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# TGF-β Increases Glioma-Initiating Cell Self-Renewal through the Induction of LIF in Human Glioblastoma

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## SUMMARY

**Glioma-initiating cells (GICs) are responsible for the initiation and recurrence of gliomas. Here, we identify a molecular mechanism that regulates the self-renewal capacity of patient-derived GICs. We show that TGF-β and LIF induce the self-renewal capacity and prevent the differentiation of GICs. TGF-β induces the self-renewal capacity of GICs, but not of normal human neuroprogenitors, through the Smad-dependent induction of LIF and the subsequent activation of the JAK-STAT pathway. The effect of TGF-β and LIF on GICs promotes oncogenesis in vivo. Some human gliomas express high levels of LIF that correlate with high expression of TGF-β2 and neuroprogenitor cell markers. Our results show that TGF-β and LIF have an essential role in the regulation of GICs in human glioblastoma.**

## INTRODUCTION

Glioma is the most frequent primary tumor of the brain and can be classified into four clinical grades on the basis of its histology and prognosis. Grade IV gliomas (glioblastoma multiforme, GBM) are highly malignant and usually refractory to radio- and chemotherapy. Despite progress in the understanding of the molecular mechanisms involved in the genesis and progression of glioma, prognosis and treatment of this tumor type continue to be dismal (Furnari et al., 2007; Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002). Recently, a subpopulation of tumor cells with stem cell-like properties has been identified in gliomas. This cell population, called cancer stem cells, glioma stem cells, brain tumor-initiating cells, or glioma-initiating cells (GICs), is considered to be responsible for the initiation, propagation, and recurrence of tumors, indicating that more effective therapies will result from approaches aimed at targeting the stem cell-like compartment of gliomas (Lee et al., 2006; Rich, 2007; Sanai et al., 2005; Singh et al., 2004a; Stiles and Rowitch, 2008; Vescovi et al., 2006). Still, little is understood regarding the molecular characteristics and regulatory mechanisms that control GIC biology.

GICs are characterized by their highly oncogenic potential, generating tumors that reproduce the characteristics of the original tumor, their self-renewal capacity, their multilineage differentiation properties, and their ability to generate detached spherical cellular structures (neurospheres) when cultured in serum-free medium. Several markers, most of them previously described for neuroprogenitor cells, have been reported to identify GICs. Specifically, a subpopulation of cells in human glioma

### SIGNIFICANCE

A defined cell population within the tumor mass, called glioma-initiating cells (GICs), is responsible for glioma initiation and recurrence and is a critical therapeutic target. The understanding of the molecular mechanisms involved in the regulation of this type of cell is crucial in order to be able to improve on treatments against glioma. We have identified TGF-β and LIF, which are highly expressed in malignant glioma, as regulators of the self-renewal capacity of patient-derived GICs. TGF-β increases GIC self-renewal through the induction of LIF and the JAK-STAT pathway. The induction of GIC self-renewal by TGF-β and LIF promotes oncogenesis. Our work identifies LIF as a therapeutic target against GICs and provides a molecular mechanism through which anti-TGF-β therapies might be successful against glioma.

tumors expressing the cell surface protein CD133 (prominin-1) has been described to be enriched for GICs (Bao et al., 2006; Galli et al., 2004; Piccirillo et al., 2006; Singh et al., 2003, 2004b). However, some gliomas contain CD133-negative cells that have the characteristics of GICs (Beier et al., 2007), indicating that more refined markers to identify GICs are still needed.

Leukemia inhibitory factor (LIF) is a cytokine that has been extensively studied as an inducer of mouse embryonic stem cell self-renewal (Niwa et al., 1998; Williams et al., 1988). Moreover, although LIF can induce astrocytic differentiation in late-stage mouse neuroprogenitors (Bonni et al., 1997; Rajan and McKay, 1998), LIF also promotes the self-renewal capacity of human and mouse early neuroprogenitor cells (Bauer and Patterson, 2006; Molne et al., 2000; Wright et al., 2003). Interestingly, some investigators add recombinant LIF to neural stem cell culture media to improve the culture yield of neuroprogenitor cells and GICs (Beier et al., 2007; Carpenter et al., 1999; Hemmati et al., 2003; Singh et al., 2003; Yuan et al., 2004). LIF signals through the heterodimeric glycoprotein 130 (gp130)/LIF receptor (LIFR) complex activating the JAK-STAT pathway (Auernhammer and Melmed, 2000; Taga and Kishimoto, 1997).

TGF-β family members have been reported to be involved in embryonic stem cell self-renewal (James et al., 2005; Moses and Serra, 1996; Xu et al., 2008). TGF-β is a pleiotropic cytokine that binds and activates a membrane receptor serine/threonine kinase complex. Upon TGF-β binding, the receptor complex phosphorylates the transcription factors Smad2 and Smad3, which then bind to Smad4 and accumulate in the nucleus, where they regulate transcription (Massagué 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 (Massagué, 2008; Roberts and Wakefield, 2003; Seoane, 2006) and is considered a therapeutic target (Akhurst, 2006; Arteaga, 2006; Seoane, 2008; Yingling et al., 2004). Recent work is beginning to uncover the oncogenic function of TGF-β in glioma (Rich, 2003). We found that TGF-β is highly active in high-grade glioma and that elevated TGF-β activity confers poor prognosis in glioma patients. TGF-β induces cell proliferation and tumor progression through the induction of PDGF-B in human gliomas with an unmethylated *PDGF-B* gene (Bruna et al., 2007).

In this study, we aimed to understand the molecular mechanisms underlying the effect of the TGF-β pathway on the regulation of GIC self-renewal.

## RESULTS

### TGF-β Induces Patient-Derived GIC Self-Renewal

In order to study the effect of TGF-β on the self-renewal capacity of GICs, we obtained cells from surgically resected human GBM samples (see Table S1 available online). From each tumor specimen, we generated primary cultures of tumor cells (PCTCs) in the presence of serum and, in parallel, cultured tumor cells in serum-free medium in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF). Cells cultured in the EGF- and FGF-supplemented serum-free medium readily generated nonadherent, multicellular spheres (neurospheres) as described previously (Galli et al., 2004; Günther et al., 2007; Lee et al., 2006; Singh et al., 2003) (Figure 1A). Neurospheres originating from the tumor specimens expressed high levels of the neuroprogenitor cell markers Musashi-1, Sox2, and nestin (Figure 1B) and underwent multilineage differentiation acquiring the expression of GFAP (astrocytic marker), Tuj1 (neuronal marker), and O4 (oligodendrocytic marker) when cultured in the presence of serum (Figure S1). Moreover, tumor-derived neurospheres were highly oncogenic as compared to PCTCs. We performed in vivo limiting dilution assays implanting decreasing amounts of cells from neurospheres and from PCTCs in the brains of immunocompromised mice. Tumor growth was assessed by magnetic resonance imaging (MRI). Neurosphere cells generated invasive and infiltrative tumors detectable by MRI. In contrast, mice injected with $1 \times 10^5$ PCTCs did not develop tumors during the studied time frame (Figure 1C). Tumors generated in mice were confirmed to have histopathological characteristics of GBM and had the same levels and expression pattern of neuroprogenitor markers (Musashi-1, Sox2, and nestin) as the original tumors (Figure S2). Thus, neurospheres generated from human GBM specimens expressed neuroprogenitor cell markers, showed multilineage differentiation potential, and were highly oncogenic, generating tumors with the same characteristics as the original human tumors. All of these properties indicated that neurospheres obtained from patient-derived GBMs are enriched for GICs.

We decided to assess the effect of TGF-β on GIC self-renewal following a well-described protocol based on the ability of GICs to generate neurospheres (Reynolds and Weiss, 1996; Seaberg and van der Kooy, 2002). Patient-derived neurospheres from three different glioma patients were dissociated into single cells and treated with TGF-β or left untreated for 7 days in the absence of growth factors, and the newly formed neurospheres and the total number of cells were then counted. Treatment with TGF-β enhanced the number of neurospheres and increased the total number of cells. The effect of TGF-β was blocked when a TGF-β receptor I (TβRI) inhibitor was added concomitantly with TGF-β (Figures 1D–1F). Moreover, there was a dose-dependent effect of TGF-β on the number of neurospheres and cells, and the effect of TGF-β on self-renewal was observed even when cells were plated at very low density (Figure S3). These results showed that the TGF-β pathway increases GIC self-renewal.

