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TGF-β Receptor Inhibitors Target the CD44^{high}/Id1^{high} Glioma-Initiating Cell Population in Human Glioblastoma

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SUMMARY

Glioma-initiating cells (GICs), also called glioma stem cells, are responsible for tumor initiation, relapse, and therapeutic resistance. Here, we show that TGF-β inhibitors, currently under clinical development, target the GIC compartment in human glioblastoma (GBM) patients. Using patient-derived specimens, we have determined the gene responses to TGF-β inhibition, which include inhibitors of DNA-binding protein (Id)-1 and -3 transcription factors. We have identified a cell population enriched for GICs that expresses high levels of CD44 and Id1 and tend to be located in a perivascular niche. The inhibition of the TGF-β pathway decreases the CD44^{high}/Id1^{high} GIC population through the repression of Id1 and Id3 levels, therefore inhibiting the capacity of cells to initiate tumors. High CD44 and Id1 levels confer poor prognosis in GBM patients.

INTRODUCTION

Work over the last several years has shown that the TGF-β signaling pathway has an important role in cancer (Massague, 2008), and this has prompted the development of anticancer strategies based on inhibition of this pathway (Seoane, 2008; Yingling et al., 2004). However, due to the complexity of the TGF-β signaling pathway, it is crucial to understand how TGF-β inhibition affects tumor biology in order to assess and improve the therapeutic potential and clinical testing of anti-TGF-β compounds.

TGF-β is a cytokine that signals through a heterodimeric receptor complex formed by the type I (TβRI) and the type II (TβRII) receptors. Upon ligand binding, the receptor complex phosphorylates Smad transcription factors, which then shuttle into the nucleus to regulate transcription (Massague et al., 2005; Schmierer and Hill, 2007). Functionally, the TGF-β pathway is relevant in embryonic development and tissue homeostasis, and exhibits a pleiotropic response determined by the cellular context and state (Massague, 2008).

The TGF-β pathway acts as an oncogenic factor in advanced tumors, including high-grade gliomas (Massague, 2008). Glioma is the most common tumor of the brain, and its most aggressive form, grade IV glioma, also called glioblastoma (GBM), is one of the deadliest cancers with a median survival of around 14 months (Stupp et al., 2005). In glioma, elevated TGF-β activity

Significance

GBM is one of the most aggressive and recalcitrant tumors and GICs are responsible for therapeutic failures. Still, not many therapeutic approaches have been described to target GICs. Through the study of patient-derived tumor specimens, we have observed that the inhibition of the TGF-β pathway targets a cell population characterized by high expression of CD44 and Id1, which is enriched for GICs. The depletion of the CD44^{high}/Id1^{high} GIC population by TGF-β inhibitors prevents tumor initiation and recurrence. Our work shows that Id1 and Id3 are therapeutic targets against GICs and GBM, and that combined CD44 and Id1 expression confers poor prognosis in patients. Furthermore, our results have clear implications on the clinical development of TGFβ inhibitors as compounds targeting GICs.

confers poor prognosis in patients (Bruna et al., 2007), and shows a diverse oncogenic response that includes the induction of angiogenesis, immunosuppression, cell invasion, and cell proliferation (Bruna et al., 2007; Rich, 2003). In addition, TGF-β can increase the self-renewal capacity of glioma-initiating cells (GICs) through the induction of LIF or Sox2 (Ikushima et al., 2009; Penuelas et al., 2009).

In tumors, cancer-initiating cells (CICs), including GICs, are a cellular subpopulation that have characteristics of normal stem cells, exhibit sustained self-renewal, and can generate secondary tumors that reproduce the characteristics and cellular diversity of the original tumor. CICs are considered to be responsible for tumor initiation, propagation, recurrence, and chemo- and radioresistance (Bao et al., 2006; Dick, 2008; Gupta et al., 2009a; Visvader and Lindeman, 2008; Zhou et al., 2009). All these characteristics indicate that CICs are critical therapeutic targets, and that the understanding of the biology of CICs is crucial to improving anticancer treatments. A number of cell surface markers, including CD133 and CD44, have proved useful for the isolation of subsets of cells enriched for CICs in different tumor types (Visvader and Lindeman, 2008). In glioma, CD133 has been frequently used to identify GICs, although some gliomas contain CD133-negative GICs (Bao et al., 2006; Beier et al., 2007; Galli et al., 2004; Singh et al., 2004). In contrast, the CD44 compartment has not been extensively studied in glioma. In breast cancer, a link between TGF-β and the CD44^{high} population has been described. TGF-β has been shown to increase the CD44^{high} cell population enriched for CICs through the induction of an epithelial-mesenchymal transition (EMT) (Gupta et al., 2009b; Mani et al., 2008).

Inhibitors of DNA-binding proteins (Ids) are transcription factors that antagonize the DNA-binding capacity of basic helix-loop-helix factors (Perk et al., 2005). Ids regulate cell cycle and cell differentiation, and have an important role in the control of stem cell self-renewal (Perk et al., 2005; Ying et al., 2003). Very recently, Id1 has been shown to be expressed in B1 type adult neural stem cells, having an important role in the regulation of the self-renewal capacity of these cells (Nam and Benezra, 2009). In cancer, Id1 is found upregulated in several tumors (Perk et al., 2006), and has been described to be involved in metastasis (Gupta et al., 2007). TGF-β family members regulate the expression of Id1. In normal epithelial cells, TGF-β represses and BMP induces Id1 transcription (Massague, 2008). However, in endothelial cells and some tumor cells, TGF-β is able to induce Id1 expression (Goumans et al., 2002; Padua et al., 2008).

In this study, we investigated the molecular mechanisms of the antitumor effect of the inhibition of the TGF-β pathway in GBM.

RESULTS

The TβRI Inhibitor Gene Responses in GBM Include Id1 and Id3

With the purpose of understanding the effect of the inhibition of the TGF-β pathway in GBM, we decided to identify the gene responses to the TβRI inhibition in patient-derived primary cultures of tumor cells (PCTCs). In order to inhibit the TβRI, we used a highly selective compound that acts as an ATP competitor, LY2109761, a derivative of which is now in a phase I-II clinical trial in our hospital (Vall d'Hebron University Hospital, Barcelona, Spain). We obtained cells from surgically resected human GBM samples and generated 11 PCTCs from 11 different patients. Shortly after setting up the cultures, cells were treated with the TβRI inhibitor for 3 hr and the RNA was collected. We used the lowest concentration of inhibitor required to prevent the phosphorylation of Smad2 (2 μM) (see Figure S1A available online).We performed a transcriptomic analysis and determined the transcripts that were modulated by the treatment with the TβRI inhibitor (Figure 1A). A signature of TGF-β inhibition was obtained with six transcripts that were regulated by the TβRI inhibitor with a fold change over 1.4 or below 0.6, and a $p < 0.001$. We validated the gene responses that were the most significantly regulated: *ID3*, *ID1*, *Smad7*, and *RhoB* (Figure 1B). All four genes were modulated by the TβRI inhibitor in almost all PCTCs. In particular, *ID3* and *ID1* were downregulated in all PCTCs with the exception of PCTC2. Interestingly, PCTC2 was generated from the only GBM of our set that presented cysts (Figure S1B).

