM, n=5 in duplicates or triplicates) in LTC and GIC. C. Representative immunoblots (SMAD2, pSMAD2, SMAD3, pSMAD3, SMAD5, pSMAD1/5/8, GAPDH) of LN-308 cells after transfection with siRNA targeting TGF-βRII (72 h) or ALK-5 (48 h) or after pharmacological inhibition of ALK-5 by SD-208 (1 µM, 24 h) or respective controls (non-targeting siRNA or DMSO). D. Representative immunoblots of LN-308 cells with siRNA targeting SMAD2 (48 h), SMAD3 (48 h), SMAD2 and SMAD3 (48h) or SMAD5 (72 h) or non-targeting control. E. Supernatants of LN-308 cells harvested 72 h after transfection with siRNA targeting TGF-βRII were assessed for VEGF levels by ELISA. F. Supernatants of U87MG, LN-308 and ZH-161 cells were analyzed for VEGF by
ELISA after exposure to ALK-5 siRNA (left, 48 h post transfection for LN-308 and ZH-161, 72 h for U87MG) or SD-208 (right, 24 h, 1 µM). G, H. Supernatants harvested after transfection with siRNA targeting SMAD2 (24 h for U87MG and ZH-161, 48 h for LN-308), SMAD3 (36 h for U87MG, 48 h for LN-308, 24 h for ZH-161), SMAD2/3 (36 h for U87MG, 48 h for LN-308, 24 h for ZH-161) (G) or SMAD5 (24 h for U87MG and ZH-161, 48 h for LN-308) (H). Shown are representative experiments performed in triplicates (student’s t-test, *p<0.05).

Figure 3. **Stimulation of VEGF release by exogenous TGF-β**. A. TGF-β pathway inducibility was examined by assessing the induction of pSMAD2, pSMAD3 or pSMAD1/5/8 via immunoblot with or without stimulation with TGF-β$_2$ (10 ng/ml) for 24 h after 24 h serum starvation. Shown are immunoblots of SMAD2, pSMAD2, SMAD3, pSMAD3, SMAD5, pSMAD1/5/8, and representative loading controls for GAPDH and β-actin. B. Transcriptional activation by TGF-β was assessed by a SBE reporter assay. Shown are relative units (RU) of SBE reporter signal after stimulation with TGF-β$_2$ (10 ng/ml) for 24 h normalized to untreated control. C,D. The supernatants of glioma cell lines maintained with or without 10 ng/ml TGF-β$_2$ for 24 h (C) or with or without hypoxia (D) after a 24 h period of serum starvation were analyzed for VEGF by ELISA. E,F. VEGF was assessed after 4, 24 and 48 h exposure to 10 ng/ml TGF-β$_2$ in U87MG or ZH-161 on mRNA level (E) or protein level using cell culture supernatants as in A (F). G,H. U87MG or ZH-161 cells were analyzed for VEGF after 24 h stimulation with TGF-β$_2$ at 0, 0.1, 1 or 10 ng/ml on mRNA level (G) and in cell culture supernatants (H).

Figure 4. **TGF-β-dependent increase of VEGF is mediated by ALK-5 and involves different SMAD signaling pathways**. A. U87MG or ZH-161 cells were treated without or with SD-208 (1 µM, 25 h) or siRNA targeting ALK-5 (72 h for U87MG, 48 h for ZH161) and
24 h before harvesting with or without TGF-β₂ (10 ng/ml). Total SMAD2, pSMAD2, SMAD3, pSMAD3, SMAD5, pSMAD1/5/8 or GAPDH were analyzed by immunoblot. Supernatants were analyzed for VEGF by ELISA. B. Levels of total SMAD2, pSMAD2, SMAD3, pSMAD3, SMAD5, pSMAD1/5/8 or GAPDH were assessed by immunoblot after silencing of SMAD2 (24 h), SMAD3 (36 h for U87MG, 24 h for ZH-161), SMAD2 and SMAD3 (ZH-161, 24 h) or SMAD5 (24h) without or with additional treatment with TGF-β₂ (10 ng/ml, 12-24 h). C. Effect of silencing of SMAD2, SMAD3 or SMAD5 or respective controls on transcriptional activation in U87MG or ZH-161 cells without or with treatment with TGF-β₂ (10 ng/ml, 12 h) was examined by SBE reporter assay. Shown are RU of SBE reporter signal normalized to untreated control-transfected samples. D. Supernatants from cells treated as in B were assessed for VEGF by ELISA.