### TGF-β Induces LIF Expression in Human GBM Cells

We decided to discern the molecular mechanisms responsible for the effect of TGF-β on GICs. We looked for TGF-β gene responses in GBM cells that could be involved in the regulation of GIC self-renewal. In our previous work (Bruna et al., 2007), we performed a transcriptomic analysis of the U373MG glioma cell line treated with TGF-β and/or a TβRI inhibitor. *LIF* was among the 63 TGF-β gene responses in U373MG cells that were dependent on TβRI activity. The LIF-LIFR/gp130-JAK-STAT signaling pathway has been implicated in stem cell self-renewal in both embryonic stem cells (Niwa et al., 1998; Williams et al., 1988) and neuroprogenitor cells (Bauer and Patterson, 2006; Molne et al., 2000; Wright et al., 2003), and we hypothesized that LIF could be involved in GIC self-renewal and

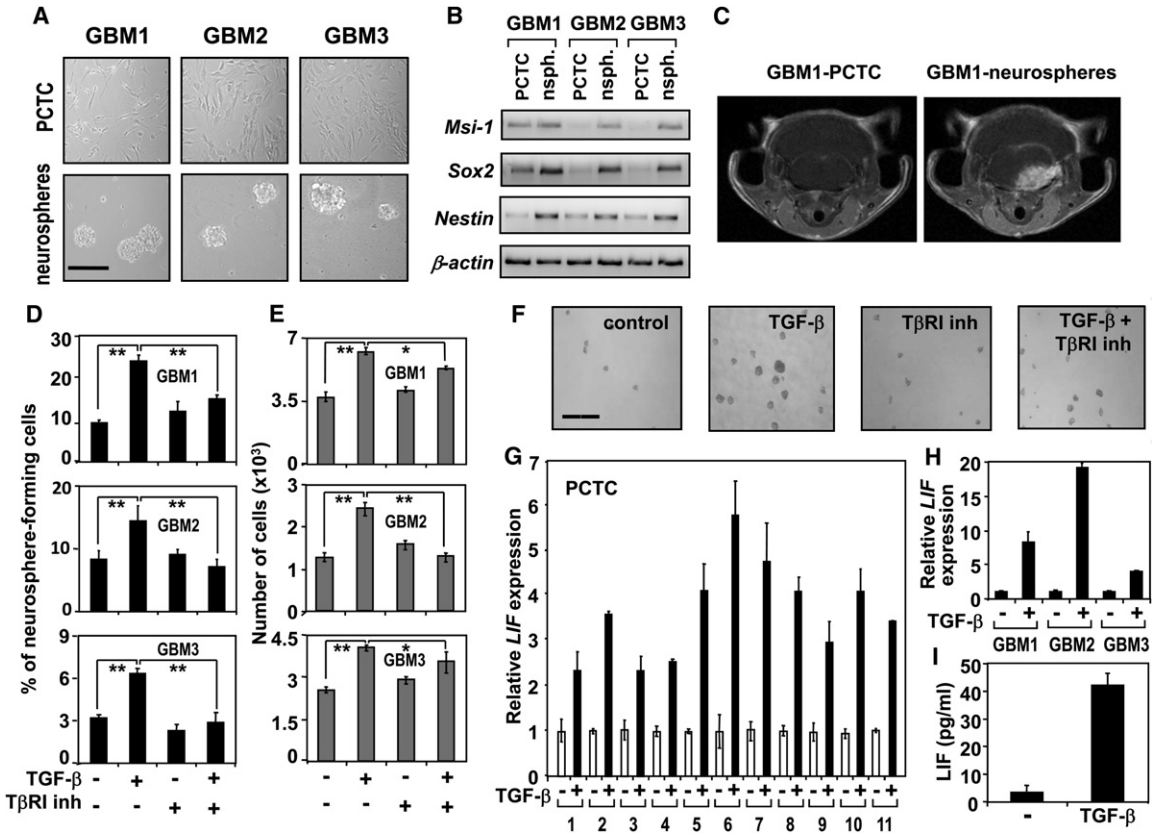


**Figure 1. Effect of TGF-β on Patient-Derived Glioma-Initiating Cell Self-Renewal**

(A) Representative images of primary cultures of tumor cells (PCTCs) and glioblastoma multiforme (GBM) neurospheres generated from specimens of three different GBM patients. Scale bar = 300 μm.

(B) *Musashi-1* (*Msi-1*), *Sox2*, *Nestin*, and β-*actin* levels were determined by RT-PCR analysis of PCTCs and neurospheres from three human GBM samples.

(C) Representative MRI images of brains of immunocompromised mice inoculated with $1 \times 10^5$ PCTCs or 1000 neurosphere cells from GBM1.

(D and E) Cells from the indicated GBM neurospheres were incubated in the absence of growth factors with 100 pM TGF-β1 and/or 2 μM TβRI inhibitor (TβRI inh) for 7 days, and the percentage of neurosphere-forming cells (D) and the total number of cells (E) were determined. *$p < 0.05$; **$p < 0.005$.

(F) Representative images of GBM1 neurospheres treated as indicated in (D and E). Scale bar = 400 μm.

(G) PCTCs from 11 human GBM samples (GBM1–11) were treated with 100 pM TGF-β1 or left untreated for 3 hr, and the levels of *LIF* expression were determined by qRT-PCR. β-*actin* was used as an internal normalization control.

(H) Cells from GBM neurospheres were treated with 100 pM TGF-β1 or left untreated for 3 hr, and the levels of *LIF* expression were determined by qRT-PCR.

(I) Secreted LIF protein levels were determined by ELISA in GBM1 neurospheres after 48 hr treatment with 100 pM TGF-β1.

Error bars represent mean ± standard deviation (SD).

mediating the effect of TGF-β on GICs. We first determined whether the TGF-β-mediated induction of the *LIF* transcript was observed in patient-derived tumor cells. A panel of PCTCs derived from 11 different human GBMs was treated with TGF-β for 3 hr, and *LIF* mRNA levels were determined. TGF-β induced LIF in all PCTCs assayed (Figure 1G). These results indicated that the induction of LIF by TGF-β is a common phenomenon that takes place in most human GBMs. In order to assess the effect of LIF on PCTCs, we treated PCTCs from GBMs 1, 2, and 3 with LIF. Cell proliferation was not affected by treatment with LIF either when cells were counted after 7 days of treatment or by BrdU incorporation assays (Figure S4). In patient-derived neurospheres, TGF-β was able to induce the *LIF* transcript (Figure 1H), and this effect was dependent on TβRI activity since LIF induction by TGF-β was blocked by the presence of a TβRI inhibitor (Figure S5). Three members of the TGF-β family (TGF-β1, TGF-β2, and TGF-β3) were able to induce LIF in patient-derived neurospheres (Figure S5), and, as expected, the induction of the *LIF* transcript by TGF-β resulted in an increase in LIF protein secretion as measured by ELISA in neurosphere-conditioned medium (Figure 1I).

## TGF-β Induces LIF Expression via an Activated Smad Complex Binding to the *LIF* Promoter

To study the transcriptional regulation of *LIF* by TGF-β, we cloned the human *LIF* promoter into the pGL2 basic reporter construct. TGF-β was able to transactivate reporter constructs containing the −634/+32 and −276/+32 regions of the *LIF* promoter. The −73/+32 fragment of the *LIF* promoter lost the transcriptional response to TGF-β, indicating that the TGF-β-responsive element was included in the −276/−73 region (Figures 2A and 2B). This region contains a single Smad-binding element (SBE,

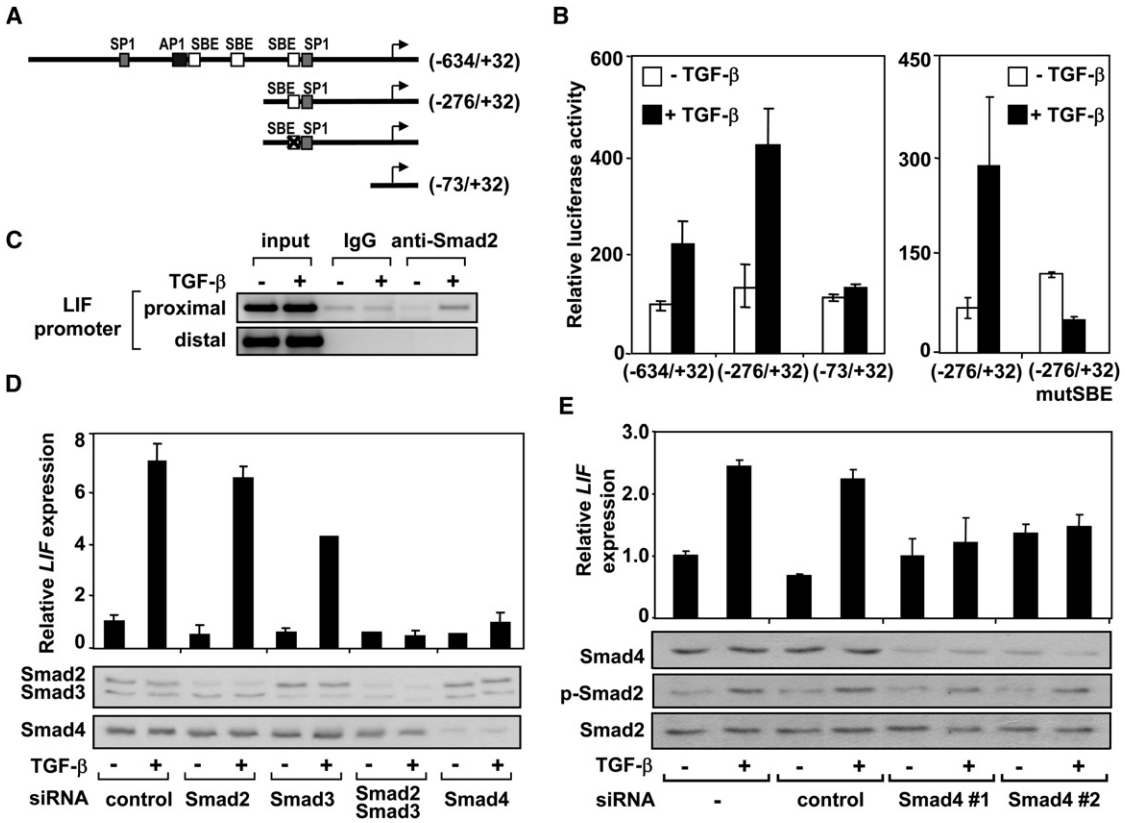


**Figure 2. TGF-β Induces *LIF* Transcription through an Activated Smad Complex**

(A) Scheme of *LIF* luciferase reporter constructs.