Next, we assessed whether Id1 and Id3 could be targets of the TβRI inhibitor in vivo by use of a preclinical model based on the orthotopical inoculation of patient-derived GICs in immunocompromised mice. We generated neurosphere cultures enriched for GICs from two GBM patient samples and inoculated them in the brain of NOD-SCID mice. As we and others have described (Penuelas et al., 2009; Visvader and Lindeman, 2008), GICs generated tumors with similar characteristics to the original tumor from the patient (Figure S1C). Mice inoculated with patient-derived neurospheres were treated orally with the TβRI inhibitor twice a day for 30 days and tumor progression was monitored by magnetic resonance imaging (MRI). Mice treated with the TβRI inhibitor generated smaller tumors and had a longer survival rate than mice treated with the vehicle (Figures 1C and 1D; Figure S1D). We determined the levels of Id1 in the tumors and found that the TβRI inhibitor-treated tumors exhibited lower levels of Id1 than control tumors (Figure 1E). In order to exclude the Id1-positive endothelial cells, we performed costaining of Id1 and the endothelial cell marker CD31 (Figure 1E). We also performed Id3 analysis and found that the difference of Id3 levels between mice treated or untreated with the TβRI inhibitor was not statistically significant (Figure S1E and data not shown). The effect of the TβRI inhibitor on *ID1* was confirmed by tumor microdissection and posterior analysis of mRNA by qRT-PCR (Figure 1F). Globally, our results showed that Id1 is a target of the TβRI inhibitor in human GBM.

Ids have been shown to be involved in stem cell biology (Nam and Benezra, 2009; Ying et al., 2003), and we therefore hypothesized that Id1 downregulation by the TβRI inhibitor could be relevant for the maintenance of GICs. We first determined the levels of Id1 and Id3 in PCTCs and GBM neurospheres, and found that GBM neurospheres expressed higher levels of Id1 and Id3 than did PCTCs (Figure S2). We then assessed the effect of the TβRI inhibitor on GBM neurospheres. We dissociated neurospheres into single cells, plated them at low density, treated them with the TβRI inhibitor for 7 days, and then counted the newly formed neurospheres. Treatment of GBM neurospheres with the TβRI inhibitor decreased the levels of p-Smad2 (Figure 2A) and the number of neurosphere-forming cells (Figure 2B). Alternatively, neurospheres were treated for 7 days with the TβRI inhibitor, then dissociated and replated in the absence of treatment for another 7 days, and the newly formed neurospheres

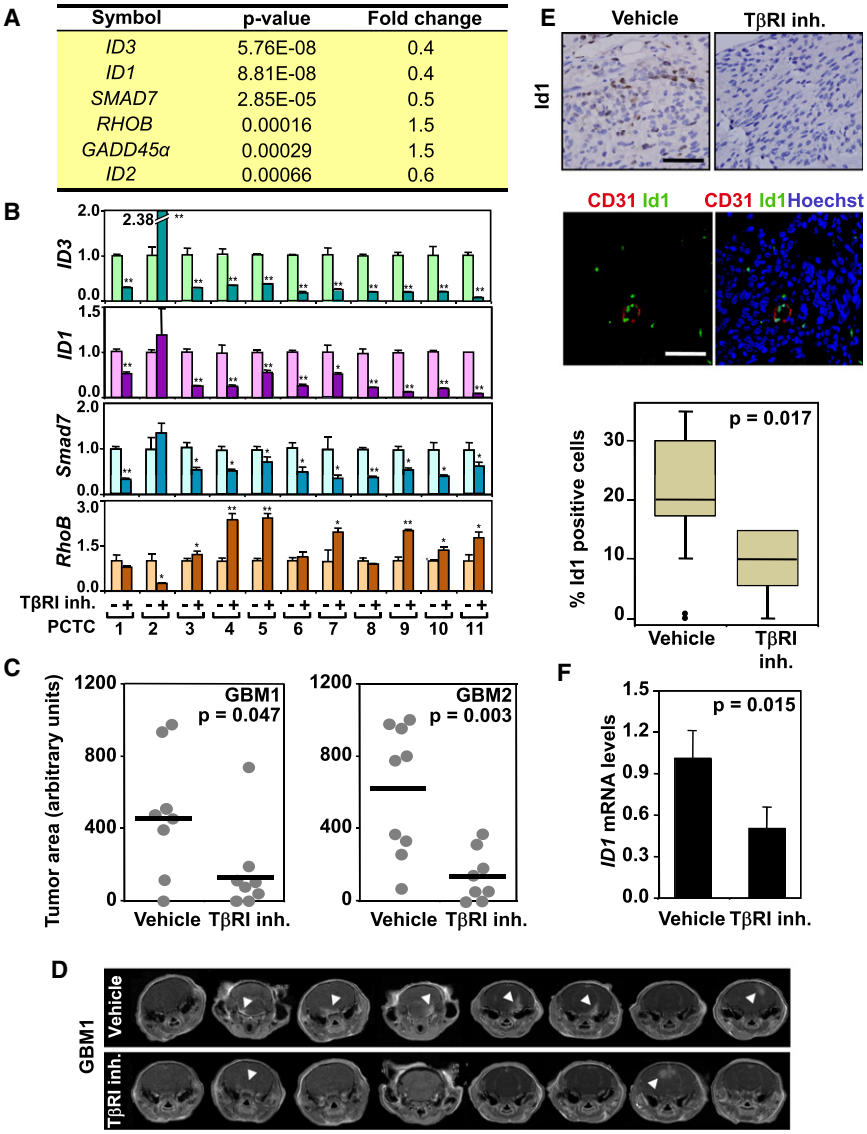

Figure 1. Id1 Is a Target of the TβRI Inhibitor in PCTCs and in a Patient-Derived Mouse Model for Glioma

(A) The genes were regulated by the treatment with 2 μM TβRI inhibitor for 3 hr in 11 human glioma PCTCs with a fold change over 1.4 or below 0.6 and a $p < 0.001$.