Figure 5. **TGF-β-dependent regulation of VEGF release in glioblastoma.** Proposed model for the regulation of VEGF by TGF-β (straight arrows) and HIF (dotted arrows). TGF-β released from cells of the tumor microenvironment, e.g., endothelial, immune and microglial cells, signals via TGF-βRII and ALK-5 leading to phosphorylation of SMAD2, SMAD3 and SMAD1/5/8 and increased VEGF release. BMP-dependent pSMAD1/5/8 signaling (dashed arrows) destabilizes HIF and thereby indirectly reduces VEGF. TGF-β-dependent SMAD1/5/8 signaling requires, beyond TGF-βRII, a non-ALK-5-type-I-receptor (TGF-βRI-x). Overall, VEGF release is modulated in a cell-specific and context-dependent manner, with a predominance of either hypoxia-, TGF-β- or BMP-driven signaling: for ZH-161 GIC, exhibiting low endogenous TGF-β levels but stable endogenous HIF-1α, VEGF release is highly resistant to TGF-β pathway inhibition at baseline conditions, but sensitive when TGF-β is abundant. In contrast, LN-308 cells, characterized by high endogenous TGF-β and pSMAD2/3, are highly sensitive for TGF-β pathway inhibition via ALK-5 and SMAD2/3, but not for inhibition of SMAD5 at baseline. In U87MG cells, the duality of pSMAD1/5/8
signaling as driven both by TGF-β and BMPs becomes apparent: SMAD5 at baseline conditions with low endogenous TGF-β negatively regulates VEGF, probably via BMP-driven destabilization of HIF, but positively regulates VEGF when TGF-β is abundant.
Supplementary Methods

Cell culture

The 9 long-term glioblastoma cell lines (LTC)\textsuperscript{17} were maintained in Dulbecco`s modified Eagle`s medium supplemented with 10% fetal calf serum (FCS) and last subjected to authentication tests at the German Biological Resource Centre DSMZ in Braunschweig, Germany, in November 2013. The 4 glioma-initiating cell (GIC) lines were established after informed consent and approval of the local ethics committees and characterized in our laboratories in Tübingen and Zurich\textsuperscript{18,19} as well as via comparative genomic hybridization by R. Weber (Department of Genetics, University of Hannover) and maintained in Neurobasal Medium supplemented with B-27 (20 \(\mu\)l/ml) and Glutamax (10 \(\mu\)l/ml) from Invitrogen (Basel, Switzerland), fibroblast growth factor (FGF)-2, epidermal growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, PA) and heparin (32 IE/ml; Ratiopharm, Ulm, Germany). hCMEC/D3, a human brain endothelial cell line previously characterized\textsuperscript{20}, was maintained in EndoPrime medium supplemented with Endoprime Supplement, EGF, VEGF and fetal bovine serum (FBS) (PAA laboratories, Pasching, Austria). We instituted a 24 h incubation in serum-free medium of 24 h for preparation of baseline analyses as well as before any treatment steps. Serum deprivation was applied to remove serum as a confounder for LTC. We verified that this switch of culture conditions did not affect TGF-\(\beta\) activity as assessed by pSmad2 levels (data not shown).