(B) A172 glioma cells were transfected with the −634/+32, −276/+32, or −276/+32 mutated Smad-binding element (mutSBE) or −73/+32 *LIF* luciferase reporter constructs. Cells were then treated with 100 pM TGF-β1 for 20 hr, and luciferase activity was analyzed.

(C) U373MG cells were treated with 100 pM TGF-β1 for 3 hr, and chromatin immunoprecipitation (ChIP) assays were performed with the indicated antibodies and the indicated PCR primers.

(D and E) *LIF* mRNA levels were determined by qRT-PCR in U373MG cells (D) or GBM1 neurospheres (E) treated with 100 pM TGF-β1 for 3 hr after siRNA-mediated knockdown of the indicated Smad family members. Immunoblotting was performed with the indicated antibodies against Smads.

Error bars represent mean ± SD.

5′-GTCT-3′) close to an SP1 binding site (Figure 2A). We mutated the SBE and observed that the response to TGF-β was blunted (Figure 2B), indicating that an activated Smad complex binds to the proximal SBE in the *LIF* promoter to induce transcription. We performed chromatin immunoprecipitation (ChIP) assays and observed that endogenous Smad2 bound to the proximal region of the *LIF* promoter and not to a distal region 4 kb upstream of the transcription start site in TGF-β-treated cells (Figure 2C). Finally, to prove that Smads are involved in the induction of LIF expression by TGF-β, we knocked down Smad2, Smad3, both Smad2 and 3, and Smad4 using two independent siRNAs against each of the Smads. LIF induction by TGF-β was decreased when Smad4 or both Smad2 and Smad3 were downregulated, indicating that an activated Smad complex is required for the *LIF* transcriptional response to TGF-β (Figure 2D; Figure S6). Smad2 and Smad3 are redundant in this process since knockdown of each Smad in isolation did not significantly affect TGF-β-induced LIF levels (Figure 2D; Figure S6). As expected, the induction of LIF by TGF-β in patient-derived neurospheres was also Smad dependent. Knockdown of Smad4 with two independent siRNAs in human GBM neurospheres abolished the LIF response to TGF-β (Figure 2E).

## TGF-β Induces the JAK-STAT Pathway through the Induction of LIF in Patient-Derived Neurospheres

In order to discern whether the LIF signaling pathway is functional in GBM neurospheres, we treated the neurospheres with recombinant LIF and determined the phosphorylation levels of the downstream substrate of the LIF receptor complex, STAT3. Recombinant LIF induced a rapid phosphorylation of STAT3 at Tyr705 and did not increase the phosphorylation of STAT3 at Ser727. The latter residue was already phosphorylated in untreated cells (Figure S7). Moreover, the induction of p-STAT3 at Tyr705 (hereafter, p-STAT3) by LIF was prevented by the presence of a specific pharmacological inhibitor of JAK, tetracyclic pyridone 6 (P6) (Pedranzini et al., 2006; Thompson et al., 2002) (Figure 3A). Importantly, TGF-β induced STAT3 phosphorylation in GBM neurospheres, and the TβRI inhibitor prevented that effect (Figure 3B). We decided to assess whether LIF was mediating the induction of p-STAT3 by TGF-β. For this purpose, we used a neutralizing antibody against LIF to

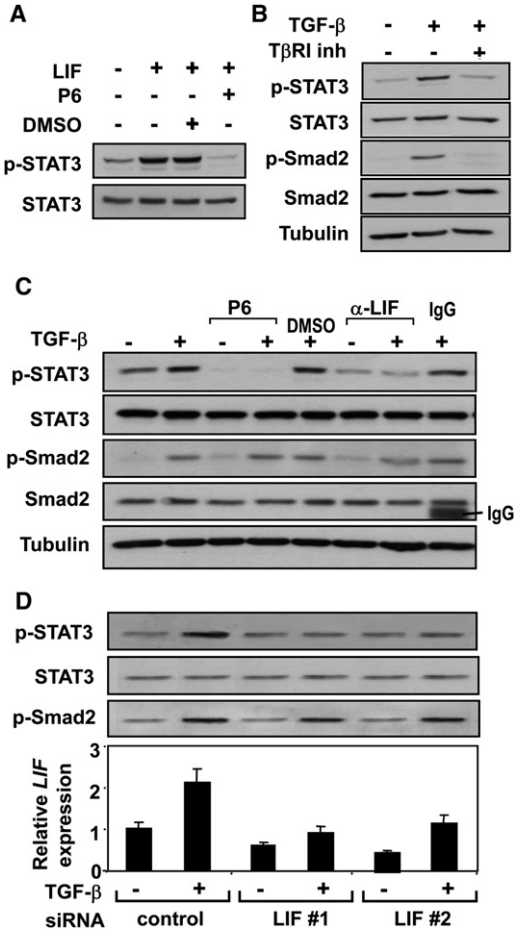


**Figure 3. TGF-β Induces the LIF-JAK-STAT Pathway in Patient-Derived GBM Neurospheres**

(A) GBM1 neurospheres were treated with 20 ng/ml LIF and/or 0.5 μM P6 (a specific pharmacological inhibitor of JAK) for 15 min, and the levels of p-STAT3 and STAT3 were determined by immunoblotting.

(B) GBM1 neurospheres were treated with 100 pM TGF-β1 and/or 2 μM TβRI inhibitor for 4 hr in the absence of EGF and FGF, and levels of p-STAT3, STAT3, p-Smad2, Smad2, and α-tubulin were determined by immunoblotting.

(C) GBM1 neurospheres were treated with 100 pM TGF-β1, 0.5 μM P6, or 10 μg/ml anti-LIF neutralizing antibody for 4 hr as in (B), and the levels of p-STAT3, STAT3, p-Smad2, Smad2, and α-tubulin were determined by immunoblotting.

(D) GBM1 neurospheres were transfected with two independent siRNAs against LIF and treated with 100 pM TGF-β1 or left untreated for 4 hr. p-STAT3, STAT3, and p-Smad2 levels were determined by immunoblotting. qRT-PCR analysis was performed to determine *LIF* levels, with β-*actin* used as an internal normalization control. Error bars represent mean ± SD.

specifically block the effect of secreted LIF in TGF-β-treated cells. The presence of the LIF-neutralizing antibody decreased the induction of p-STAT3 by TGF-β. Moreover, we observed that the induction of p-STAT3 by TGF-β was dependent on JAK activity since p-STAT3 levels in TGF-β-treated cells were repressed by treatment with the JAK inhibitor P6 (Figure 3C). Additionally, we decided to knock down LIF expression using RNA interference in order to confirm that LIF was mediating the induction of p-STAT3 by TGF-β. GBM neurospheres were transfected with two independent siRNAs against LIF, and the induction of p-STAT3 by TGF-β was observed to decrease (Figure 3D). Altogether, these results indicated that TGF-β activates the JAK-STAT pathway in patient-derived neurospheres via the induction of LIF secretion acting through an autocrine/paracrine loop.