(B) *ID3*, *ID1*, *Smad7*, and *RhoB* transcript levels were determined by qRT-PCR analysis. *GAPDH* RNA levels were used as an internal normalization control. *$p < 0.05$; **$p < 0.001$. Data are presented as means ± SD.

(C and D) Cells from GBM1 and GBM2 neurospheres were inoculated in the brain of immunocompromised mice. Twenty days after surgery, mice were orally treated twice a day with 100 mg/kg of TβRI inhibitor for 30 days. (C) Tumor area was quantified. (D) Images from the entire mouse brains were obtained by MRI (arrowheads indicate tumors).

(E) Immunohistochemistry for Id1 (upper panel) and double immunofluorescence for Id1 and CD31 (middle panel) were performed in tumors generated by GBM neurospheres. For immunofluorescence, nuclei were counterstained with Hoechst. Scale bar, 100 μm. Representative images are shown and percentage of Id1-positive cells was calculated (lower panel). Data are presented as means ± SD.

(F) Cells from GBM1 neurospheres were inoculated in the brain of immunocompromised mice. Forty days after surgery, mice were orally treated twice a day with 100 mg/kg of TβRI inhibitor for 10 days. Subsequently, 10 μm slides from mouse brains were stained with H&E and tumors were localized and microdissected. *ID1* transcript levels were determined by qRT-PCR ($n = 4$ for vehicle; $n = 3$ for TβRI inhibitor). Data are presented as means ± SD. See also Figure S1.

were then counted. The decrease in neurosphere-forming cells was maintained upon serial plating in the absence of treatment, showing that the TβRI inhibitor decreases the neurosphere self-renewal capacity (Figure 2C). The effect of the TβRI inhibitor on GBM neurospheres from four different patients coincided with a decrease in the mRNA levels of *ID1* and *ID3* (Figure 2D).

As expected, TGF-β induced Id1 expression in human GBM neurospheres. TGF-β-mediated Id1 induction was prevented by the treatment with two unrelated TβRI inhibitors, LY2109761 and SB431542 (Figures 3A and 3B; Figure S3). We then decided to discern the mechanism through which TGF-β regulates Id1 expression. To study the transcriptional regulation of Id1 by TGF-β, we used a reporter construct containing the −1046/+86 promoter region of the human *ID1* gene. TGF-β and the TβRI inhibitor were able to regulate the *ID1* reporter construct, indicating that TGF-β regulated *ID1* at the level of transcription (Figure 3C). Moreover, we were able to map the TGF-β-responsive element to the −1046/−863 promoter region, since the −863/+86 region did not respond to TGF-β and the −1046/−863 promoter region was sufficient to be regulated by TGF-β and the TβRI inhibitor (Figure 3C). The −1046/−863 promoter region is the same region that has been described to mediate the induction of *ID1* transcription by BMP and the repression of *ID1* transcription by TGF-β in epithelial cells (Kang et al., 2003; Lopez-Rovira et al., 2002). To assess whether the transcriptional regulation of *ID1* was Smad dependent, we knocked down Smad4 by RNA interference. TGF-β-mediated induction of Id1 was reduced when Smad4 was knocked down, indicating that Id1 is regulated by the TGF-β-Smad pathway (Figure 3D). Moreover, chromatin immunoprecipitation (ChIP) assays showed that Smad4 bound the TGF-β-responsive element in the *ID1* promoter after TGF-β treatment (Figure 3E). These results indicate that TGF-β regulates *ID1* transcription through a mechanism similar to that described in epithelial cells. However, while TGF-β induces *ID1* transcription in GBM neurospheres, TGF-β represses *ID1* transcription in epithelial cells (e.g., a human keratinocyte cell line, HaCaT) (Figure 3F).

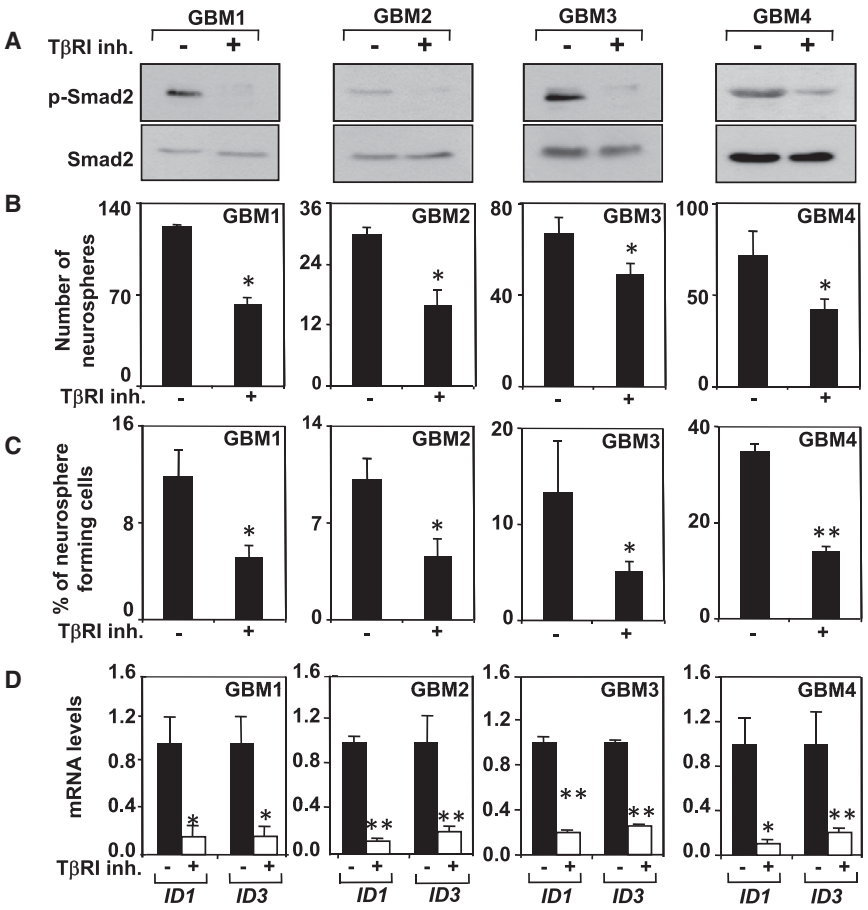

Figure 2. Id1 and Id3 Expression and Neurosphere Formation Are Regulated by the TβRI Inhibitor in Patient-Derived Neurospheres

(A) Cells from the indicated GBM neurospheres were incubated with 2 μM TβRI inhibitor for 8 hr and the levels of p-Smad2 and total Smad2 were determined by immunoblotting.