Preparation of whole cell lysates and immunoblot analyses

Whole cell lysates were prepared in lysis buffer containing 25 mM Tris-HCL, 120 mM NaCl, 5 mM EDTA and 0.5% NP-40 supplemented with 2 \(\mu\)g/mL aprotinin, 10 \(\mu\)g/mL leupeptin and 100 \(\mu\)g/mL phenylmethylsulfonyl fluoride (Sigma Aldrich) and phosphatase inhibitor cocktails 2 and 3 (Sigma Aldrich). Protein levels were determined using a Bradford-based protein assay (Biorad, Munich, Germany). After SDS-PAGE, proteins were transferred to
nitrocellulose membranes (Biorad, Munich, Germany), blocked in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20 following antibody incubation. The following antibodies were used: anti-SMAD2 (3122S), anti-pSMAD2 (3108S), anti-SMAD3 (9513S), anti-pSMAD3 (9520S), anti-SMAD5 (9517S), anti-pSMAD1/5/8 (9511S) (all Cell Signaling, Boston, MA) anti-GAPDH (EB07069, Everest Biotech, Oxfordshire, UK), anti-β-actin (sc-1616, santa cruz biotechnology, Santa Cruz, CA). Visualization of protein bands was accomplished using horseradish peroxidase (HRP)-coupled secondary antibodies (Santa cruz Biotechnology) and enhanced chemoluminescence (Pierce/Thermo Fisher, Madison, WI).

**Real-time PCR (RT-PCR)**

Total mRNA extraction was done using the NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany) system including DNase treatment. cDNA was prepared using Superscript reverse transcriptase II (Invitrogen) and random primer 9 (New England BioLabs, Ipswich, MA) or the iScript™ cDNA synthesis kit (Biorad Laboratories, Reinach, Germany). For real-time PCR (RT-PCR), gene expression was measured using the Real Time PCR System 7300 (Applied Biosystems, Foster City, CA) with SYBR Green Master Mix (AppliChem, Darmstadt, Germany) and primers at optimized concentrations. The conditions for RT-PCR were 40 cycles, 95°C/15s, 60°C, 1 min. Relative quantification of gene expression was determined by comparison of threshold values. The following primers were used: GAPDH (forward 5’-CTC TCT GCT CCT CCT GTT CGA C-3’, reverse 5’-TGA GCG ATG TGG CTC GGC T-3’), VEGF (forward 5’-GAG ACC CTG GTG GAC ATC TT-3’, reverse 5’-TTG ATC CGC ATA ATC TGC AT-3, Microsynth, Balgach, Switzerland), ALK-1 (Lot no. 98730849, Qiagen, Hilden, Germany), ALK-5 (Lot no. 6734902_1855089_88442_88443_88444, Qiagen), PAI-1 (forward 5´-CAGAAAGTGAAGATCGAGGTGAAC-3’, reverse 5´-GGAAGGTCTGTCCATGATGAT-3’, TGF-β1 (forward 5’-GCCCTGGACACCAACTATTG-3’, reverse 5’-CGTGTCAGGCTCCCAAATG-3’), TGF-β2,
Flow cytometry

LTC were detached using Accutase (PAA Laboratories), GIC were mechanically dissociated and blocked with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Selected experiments regarding ALK-1 detection in adherent cells were confirmed using mechanical dissociation of cells using a cell scraper to exclude receptor loss by accutase treatment. The cells were incubated for 30 min at 4°C using the following primary antibodies: anti-human TGF-βRI/ALK-5 (AF3025, R&D systems, Minneapolis, MN), anti-TGF-βRII (ab78419, abcam, Cambridge, UK), anti-human ALK-1 (PF-03446962, Pfizer, New York, NY) or appropriate isotype controls (normal goat IgG, sc-2028, Santa Cruz Biotechnology, Santa Cruz, CA), mouse IgG1 (557273, Becton Dickinson, Franklin Lakes, NJ), human IgG (Sigma Aldrich, Buchs, Switzerland). After 2 washing steps with PBS containing 0.5% BSA, cells were incubated at 4°C with the following secondary antibodies: anti-goat IgG-FITC (F2016, Sigma Aldrich), anti-mouse IgG1-PE (RMG1-1, Biolegend, London, UK), anti-human Alexa Fluor 488 antibody (Invitrogen). Subsequently, the cells washed twice with PBS containing 0.5% BSA and subjected to flow cytometry analysis. A Cyan® Dako flow cytometer was used and data were analyzed via Summit® software version 4.3 (Beckmann Coulter, Krefeld, Germany). Signal intensity was calculated as the ratio of the mean fluorescence of the specific antibody and the isotype control antibody (specific fluorescence index, SFI). A SFI of 1.3 was arbitrarily defined as a significant surface expression.