## LIF Mediates the Induction of GIC Self-Renewal by TGF-β

We decided to evaluate whether LIF and the JAK-STAT pathway mediate the increase of GIC self-renewal by TGF-β. For this purpose, we used LIF-neutralizing antibodies, the pharmacological JAK inhibitor P6, and siRNAs against LIF to specifically block the effect of secreted LIF in cells treated with TGF-β. Neurospheres were dissociated into single cells and treated with recombinant LIF, TGF-β, anti-LIF antibody, and/or P6. The newly formed neurospheres and the total number of cells were counted. Recombinant LIF increased the amount and size of the newly formed neurospheres as well as the total number of cells, indicating that LIF induces GIC self-renewal (Figures 4A–4C). Treatment with the LIF-neutralizing antibody decreased the induction of GIC self-renewal by TGF-β. Moreover, P6 also repressed the effect of TGF-β on GIC self-renewal, indicating that the TGF-β effect on self-renewal was dependent on JAK activity (Figures 4A and 4B). TGF-β and LIF did not affect BrdU incorporation of treated neurospheres (Figure S8). We next confirmed that the effect of TGF-β on GIC self-renewal is mediated by LIF using RNA interference. We observed that knockdown of LIF using two independent siRNAs previously shown to block p-STAT3 induction by TGF-β (see Figure 3D) also prevented the induction of GIC self-renewal by TGF-β (Figure 4D).

We then performed a different type of experiment to assess GIC self-renewal. Neurospheres were dissociated into single cells and treated with LIF, TGF-β, anti-LIF, and/or P6 as indicated for 7 days. The newly formed neurospheres were then dissociated, the same number of isolated cells was plated into 96-well plates in the absence of treatment, and the number of cells capable of generating neurospheres was determined. This process was performed for over three passages. Following this protocol, we observed that TGF-β and LIF increased the number of neurosphere-forming cells and hence the self-renewal capacity of GICs, and that the TGF-β effect was dependent on the induction of LIF and the JAK-STAT pathway (Figure S9).

To further validate the effect of TGF-β and LIF on GIC self-renewal, we performed soft agar assays. GBM neurospheres were dissociated, and the same number of cells was treated with TGF-β or LIF for 7 days. After treatment, dissociated neurospheres were plated in the absence of treatment in soft agar, and the number of generated colonies was determined. Cells previously treated with TGF-β or LIF generated an increased number of colonies in soft agar suspension cultures as compared to untreated cells (Figure 4E; Figure S10).

Next, we decided to analyze the levels of CD133-positive cells in the GBM neurospheres. Our three patient-derived neurospheres had a percentage of CD133-positive cells ranging from 3% to 20% (Figure 4F; Figure S11). Treatment of the neurospheres with TGF-β or LIF for 7 days doubled the percentage of CD133-positive cells present in GBM1 neurospheres (Figure 4F).

Overall, our data indicate that TGF-β induces the self-renewal capacity of patient-derived GICs through the LIF-JAK-STAT pathway.

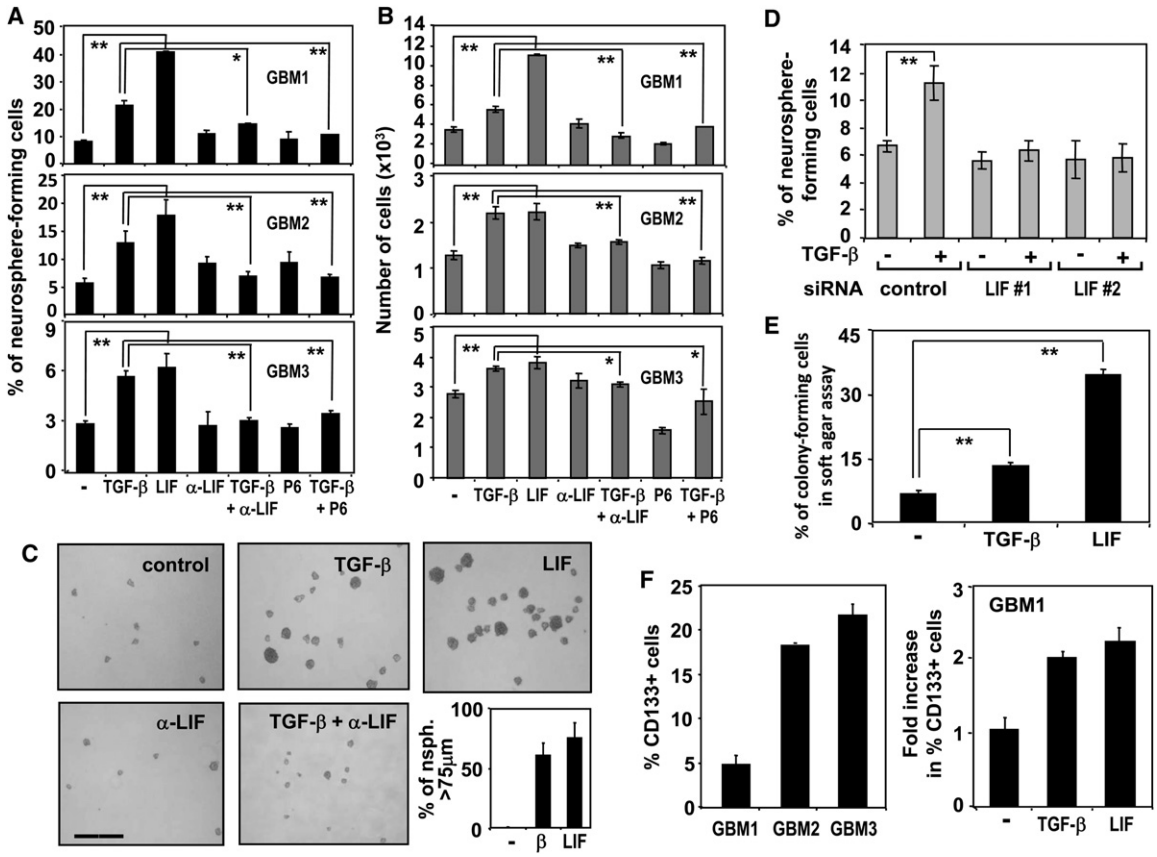


**Figure 4. LIF Mediates the Increase of Glioma-Initiating Cell Self-Renewal by TGF-β**

(A and B) Cells from GBM1, GBM2, or GBM3 neurospheres were treated with 100 pM TGF-β1, 20 ng/ml LIF, and/or 10 μg/ml anti-LIF neutralizing antibody and 0.5 μM P6 in the absence of EGF and FGF, and the number of newly formed neurospheres (A) and the total number of cells (B) were determined. *$p < 0.05$; **$p < 0.005$.

(C) Representative images of GBM1 neurospheres treated as indicated in (A and B). Scale bar = 400 μm. Lower right: the percentage of neurospheres larger than 75 μm was determined.

(D) GBM1 neurospheres were transfected with two independent siRNAs against LIF as in Figure 3D and incubated in the absence of growth factors with 100 pM TGF-β1 for 7 days, and the percentage of neurosphere-forming cells was determined. **$p < 0.005$.

(E) Cells from GBM1 neurospheres were treated with 100 pM TGF-β1 or 20 ng/ml LIF for 7 days and plated in soft agar. The number of colonies was determined after staining with 0.01% crystal violet. **$p < 0.005$.

(F) The percentage of CD133-positive cells from the indicated GBM neurosphere cultures was determined by flow cytometry. Cells from GBM1 neurospheres were incubated with 100 pM TGF-β1 or 20 ng/ml LIF for 7 days, and the percentage of CD133-positive cells was determined.

Error bars represent mean ± SD.

## TGF-β Prevents GIC Differentiation via LIF

GBM-derived neurospheres tend to differentiate either by culturing in the presence of serum or, alternatively, by culturing in the absence of growth factors. During the differentiation process, cells tend to acquire markers of differentiation (GFAP, S100β, Tuj1, O4, Olig2), lose the expression of neuroprogenitor markers (Musashi-1, Sox2, nestin), and become attached to the culture plate, losing the spherical shape. We decided to assess the effect of TGF-β and LIF on differentiation following the two mentioned differentiation protocols.

Neurospheres cultured in the presence of serum and TGF-β or LIF suffered a delay in differentiation. A time-course experiment showed that cells treated with TGF-β or LIF acquired the markers of differentiation GFAP, Tuj1, and Olig2 (Figure 5A) and S100β, Tuj1, and O4 (Figure 5B) later than untreated cells. In addition, TGF-β- or LIF-treated neurospheres maintained the expression of neuroprogenitor markers (Musashi-1, Sox2, and nestin) and became less attached to the culture plate, maintaining the spherical morphology (Figure 5B). Moreover, when neurospheres were induced to differentiate in the absence of EGF and FGF for 7 days, TGF-β and LIF also retained the expression of Musashi-1, Sox2, and nestin as detected by immunocytochemical assays and as quantified by qRT-PCR, and the cells maintained the spherical, detached morphology (Figure S12). Overall, our results indicated that TGF-β and LIF not only regulate GIC self-renewal but also prevent the differentiation of GICs.