(B) Equal numbers of dissociated cells from the indicated GBM neurospheres were seeded at low density and incubated with 2 μM TβRI inhibitor for 7 days, after which the number of neurospheres was determined. Data are presented as means ± SD.

(C) Cells from the indicated GBM neurospheres were incubated in the presence of 2 μM TβRI inhibitor for 7 days. Subsequently, cells were dissociated, counted, and equal numbers of dissociated cells were replated at low density without treatment. After 7 days, the percentage of neurosphere-forming cells was determined. Data are presented as means ± SD.

(D) Cells from the indicated GBM neurospheres were incubated with 2 μM TβRI inhibitor for 8 hr and the levels of *ID1* and *ID3* expression were determined by qRT-PCR. *$p < 0.05$; **$p < 0.001$. Data are presented as means ± SD. See also Figure S2.

Interestingly, TGF-β has been described to induce Id1 in endothelial cells and some tumor cell lines (Goumans et al., 2002; Padua et al., 2008).

We decided to address the mechanism of the differential Id1 response to TGF-β in GBM neurospheres and epithelial cells. A previous report (Kang et al., 2003) showed that Id1 transcription is repressed by TGF-β in epithelial cells through an ATF3-Smad complex that binds to the *ID1* promoter. The authors showed that ATF3 acted as a transcriptional repressor, and was recruited to the *ID1* promoter by the Smad complex. Interestingly, they showed that ATF3 was induced by TGF-β and TNF-α. We found that GBM neurospheres did not express ATF3, and, moreover, that TGF-β did not induce ATF3 (Figure 3F). However, when GBM neurospheres were treated with TNF-α, ATF3 was induced as described by Kang et al. (2003), and TGF-β lost its ability to induce Id1 (Figure 3G). Overall, our results show that the dual response of Id1 to TGF-β depends on the activity of the TNF-α pathway. Hence, the crosstalk between the TGF-β and the TNF-α pathways through a downstream mediator (most likely ATF3) dictates the transcriptional regulation of Id1 in GBM neurospheres.

The TβRI Inhibitor Targets a CD44^{high}/Id1^{high} Cell Compartment Enriched for GICs

We determined the expression of Id1 in neurospheres by immunofluorescence and observed that the expression pattern was heterogeneous, with some cells expressing high levels of nuclear Id1 (Figure 4A; Figure S4A). In order to assess in which cell compartment Id1 was expressed, we analyzed by coimmunofluorescence the expression of well-described markers of GICs (CD44, SSEA-1, and CD133) and Id1 in four different patient-derived neurospheres. High expression of Id1 colocalized with high expression of CD44, but not with SSEA-1 or CD133 (CD133 was not expressed in GBM1 neurospheres) (Figure 4A; Figure S4B). We quantified the expression of CD44 and Id1, and observed a direct correlation between CD44 and Id1 levels (Figure 4A). Interestingly, in GBM specimens (Figure S4C) and GBM neurospheres (Figure 4B), we could distinguish two discrete populations expressing different levels of CD44. The CD44^{high} and CD44^{low} subpopulations of neurospheres from four different patients were sorted by flow cytometry, and Id1 expression levels were determined. Id1 and Id3 were detected at much higher levels in the CD44^{high} than in the CD44^{low} compartment (Figures 4B and 4C). Interestingly, this was not the case, however, for Id2, another member of the Id family of transcription factors (Figure 4B).

We addressed whether the CD44^{high} cell population in glioma was enriched for GICs. First, we observed that, when we induced differentiation of patient-derived neurospheres through treatment with serum, the CD44^{high} compartment disappeared (Figure 5A). Next, we sorted the CD44^{high} and CD44^{low} cells and serial plated them at low density. CD44^{high} cells generated more neurospheres than the CD44^{low} compartment (Figure 5B), indicating that the CD44^{high} cells had higher self-renewal capacity. The small number of neurospheres