Enzyme-linked immunosorbent assay

(forward 5′-AAGCTTACACTGTCCCTGCTGC-3′, reverse 5′-TGTGGAGGTGCCATCAATACCT-3′<sup>45</sup>, Microsynth), TGF-βRII (Lot no. 98554236, Qiagen).
Cell supernatants from LTC were collected at subconfluency, seeded at $2.6 \times 10^4$ cells/cm$^2$, and from GIC, seeded in suspensions of $0.6 \times 10^6$/ml, respectively, after the indicated time periods. For transfection experiments, LTC were seeded at higher densities between $6.6$ and $7.5 \times 10^4$ cells/cm$^2$. For LTC a 24 h period of serum deprivation was included prior the indicated time periods. Cellular debris was removed by centrifugation. Results of ELISA were expressed normalized to the cell number seeded with $2.6 \times 10^4$ cells/cm$^2$ (LTC) or $0.6 \times 10^6$/ml (GIC). For experiments with previous transfection, an additional correction for overall protein concentration in the supernatant was done for LTC. Since the B27 supplement of the medium perturbs protein measurement in the supernatant, cell counts were obtained for GIC cultures before harvesting the supernatants and results were normalized to the cell count of control-transfected cells.

**Luciferase reporter assay**

Transcriptional activity was measured by a dual luciferase reporter gene assay. Cells were co-transfected using Metafectene Pro transfection reagent (Biontex, Martinsried, Germany) for LTC and with electroporation (Neon transfection system, Invitrogen) for GIC with SBE-Luc plasmid$^{21}$ and renilla reniformis-CMV (pRL-CMV) control plasmid (Promega, Madison, WI) at a ratio of 7.5:1. Firefly/renilla luciferase activities were determined sequentially and normalized to Renilla activity. Results were further normalized to untreated control cells as indicated.

**Database interrogations**

Correlation analyses of genes were downloaded from the R2 microarray analysis and visualization platform using normalized Affymetrix gene expression data in The Cancer Genome Atlas (TCGA)-540 database (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi, available on 15 February 2014).
Statistical analysis

Data are commonly derived from experiments performed three times with similar results. If not otherwise indicated, results of representative experiments are shown. Statistical significance was assessed using two-sided unpaired Student’s t-test. Means, standard error of the mean (SEM) and correlation (r=Spearman’s coefficient) were calculated using the software of GraphPad Prism Version 5 (San Diego, CA). A p-value of p=0.05 was considered to be statistically significant.
Supplementary Figure 1. The induction of VEGF by TGF-β is independent of culture conditions

U87MG or ZH-161 cells were passaged in parallel in their native culture conditions or after 2 passages in GIC conditions (NB) for U87MG and LTC conditions (DM) for ZH-161. VEGF mRNA induction by TGF-β2 (10 ng/ml, 24 h) was assessed by RT-PCR (A) and protein release by ELISA (B, results adjusted to cell count performed after harvesting the supernatants).
Supplementary Table 1: Correlation analyses of gene expression data in the Tumor Glioblastoma - TCGA - 540 database:

Correlation analyses of genes were derived from normalized Affymetrix gene expression data in the TCGA - 540 database and downloaded from the R2 microarray analysis and visualization platform. The affymetrix code for the probeset is given in brackets.

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