## Effect of TGF-β and LIF on Normal Human Neuroprogenitors

Our data indicated that TGF-β and LIF regulate GIC self-renewal and differentiation. Still, we wanted to address whether this effect is specific to tumor cells or is also present in normal

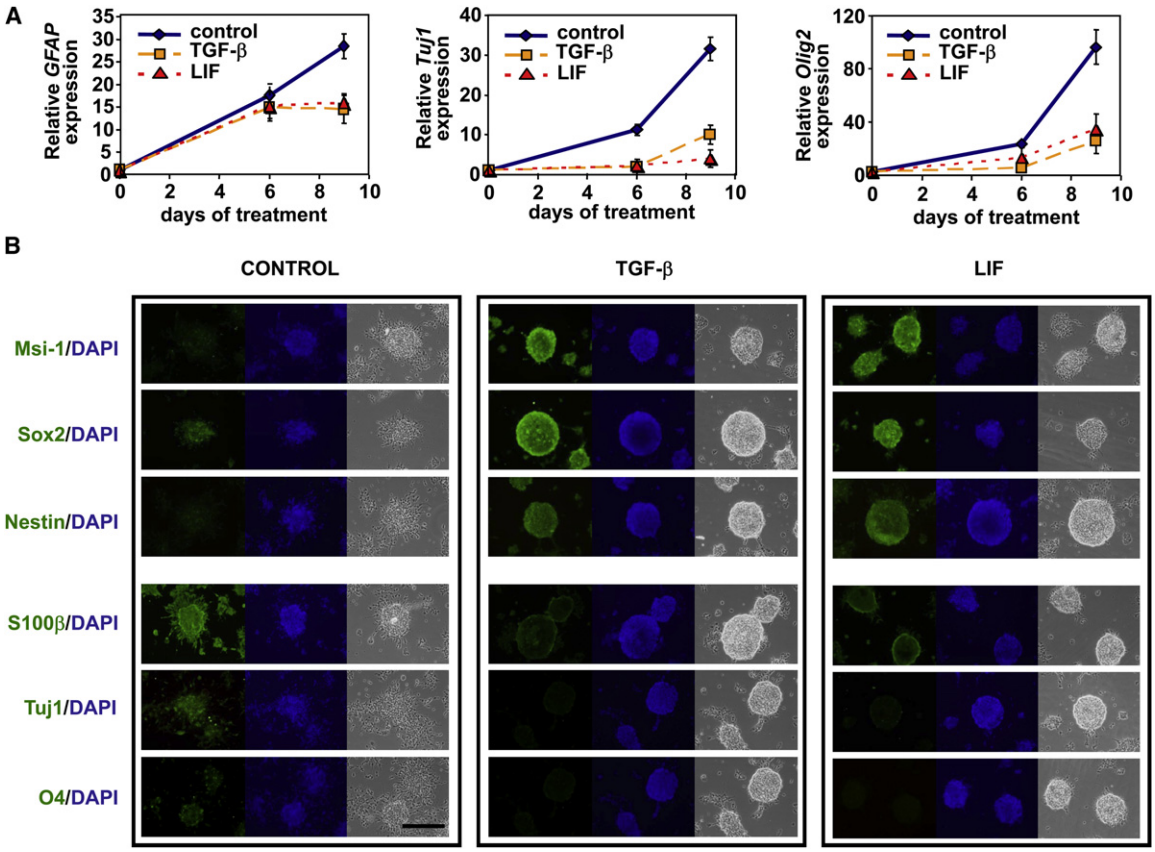


**Figure 5. TGF-β and LIF Prevent Differentiation of GBM Neurospheres**

(A) qRT-PCR analysis was performed to determine the mRNA levels of the differentiation markers *GFAP*, *Tuj1*, and *Olig2* in GBM1 neurospheres after 7 days of the indicated treatments in the presence of 0.05% FBS. *GAPDH* mRNA levels were used as an internal normalization control. Error bars represent mean ± SD. (B) Immunocytochemistry for the indicated proteins was performed in GBM1-derived neurospheres treated with 100 pM TGF-β1 or 20 ng/ml LIF for 7 days in the presence of 0.05% FBS on poly-L-lysine-coated coverslips. Nuclei were counterstained with DAPI. Scale bar = 300 μm.

neuroprogenitor cells. To answer this question, we obtained neuroprogenitor cells from five different human fetal cerebral cortex samples (8–16 weeks postconception). As described previously, human neuroprogenitors generated neurospheres when grown in serum-free medium supplemented with EGF and FGF, and these neurospheres expressed Musashi-1, Sox2, and nestin similarly to GBM neurospheres (Figure 6A). First, we determined whether TGF-β induced LIF in normal human neuroprogenitors. Normal neurospheres obtained from the five human fetal cerebral cortex samples did not induce LIF in response to TGF-β (TGF-β1, TGF-β2, or TGF-β3) to the same extent as GBM neurospheres (Figures 6B and 6C). Moreover, TGF-β did not induce LIF in mouse neuroprogenitors obtained from mouse embryos or from the subventricular zone of adult mice (data not shown). We analyzed the levels of TβRI, TβRII, Smad2, Smad3, Smad4, LIFR, and gp130 in GBM PCTCs, GBM neurospheres, and three of the normal neuroprogenitor neurosphere samples in order to determine the levels of the mediators of TGF-β and LIF signaling pathways and to assess whether the lack of one of the mediators of TGF-β was responsible for the inability of TGF-β to induce LIF. The levels of expression of the analyzed factors varied among samples, but none was absent in normal neuroprogenitors (Figure S13). Moreover, TGF-β induced p-Smad2 in normal neuroprogenitors at the same level as in GBM neurospheres, indicating that the difference between the two cell types resides downstream of p-Smad2 (Figure 6D). In addition, Smad7 expression, a well-defined TGF-β response, was induced by TGF-β in both neuroprogenitors and GBM neurospheres (Figure 6B). Thus, the observed difference between GBM neurospheres and neuroprogenitors in the LIF response to TGF-β resides downstream of p-Smad2 and is specific to the LIF transcriptional response.

TGF-β did not increase the self-renewal capacity of normal neuroprogenitors, and the number of neuroprogenitor neurospheres was not increased by treatment with TGF-β (Figure 6E). LIF, on the other hand, did increase the number and size of newly formed neurospheres as well as the total number of cells (Figure 6E), in agreement with previous reports (Bauer and Patterson, 2006; Wright et al., 2003). Thus, LIF has the same effect on self-renewal in normal and GBM neurospheres. In contrast, there is a difference in the TGF-β effect on the self-renewal capacity of normal and tumoral neurospheres that coincides with the inability of TGF-β to induce LIF in normal neuroprogenitors to the same level as in GBM neurospheres.

Next, we evaluated the effect of TGF-β and LIF on the capacity of normal human neuroprogenitors to differentiate in response to

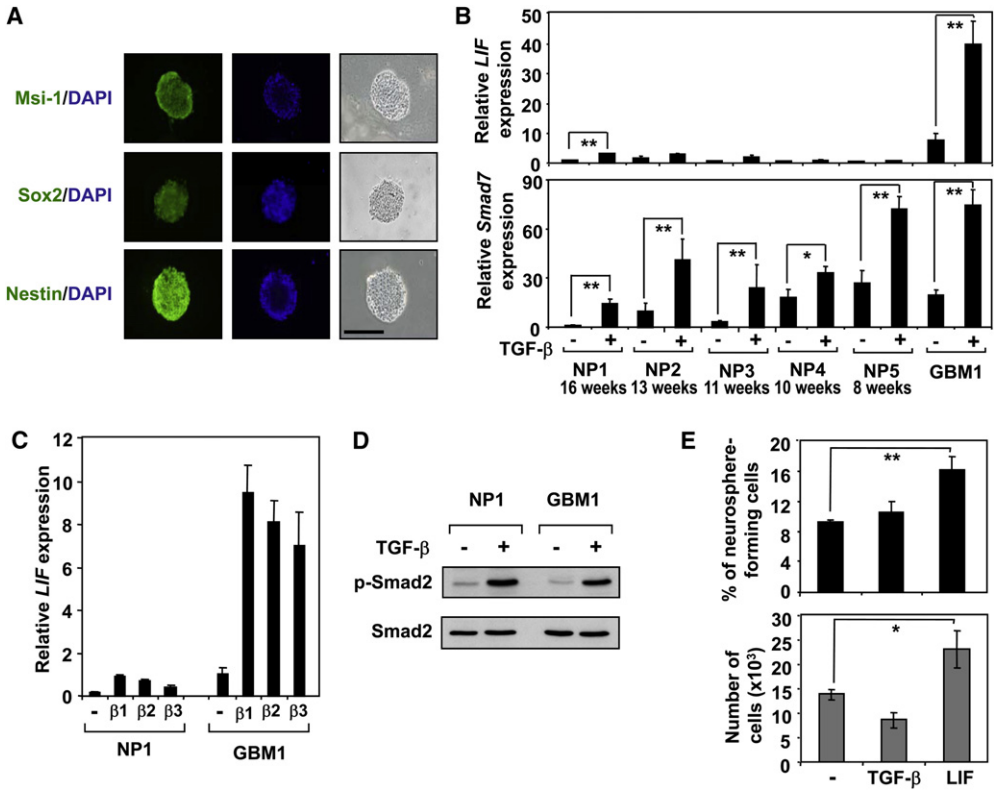


**Figure 6. TGF-β Effect on the Self-Renewal Capacity of Normal Human Neuroprogenitors**

(A) Immunocytochemistry for the indicated neuroprogenitor markers was performed in human neuroprogenitor neurospheres. Nuclei were counterstained with DAPI. Scale bar = 200 μm.