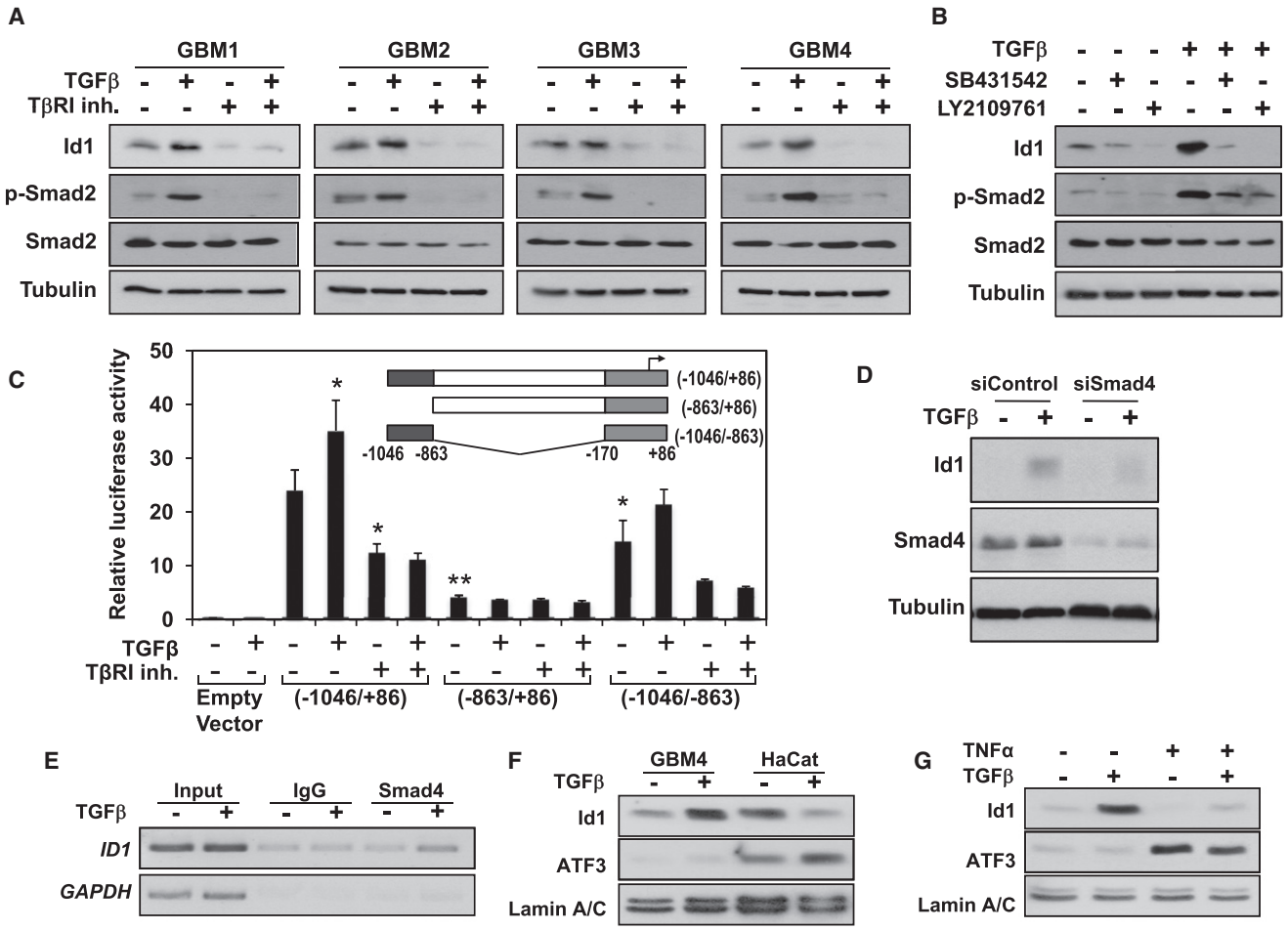

Figure 3. TGF-β Pathway Regulates Id1 Expression through an Activated Smad Complex

(A) Cells from GBM neurospheres were treated with 2 μM TβRI inhibitor for 8 hr with 100 pM TGF-β1 added, or not, during the last 3 hr, as indicated. The levels of Id1, p-Smad2, Smad2, and tubulin were determined by immunoblotting.

(B) Cells from GBM4 neurospheres were treated with 100 pM TGF-β1 during 3 hr and/or 2 μM TβRI inhibitors SB431542 and LY2109761 for 8 hr, or left untreated, and the levels of Id1, p-Smad2, Smad2, and tubulin were determined by immunoblotting.

(C) GBM4 neurospheres were transfected with the indicated constructs and treated with 100 pM TGF-β1 and/or 2 μM TβRI inhibitor during 36 hr. Luciferase analysis was performed. *$p < 0.05$; **$p < 0.001$ compared with their respective controls. Data are presented as means ± SD.

(D) GBM4 neurospheres were transfected with the indicated siRNA. After 48 hr, cells were treated with 100 pM TGF-β during 3 hr, or left untreated. Levels of Id1, Smad4, and tubulin were determined by immunoblotting.

(E) Cells from GBM4 neurospheres were treated with 100 pM TGF-β for 45 min, after which cells were lysed and ChIP performed with the indicated antibodies. PCR analysis was performed with specific primers for the *ID1* and *GAPDH* promoters.

(F) GBM4 neurospheres and HaCaT cells were incubated in the presence or absence of TGF-β (100 pM) for 3 hr. Nuclear protein extraction was performed and the levels of Id1, ATF3, and lamin A/C were determined by immunobloting.

(G) GBM4 neurospheres were treated with TNF-α (10 ng/ml) or TGF-β (100 pM) for 4 and 3 hr, respectively. Nuclear protein extraction was performed and the levels of Id1, ATF3, and lamin A/C were determined by immunoblotting. See also Figure S3.

generated by CD44low cells expressed low levels of Id1 and Id3 (Figure S5A).

We then analyzed the tumor-initiating capacity of the CD44high and the CD44low compartments. We sorted tumor cells based on the expression of CD44, and implanted different numbers of cells in the right striatum of NOD-SCID mice. Tumor progression was monitored by MRI. Cells expressing high levels of CD44 were much more tumorigenic than the CD44low-expressing cells. Only one out of seven mice inoculated with 100,000 CD44low GBM1 neurosphere cells developed tumors, whereas nine out of nine mice generated tumors when they were inoculated with the same number of CD44high cells (Figures 5C and 5D). Moreover, mice inoculated with 10,000 or 1000 CD44high cells generated tumors, whereas those inoculated with the same number of CD44low cells never generated tumors. A similar result was obtained with cells from two other patients (Figure 5D). Tumors generated by the CD44high compartment reproduced the histopathological characteristics of the tumor of the patient, including the same cellular heterogeneity (Figure 5E). For example, tumors generated in the mouse contained the same percentage (~70%)

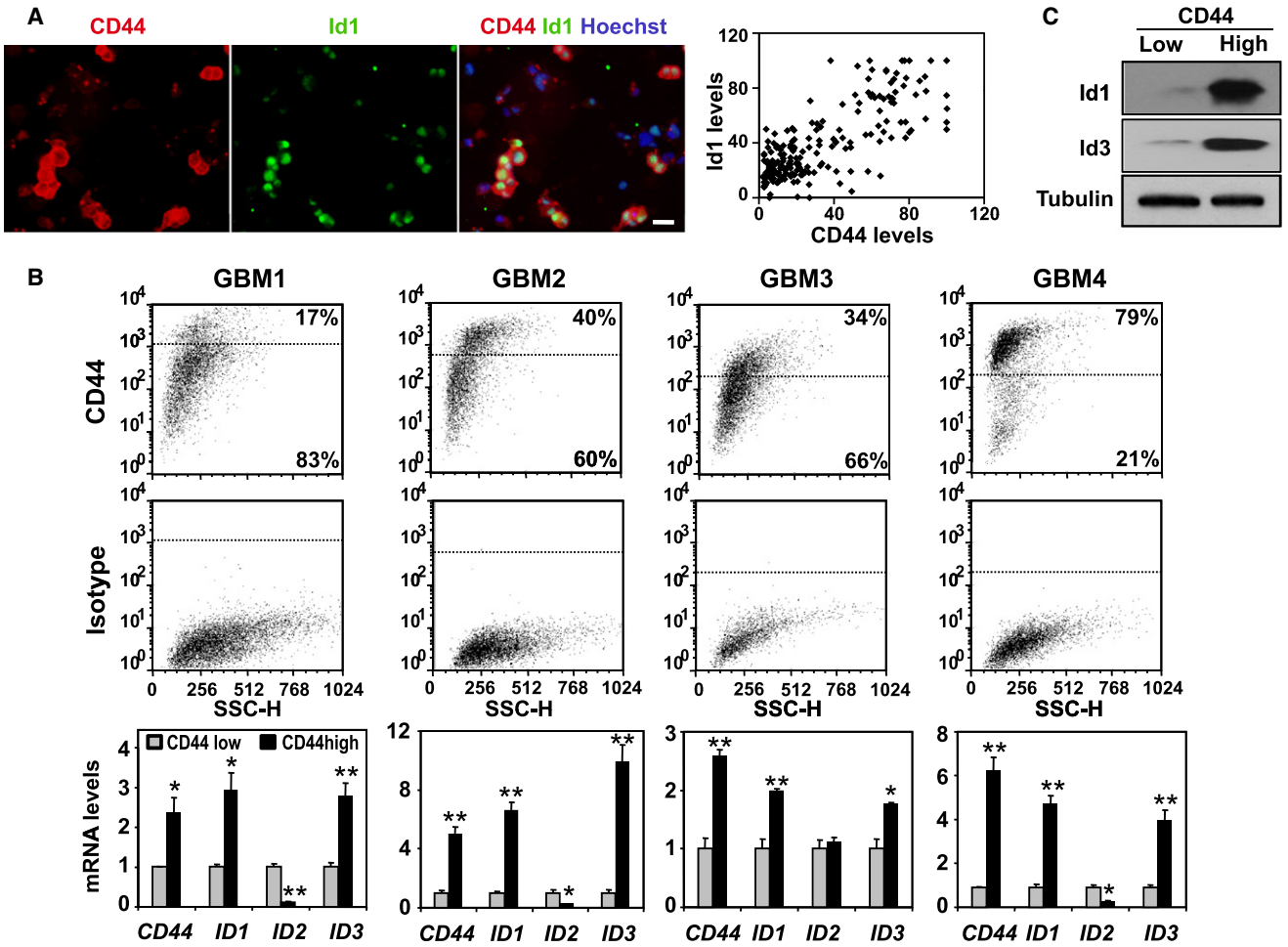

Figure 4. Patient-Derived Neurospheres Have a CD44high/Id1high Cell Population

(A) Cells from GBM1 neurospheres were dissociated and seeded on coverslips. Immunocytochemistry for Id1and CD44 was performed and nuclei were counterstained with Hoechst. Scale bar, 10 μm. Quantification of Id1 and CD44 expression levels per cell is shown.

(B) FACS analysis of CD44 levels was performed in GBM neurospheres (upper panel). Isotype control is shown. Cells with high or low levels of CD44 from the indicated GBM neurospheres were sorted by FACS and *CD44*, *ID1*, *ID2*, and *ID3* transcript levels were determined by qRT-PCR (lower panels). *$p < 0.01$; **$p < 0.001$ compared to $CD44^{low}$. Data are presented as means ± SD.

(C) Cells from GBM1 neurospheres were sorted by FACS according to CD44 levels, and the levels of Id1, Id3, and tubulin were determined by immunoblotting. See also Figure S4.

of Sox2-positive cells as the tumor of the patient (Figure 5E). All our results indicate that the $CD44^{high}$ compartment is enriched for GICs, as has been shown in other tumor types.

Remarkably, neurospheres from three patients treated with TGF-β for 7 days increased the $CD44^{high}$ compartment, while neurospheres treated with the TβRI inhibitor for the same time decreased the $CD44^{high}$ compartment (Figure 5F). A time-course of TGF-β treatment showed that CD44 began to be induced by TGF-β after 24 hr of treatment, and peaked at 7 days (Figure S5B). Moreover, inhibition of protein translation through the treatment with cycloheximide prevented the induction of CD44 by TGF-β, indicating that this phenomenon requires protein synthesis (Figure S5C). Thus, the regulation of CD44 by TGF-β was slow and indirect, suggesting that TGF-β induced CD44 as a result of a transdifferentiation process.

Altogether, our results demonstrate that TGF-β regulates the $CD44^{high}$ compartment, which expresses high levels of Id1 and is enriched for GICs.

The Effect of the TβRI Inhibitor on the $CD44^{high}$ Cell Population and the Tumor-Initiating Capacity of GICs Is Mediated by the Decrease of Id1 and Id3 Expression

Next, we assessed whether the regulation of Id1 and Id3 levels by TGF-β mediated the effect of TGF-β on the $CD44^{high}$ population and, hence, on the tumor-initiating capacity of neurospheres. We specifically decreased the levels of Id1 and Id3 by RNA interference (Figure 6A; Figure S6A). Knockdown of Id1 or Id3 separately did not have a major effect (Figure S6B), while cells expressing low levels of Id1 and Id3 had a reduced self-renewal capacity after serial plating (Figure S6C). This indicated functional redundancy between Id1 and Id3. Control and Id1 and

Id3-knockdown neurospheres were treated with TGF-β and the TβRI inhibitor, and the CD44^{high} population was determined. Neurospheres expressing lower levels of Id1 and Id3 had a decreased amount of CD44^{high} cells than control cells (Figures 6A and 6B). Interestingly, the CD44^{high} population in cells expressing shRNAs targeting Id1 and Id3 was similar to that in TβRI inhibitor-treated cells (Figure 6B). Moreover, the treatment of the Id-knockdown cells with the TβRI inhibitor did not significantly decrease the CD44^{high} population, showing that the effect of the TβRI inhibitor is in large part mediated by the decrease of Id1 and Id3 (Figure 6B).

We also overexpressed Id1 to a level similar to that achieved with the treatment with TGF-β (Figure 6A). The levels of Id1 overexpression were not affected by the treatment with the TβRI inhibitor (Figure 6A). Cells overexpressing Id1 presented an increased CD44^{high} population that was not regulated by the TβRI inhibitor (Figure 6B). In addition, the effect of the TβRI inhibitor on neurosphere self-renewal was blunted by the overexpression of Id1 (Figure S6D).

We then determined whether the levels of Id1 and Id3 proteins could affect the tumor-initiating capacity of the GBM neurospheres. Cells from the neurospheres infected with control lentivirus and lentivirus expressing shRNAs targeting Id1 and Id3 were inoculated in the brain of NOD-SCID mice. Tumor progression was monitored by MRI. Cells that expressed shRNAs against Id1 and Id3 generated smaller tumors, and with lower incidence than control cells (Figures 6C–6E). In addition, control neurospheres were pretreated in vitro with the TβRI inhibitor for 7 days, then dissociated and inoculated in mice. Cells pretreated with the TβRI inhibitor generated fewer and smaller tumors, similarly to the cells expressing Id1 and Id3 shRNAs (Figures 6C–6E). This indicates that the knockdown of Id1 and Id3 and the treatment with the TβRI inhibitor had a similar effect on GICs in decreasing the tumorigenic capacity of the neurospheres.

To evaluate further the role of Id1 in the response to the TβRI inhibitor, we inoculated Id1-overexpressing cells untreated or pretreated in vitro with the TβRI inhibitor. Cells overexpressing Id1 generated tumors earlier and with a higher incidence than control cells (Figures 6F–6H). Moreover, treatment with the TβRI inhibitor did not affect the tumor initiation capacity of Id1-overexpressing cells in contrast to control cells (Figures 6F–6H). These results showed that Id1 overexpression confers resistance to the TβRI inhibitor treatment, and proved that the downregulation of Id1 is required to mediate the effect of the TβRI inhibitor on GICs.