(B) Neurospheres from GBM1 and from neuroprogenitors isolated from samples of five different fetal cerebral cortices from animals 16 to 8 weeks postconception (*NP1–NP5*) were incubated with 100 pM TGF-β1 for 3 hr. *LIF* and *Smad7* mRNA levels were determined by qRT-PCR, with *GAPDH* used as an internal normalization control. *$p < 0.05$; **$p < 0.005$.

(C) Neurospheres from GBM1 and from normal human neuroprogenitors were incubated with 100 pM concentrations of the indicated TGF-β family members for 3 hr, and *LIF* mRNA levels were determined.

(D) p-Smad2 and Smad2 levels were determined by immunoblotting in GBM1 neurospheres and NP1 neurospheres after treatment with TGF-β for 3 hr.

(E) NP1 cells were incubated in the absence of growth factors with 100 pM TGF-β1 or 20 ng/ml LIF for 7 days, and the percentage of neurosphere-forming cells and the total number of cells were determined. **$p < 0.005$.

Error bars represent mean ± SD.

treatment with serum. As in GBM neurospheres, serum induced the attachment and loss of spherical morphology of neurospheres, decreased the expression of neuroprogenitor markers (Musashi-1, Sox2, and nestin), and promoted the appearance of differentiation markers (S100β, Tuj1, and O4) (Figure S14). LIF delayed the differentiation process of normal neuroprogenitors, maintaining the detached spherical morphology and expression of neuroprogenitor markers and preventing the expression of differentiation markers. In contrast, TGF-β did not have a major effect on the differentiation process (Figure S14).

## LIF Expression in Human Gliomas Correlates with TGF-β2 and Neuroprogenitor Markers

In order to evaluate whether LIF is expressed in human gliomas, we analyzed the levels of LIF in a panel of 43 glioma samples, including the samples that originated GBM1 (patient #40), GBM2 (patient #41), and GBM3 (patient #42) neurospheres. We observed that LIF was expressed in 20 and highly expressed in 7 of the 43 gliomas (Figure 7A), indicating that a large proportion of human gliomas express LIF. Since LIF is induced by TGF-β and since we found in our previous work that TGF-β2 is mainly responsible for the high TGF-β activity observed in gliomas (Bruna et al., 2007), we assessed whether TGF-β2 is implicated in LIF expression. Indeed, LIF levels correlated with TGF-β2 in our panel of gliomas, further supporting that TGF-β2 is responsible for the induction of LIF in human glioma (Figures 7A and 7B). If LIF promotes GIC self-renewal, the pool of cells expressing neuroprogenitor markers should be enriched in tumors expressing high levels of LIF. To address this hypothesis, we compared the levels of LIF with the expression of neuroprogenitor markers. LIF levels correlated with the expression of Musashi-1 and nestin, but not Sox2 (Figures 7A and 7B). The correlation was mainly based on some gliomas in which the expression of LIF, TGF-β2, and neuroprogenitor markers was high, and hence the relationship between LIF and TGF-β2, Musashi-1, and nestin might not be present in the majority of tumors. Our data indicate that in certain tumors with high levels of TGF-β2, LIF promotes GIC self-renewal,

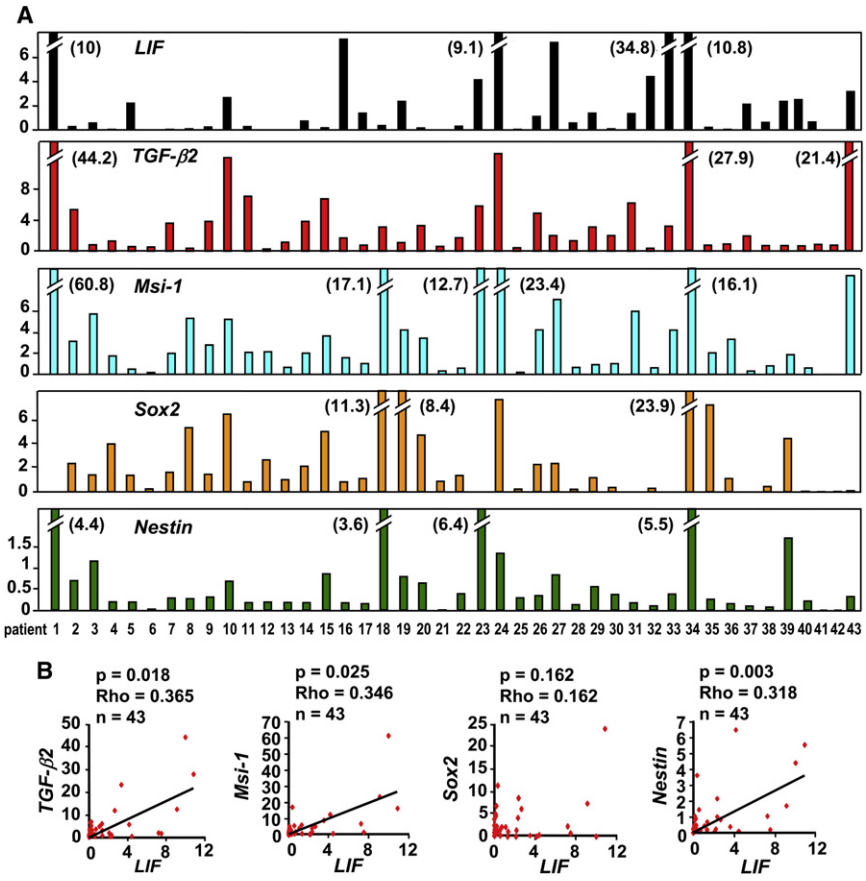


**Figure 7. LIF Expression in Human Glioma Tumors**

(A) *LIF*, *TGF-β2*, *Musashi-1* (*Msi-1*), *Sox2*, and *Nestin* transcript levels were determined by qRT-PCR analysis of 43 human glioma patient-derived tissue samples. *18S* RNA levels were used as an internal normalization control. GBM1, GBM2, GBM3, and GBM4 in other figures correspond to patients #40, #41, #42, and #43, respectively.

(B) Correlations between *LIF* and *TGF-β2*, *Msi-1*, *Sox2*, or *Nestin*. Spearman's rank correlation coefficient (Rho) with two-tailed significance is shown.

increasing the pool of cells expressing neuroprogenitor markers present in the tumor mass.

## TGF-β and LIF Induce the Oncogenic Potential of GBM Neurospheres

At this point, we decided to address whether the observed effect of TGF-β and LIF on GIC self-renewal could translate into an increase in the capacity of GBM neurospheres to initiate tumors. To address this possibility, we followed three different approaches. In a first experiment, we treated dissociated GBM neurospheres with TGF-β or LIF for 14 days. Cells from the newly formed neurospheres were orthotopically inoculated in the brain of immunocompromised mice. Neurospheres that were pretreated with TGF-β or LIF generated tumors earlier than untreated cells, and mice inoculated with neurospheres pretreated with TGF-β or LIF exhibited significantly decreased survival compared to mice inoculated with untreated neurospheres (Figure 8A). Thus, GBM neurospheres previously treated with TGF-β and LIF were more oncogenic, confirming that they were enriched in GICs.

Next, we decided to perform a loss-of-function experiment. In order to detect a physiological effect of the attenuation of the TGF-β and LIF pathways on GICs, we treated GBM neurospheres cultured at a high density (conditions where the endogenous basal TGF-β and JAK pathways are functional due to autocrine signaling) with TβRI inhibitor and JAK inhibitor. GBM neurospheres expressed and secreted functional TGF-β (Figure S15), and, when cultured at high density (100 cells/μl), GBM neurospheres were exposed to autocrine TGF-β (Figure S15). We also obtained neurospheres from a tumor that expressed high levels of TGF-β and LIF, GBM4 (patient #43) (see Figure 7). GBM4 neurospheres were as sensitive to TGF-β and LIF as GBM1, 2, and 3 neurospheres (Figure S16), and, although they were generated from a tumor with high levels of TGF-β and LIF, GBM4 neurospheres were exposed to a level of autocrine TGF-β activity similar to the rest of the GBM neurospheres (Figure S15). In all cases, treatment of GBM neurospheres cultured at high density with TβRI inhibitor and JAK inhibitor repressed the GIC self-renewal capacity (Figure 8B). Moreover, mice that were intracerebrally inoculated with GBM4 neurospheres that were previously treated with TβRI inhibitor and JAK inhibitor exhibited significantly prolonged survival (Figure 8C). These results indicated that inhibition of the TGF-β and JAK-STAT pathways decreased the self-renewal and tumorigenic potential of GICs.