Previous work from our group and others (Ikushima et al., 2009; Penuelas et al., 2009) has shown that TGF-β can regulate GICs through the induction of LIF (Penuelas et al., 2009) and Sox2 and Sox4 (Ikushima et al., 2009). Indeed, treatment of GBM neurospheres with TGF-β increased the levels of Id1, Id3, LIF, Sox2, and Sox4 (Figure S6E), indicating that TGF-β regulates a gene program that controls GIC self-renewal. Interestingly, this TGF-β gene program is interrelated, since Id1 and Id3 knockdown decreases the basal levels and the TGF-β-induced levels of LIF and Sox2 and Sox4, suggesting that Id1 and Id3 control the expression of the rest of the genes of the program (Figures S6F and S6G). In order to address how LIF, Sox2, and Sox4 regulated the CD44^{high}/Id1^{high} cell compartment, we knocked down LIF, Sox2, and Sox4 independently. We observed that a lower level of LIF, Sox2, and Sox4 induced a decrease in the CD44^{high} population (Figures S6H and S6I), indicating that these three proteins are required for the maintenance of the CD44^{high}/Id1^{high} population. Hence, TGF-β induces the expression of a set of proteins (Id1, Id3, LIF, Sox2, and Sox4) that are required to maintain the CD44^{high} population.

We analyzed the effect of the TβRI inhibitor on the CD44^{high} population compared to the CD44^{low} population. As expected, treatment of the CD44^{high} compartment with the TβRI inhibitor decreased the levels of Id1. Interestingly, some TGF-β-dependent Id1 expression was present in the CD44^{low} compartment, since the TβRI inhibitor also downregulated the relatively low levels of Id1 in the CD44^{low} population (Figure S6J). We observed that, while the TβRI inhibitor decreased cell proliferation in the CD44^{high} compartment (measured by BrdU incorporation and cell counting), there was no effect of the TβRI inhibitor on the CD44^{low} population (Figures S6K and S6L). Moreover, when neurospheres were induced to differentiate through treatment with serum, the knockdown of Id1 and Id3 did not have a major effect on cell proliferation (Figures S6M and S6N). This is in sharp contrast to the effect of the TβRI inhibitor on GBM neurospheres (see Figure 6), and indicates that the effect of the TβRI inhibitor on Id1 and Id3 has a relevant and specific role in the CD44^{high} compartment.

In Vivo Treatment of Tumors with TβRI Inhibitors Prevents Tumor Cells to Regenerate Tumors in Mice

In many cases, failure with cancer treatment is not due to lack of primary response, but to tumor recurrence in which CICs are thought to have a crucial role. If TGF-β inhibitors decrease the GIC population in tumors, this should affect tumor relapse. To test this hypothesis, we generated tumors in mice through the inoculation of GBM1 neurospheres. Forty days after inoculation of cells, mice generated tumors that were detected by MRI. At that point, mice were treated with vehicle or the TβRI inhibitor for 10 days, and sacrificed. Human tumor cells were isolated from the mouse brain through sorting of human MHC-I-positive cells (Figure 7A). Cells obtained from mice that were treated with the TβRI inhibitor showed lower levels of *ID1*, *ID3*, and *CD44* transcripts, as measured by qRT-PCR (Figure 7B). Moreover, the percentage of CD44^{high} cells was reduced in tumors from TβRI inhibitor-treated mice (Figure 7C). We then evaluated the capacity of the TβRI inhibitor-treated cells to relapse by inoculating cells isolated from mice treated or untreated with the TβRI inhibitor in secondary mice. Indeed, cells obtained from TβRI inhibitor-treated mice generated less and smaller tumors than cells obtained from vehicle-treated mice (Figures 7A, 7D, and 7E). Hence, our results indicate that in vivo treatment of tumors with TGF-β inhibitors decreases the ability of cells to reinitiate tumors in another recipient mouse, suggesting that TGF-β inhibitors could be effective against tumor recurrence.

Combined Expression of Id1 and CD44 Is a Poor Prognosis Factor in Human GBM, and Cells Expressing Id1 and CD44 Tend to Be Located in a Perivascular Niche

In order to analyze the presence and location of Id1-positive cells in human GBM, we performed an immunohistochemical analysis of Id1 in 43 cases of GBM. Id1 staining was present in endothelial cells and in a fraction of tumor cells ranging from less than 5% to