In a third approach, we overexpressed TGF-β, a constitutively active version of TβRI (TβRI-CA), and LIF in GBM1 neurospheres through the infection of cells with lentiviral vectors. Lenti-TGF-β- and lenti-TβRI-CA-infected GBM neurospheres expressed high levels of TGF-β and TβRI-CA, respectively, and both expressed high levels of p-Smad2, LIF, and p-STAT3 due to the activation of the TGF-β pathway (Figure S17). Lenti-LIF-infected GBM neurospheres expressed LIF and had higher levels of p-STAT3 (Figure S17). As expected, neurospheres overexpressing TGF-β, TβRI-CA, and LIF had increased self-renewal capacity compared to control lentivirus-infected cells (Figure 8D). Cells infected with the indicated lentivirus were inoculated in the brain of immunocompromised mice, and we performed two types of experiments. First, the overall survival of mice inoculated with lentivirus-infected cells was assessed (Figure 8E). Second, the tumor burden was analyzed and quantified by MRI 40 days after inoculation (Figures 8F and 8G). Mice inoculated with cells infected with lentivirus expressing TGF-β, TβRI-CA, or LIF had significantly shorter overall survival and generated significantly larger tumors than mice inoculated with control infected cells

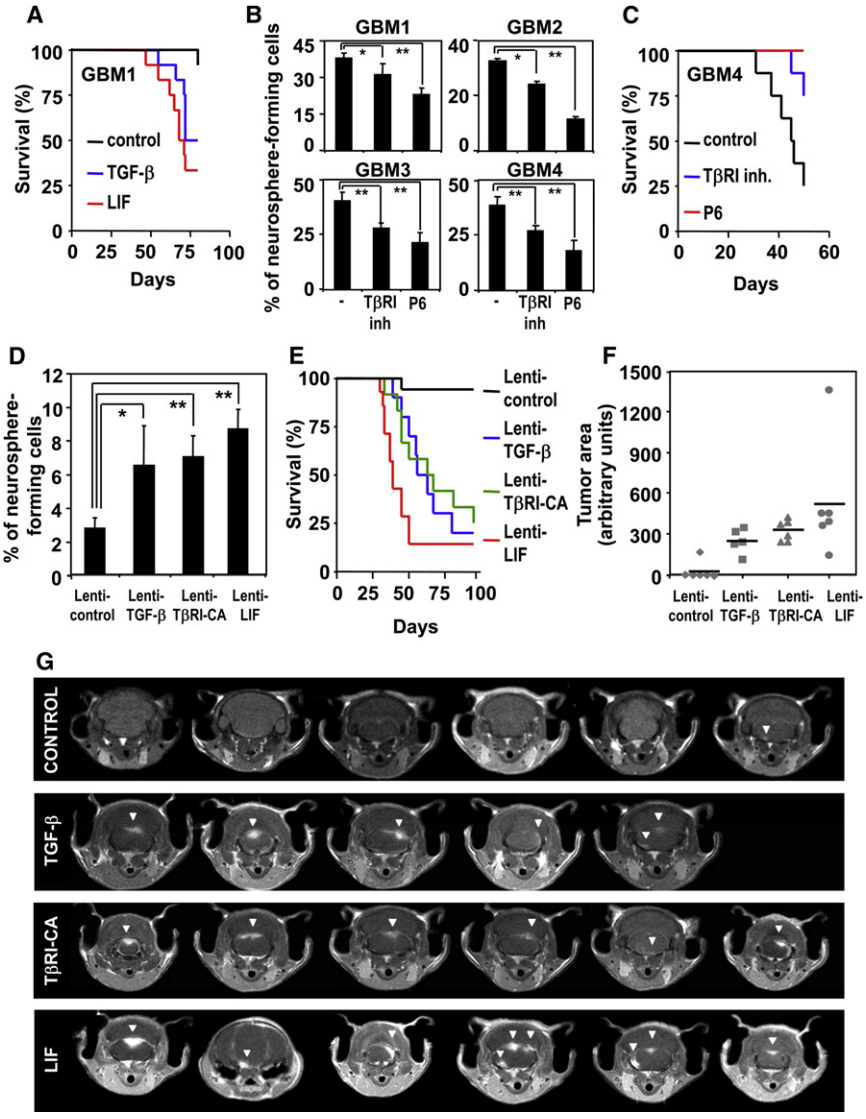


**Figure 8. The TGF-β and LIF Pathways Increase the Oncogenic Potential of GBM Neurospheres**

(A) Cells from GBM1 neurospheres, previously treated with 100 pM TGF-β1 or 20 ng/ml LIF for 14 days, were inoculated in the brains of immunocompromised mice. Animal survival was evaluated using a log-rank analysis from a Kaplan-Meier survival curve ($p = 0.021$ comparing control and TGF-β; $p = 0.002$ comparing control and LIF).

(B) Cells from the indicated GBM neurospheres plated at 100 cells/μl were treated with TβRI inhibitor or JAK inhibitor (P6) for 7 days. Neurospheres were then dissociated and plated in the absence of treatment for 7 days, and the newly formed neurospheres were counted. *$p < 0.05$; **$p < 0.005$.

(C) Cells from GBM4 neurospheres, previously treated at 100 cells/μl with 2 μM TβRI inhibitor or 0.5 μM P6 for 14 days, were inoculated in the brains of immunocompromised mice. Animal survival was evaluated using a log-rank analysis from a Kaplan-Meier survival curve ($p = 0.032$ comparing control and TβRI inhibitor; $p = 0.002$ comparing control and P6).

(D) Cells from GBM1 neurospheres infected with the indicated lentiviruses were incubated in the absence of growth factors for 7 days, and the percentage of neurosphere-forming cells was determined.

(E) Cells from GBM1 neurospheres, previously infected with lenti-control, lenti-TGF-β1, lenti-TβRI-CA, or lenti-LIF, were inoculated in the brains of immunocompromised mice. Animal survival was evaluated using a log-rank analysis from a Kaplan-Meier survival curve ($p < 0.001$ comparing lenti-control and lenti-TGF-β1; $p < 0.001$ comparing lenti-control and lenti-TβRI-CA; $p < 0.001$ comparing lenti-control and lenti-LIF).

(F and G) Cells from GBM1 neurospheres, previously infected with lenti-control, lenti-TGF-β1, lenti-TβRI-CA, or lenti-LIF, were inoculated in the brains of immunocompromised mice. Forty days after surgery, images from the entire mouse brains were obtained by MRI (G), and tumor area was quantified (F). $p = 0.0005$ comparing lenti-control and lenti-TGF-β1; $p = 0.0001$ comparing lenti-control and lenti-TβRI-CA; $p = 0.0172$ comparing lenti-control and lenti-LIF.

Error bars represent mean ± SD.

(Figures 8E–8G). Thus, the TGF-β and LIF pathways enhance the oncogenic capacity of GBM neurospheres, corroborating their effect on GIC self-renewal.

## DISCUSSION

Recent evidence indicates that GICs are responsible for the initiation, propagation, recurrence, and therapeutic failures of gliomas (Bao et al., 2006), and hence GICs are considered to be critical therapeutic targets (Sanai et al., 2005; Vescovi et al., 2006). An understanding of the molecular mechanisms involved in the regulation of GICs is crucial in order to be able to design efficient therapeutic strategies and to improve on conventional anticancer treatments, which presently have limited success against glioma. Here, we have identified a molecular pathway that regulates the self-renewal capacity of GICs.

We and others have implicated the TGF-β pathway in glioma (Bruna et al., 2007; Rich, 2003). TGF-β is a cytokine with a dual role in cancer, and while it acts as a tumor suppressor in normal epithelial cells and early-stage tumors, it becomes an oncogenic factor in advanced tumors, inducing proliferation, angiogenesis, invasion, suppression of the immune response, and metastasis (Roberts and Wakefield, 2003; Siegel and Massagué, 2003). Specifically in high-grade glioma, the TGF-β pathway acts as an oncogenic factor (Bruna et al., 2007). A highly active TGF-β pathway confers poor prognosis in human glioma patients, and TGF-β acts as a proliferative and oncogenic factor through the induction of PDGF-B in gliomas with an unmethylated *PDGF-B* gene (Bruna et al., 2007). Here, we have identified

yet another mechanism implicated in the TGF-β oncogenic response in human glioma. TGF-β increases the self-renewal capacity and prevents the differentiation of GICs isolated from patient-derived glioma specimens. Moreover, we have identified LIF as a mediator of the TGF-β effects on GICs. TGF-β induces LIF in most GBMs, and LIF in turn promotes GIC self-renewal, increasing the GIC population within the tumor mass. We have observed that the induction of GIC self-renewal by TGF-β and LIF translates into an increased oncogenic capacity. TGF-β or LIF treatment enriches GBM neurospheres for GICs and increase the capacity of neurospheres to generate tumors. In addition, neurospheres engineered to have highly active TGF-β and LIF pathways, as observed in certain human gliomas, become more tumorigenic.

LIF is a pleiotropic cytokine that binds to the LIFR/gp130 complex of receptors that can signal through the JAK-STAT pathway (Ernst and Jenkins, 2004). Using a pharmacological inhibitor of JAK, we have identified the JAK-STAT pathway as the pathway downstream of the TGF-β-LIF axis required for its effect on GIC self-renewal. The LIF-JAK-STAT pathway has been previously implicated in many processes, and depending on the cellular context, it can regulate the self-renewal capacity of early neuroprogenitors or induce astrocytic differentiation (Bauer and Patterson, 2006; Bonni et al., 1997; Molne et al., 2000; Rajan and McKay, 1998; Wright et al., 2003).

We have discerned the molecular mechanisms involved in the transcriptional induction of LIF by TGF-β. We have also identified a functional Smad-binding element on the *LIF* promoter and found that an activated Smad complex binds to the proximal *LIF* promoter close to an SP1 binding site in order to induce transcription. LIF induction by TGF-β is a Smad-dependent process since knockdown of both Smad2 and 3 or Smad4 represses TGF-β-induced LIF expression. Therefore, LIF is a typical TGF-β-Smad transcriptional response mediated by a Smad2/3-Smad4 complex.

GICs have functional and molecular similarities to normal neuroprogenitors, including their ability to generate neurospheres, the expression of certain protein markers, and their capability for multilineage differentiation. However, GICs and normal neuroprogenitor cells are two different cell types, and comparisons between them should be made with caution. TGF-β does not induce the self-renewal capacity of normal human neuroprogenitors obtained from human fetal cerebral cortex samples. LIF, on the other hand, increases the self-renewal capacity of human normal neuroprogenitor cells. This is in line with previous reports showing that LIF induces the self-renewal capacity of human normal neuroprogenitors and mouse embryonic and adult neuroprogenitors (Bauer and Patterson, 2006; Molne et al., 2000; Wright et al., 2003). Interestingly, LIF is not induced by TGF-β in human normal neuroprogenitors, even though the TGF-β pathway is functional in neuroprogenitors. TGF-β is able to induce p-Smad2 levels and Smad7 (a well-defined TGF-β target gene) in neuroprogenitors to the same extent as in GBM neurospheres. Therefore, the lack of LIF induction in response to TGF-β in normal neuroprogenitors is dependent on the ability of the Smad transcriptional complex to specifically bind to the *LIF* promoter in GICs. Our results demonstrate that LIF has the same effect on normal neuroprogenitors and GICs and that the differential response to TGF-β coincides with the inability of TGF-β to induce LIF in neuroprogenitors.

We found that LIF is expressed in human gliomas and that tumors with high levels of TGF-β2 tend to have high levels of LIF, indicating that TGF-β2 might be responsible for LIF expression in human gliomas. Furthermore, high LIF levels correlate with the expression of markers of neuroprogenitor cells (Musashi-1 and nestin) in some tumors. This indicates that tumors expressing high levels of TGF-β2 tend to express high levels of LIF and are enriched in a cell population expressing neuroprogenitor markers, corroborating the fact that in human glioma, the TGF-β2/LIF pathway promotes an increase of the GIC pool. Interestingly, we observed previously that tumors with high levels of TGF-β2 tend to be more aggressive (Bruna et al., 2007). LIF could be one of the mediators of the malignant behavior of TGF-β2-expressing tumors.

Recently, it has been reported that another member of the TGF-β family of cytokines, BMP, inhibits GIC self-renewal and promotes GIC differentiation in GBMs with no epigenetic silencing of the BMP receptor 1B (*BMPR1B*) gene (Lee et al., 2008; Piccirillo et al., 2006). Interestingly, we found that BMP was not able to induce LIF in glioma cells (data not shown). This is not the first time that TGF-β and BMP have been reported to have contrary functions. TGF-β and BMP have opposing effects on embryonic stem cell self-renewal due in part to their differential transcriptional regulation of Nanog (Xu et al., 2008). In epithelial cells, TGF-β transduces its signal through Smad2 and 3 while BMP induces phosphorylation and translocation of Smad1, 5, and 8 (Massagué et al., 2005). Hence, the LIF response to TGF-β and BMP might be dictated by the specific ability of Smad2/3 and not Smad1/5/8 to be recruited to the *LIF* promoter to induce transcription.

TGF-β might have the same effect on tumor-initiating cells of other tumor types. Interestingly, it has been reported that a $CD44^{+}CD24^{-}$ cell population in breast tumors, considered to have stem cell-like properties, have a highly active TGF-β pathway and are regulated by TGF-β (Mani et al., 2008; Shipitsin et al., 2007). This suggests that TGF-β may have an important role in maintaining the stem cell-like pool in breast cancer as well as in glioma. Further studies are needed to define whether the molecular mechanisms identified in the present work are functional in other tumor types.

GICs and tumor-initiating cells in general are critical therapeutic targets in cancer. An understanding of the exact mechanisms implicated in the regulation of tumor-initiating cell self-renewal will provide tools for the development of more successful therapeutic strategies. Together, our results shed light on GIC biology and furthermore on the TGF-β oncogenic response in glioma. The LIF-JAK-STAT pathway increases human GIC self-renewal, and compounds designed to inhibit LIF or the JAK-STAT pathway could improve therapeutic strategies against glioma by targeting GICs. Moreover, our data identify a mechanism through which anti-TGF-β compounds might be successful against glioma since they will inhibit LIF expression and decrease GIC self-renewal.

## EXPERIMENTAL PROCEDURES

### Cell Lines and Primary Cell Cultures

U373MG and A172 cells (a kind gift of J. Rich and D. Bigner) were cultured in DMEM with 10% fetal bovine serum (FBS). PCTCs and GBM neurospheres

were generated as described previously (Bruna et al., 2007; Günther et al., 2007). Briefly, tumor samples were processed within 30 min after surgical resection. Minced pieces of human glioma samples were digested with 200 U/ml collagenase I (Sigma) and 500 U/ml DNase I (Sigma) in PBS for 2 hr at 37°C with constant vigorous agitation. The single-cell suspension was filtered through a 70 μm cell strainer (BD Falcon) and washed with PBS. Finally, cells were resuspended and subsequently cultured in DMEM with 10% FBS (for PCTC culture) or in neurosphere medium (for GBM neurospheres). Normal human neuroprogenitor neurospheres were generated from human embryonic cerebral cortex tissue collected following medical terminations of pregnancy. The samples were processed and cultured as described previously (Poltavtseva et al., 2002). The neurosphere medium consisted of Neurobasal medium (GIBCO) supplemented with B27 (GIBCO), L-glutamine (GIBCO), penicillin/streptomycin, and growth factors (20 ng/ml EGF and 20 ng/ml FGF-2 [PeproTech]). Human glioma specimens and human embryonic tissues were obtained from the Vall d'Hebron Hospital. The clinical protocol was approved by the Vall d'Hebron Institutional Review Board (CEIC), with informed consent obtained from all subjects.

### Intracranial Tumor Assay

All mouse experiments were approved by and performed according to the guidelines of the Institutional Animal Care Committee of the Vall d'Hebron Research Institute in agreement with the European Union and national directives. The indicated number of cells was stereotactically inoculated into the corpus striatum of the right brain hemisphere (1 mm anterior and 1.8 mm lateral to the bregma; 2.5 mm intraparenchymal) of 9-week-old NOD/SCID mice (Charles River Laboratories). Mice were euthanized when they presented neurological symptoms or a significant loss of weight. Magnetic resonance imaging (MRI) analysis was performed and images were acquired using a 9.4 T vertical bore magnet interfaced to an AVANCE 400 system (Bruker). Under anesthesia by xylazine/ketamine, mice were given an intraperitoneal injection of gadolinium diethylenetriamine penta-acetic acid at a dose of 0.25 mmol gadolinium/kg body weight and placed in the radio frequency coil (inner diameter 35 mm). After localizer imaging on three orthogonal axes, T1-weighted images of the entire mouse brain were acquired using a spin echo sequence with TR and TE set to 800 and 5.7 ms, respectively. Other parameters used were a 2.5 cm field of view and a 256 × 256 matrix in two averages, resulting in a total scan time of approximately 6.5 min. Tumor size was quantified by measuring the number of pixels corresponding to tumor tissue in each image using the software provided by the manufacturer (Bruker); when the tumor was visible in more than one image, areas corresponding to tumor tissue were quantified together.

### Statistical Analysis

A Spearman correlation test was used to analyze relationships between *LIF* and *TGF-β2*, *Musashi-1*, *Sox2*, and *Nestin*. Data in graphs are presented as mean ± SD.

## SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, one table, and seventeen figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00042-7.

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