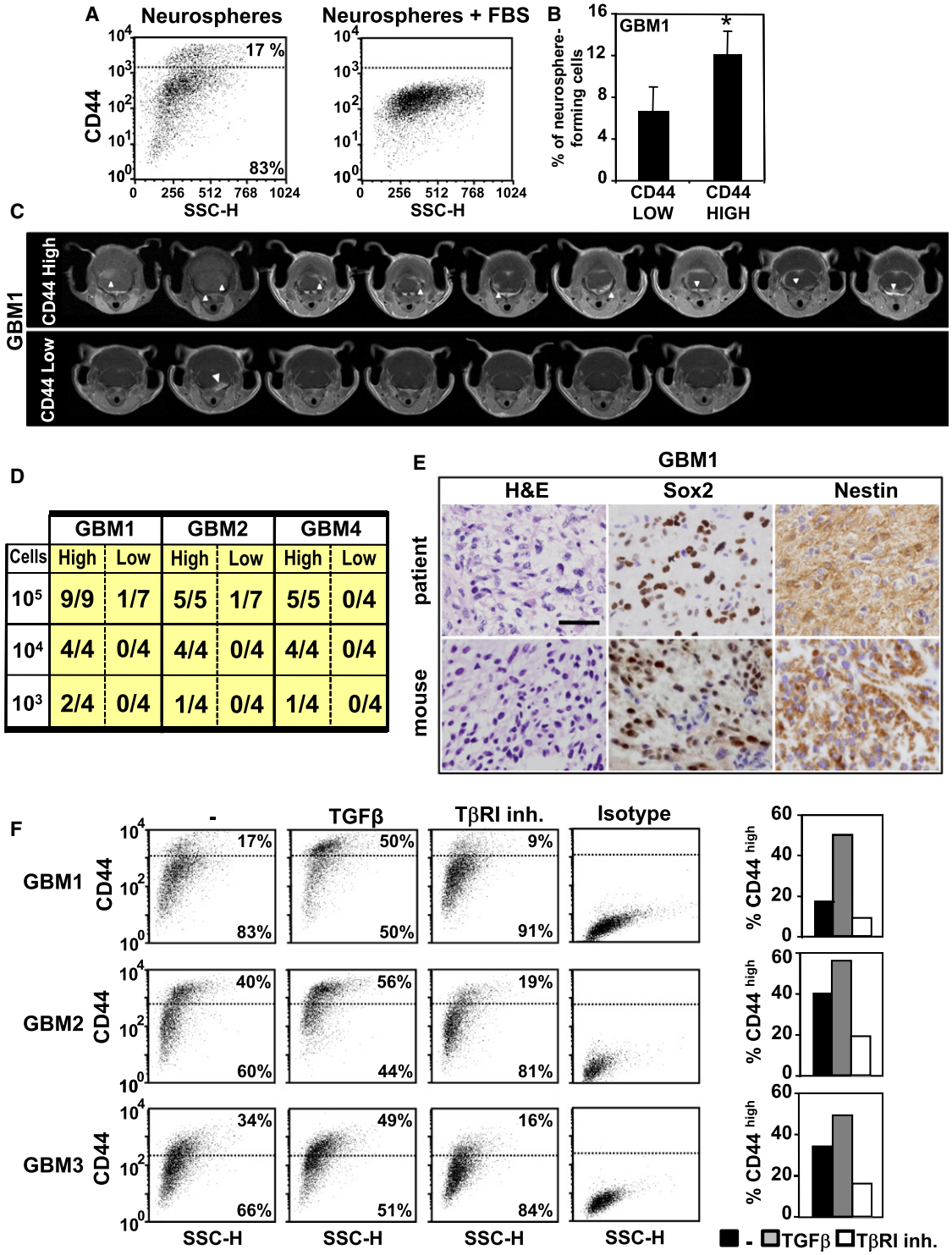

Figure 5. The CD44 high Population in Neurospheres Is Enriched for GICs and Is Regulated by the TGF-β Pathway

(A) FACS analysis of CD44 levels was performed in GBM1 neurospheres cultured in the presence or absence of 10% FBS for 10 days.

(B) GBM1 neurosphere cells with high or low levels of CD44 were sorted and seeded in neurosphere medium. After replating, cells were dissociated, counted, and a neurosphere-forming assay was seeded. The number of neurospheres was determined after 7 days to determine the percentage of neurosphere-forming cells. *$p < 0.05$. Data are presented as means ± SD.

(C–E) Neurosphere cells with high or low levels of CD44 were sorted, and the indicated number of cells was inoculated in the brain of immunocompromised mice. (C) Forty days after surgery, images from the entire mouse brains inoculated with 10^5 GBM1 cells were generated by MRI (arrowheads indicate tumors). (D) The number of mice bearing tumors, as determined by MRI versus total inoculated mice, is shown. (E) Immunohistochemistry of the indicated proteins and H&E staining of the tumors were performed. Scale bar, 50 μm.

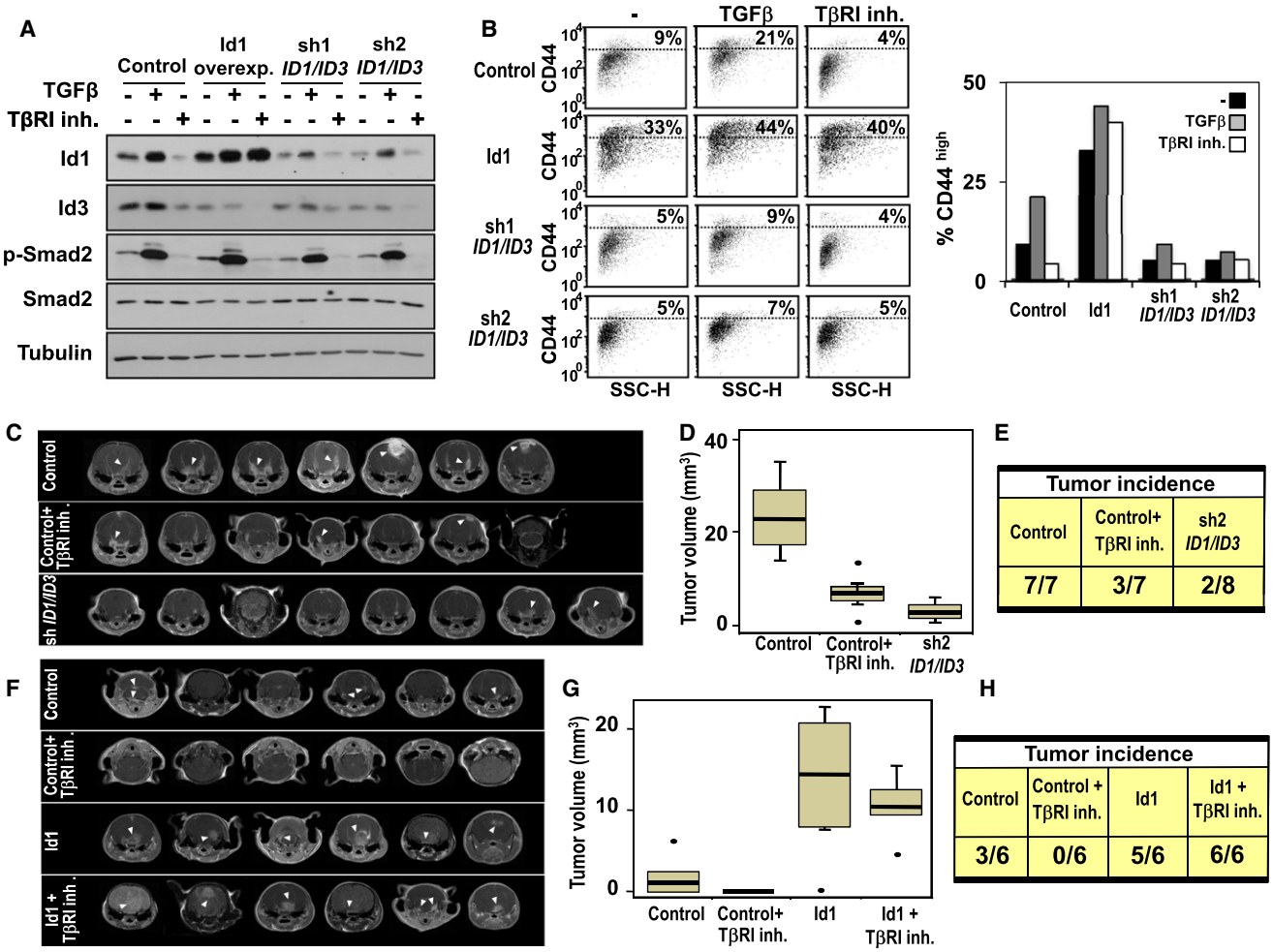

Figure 6. Id Proteins Mediate the Effect of TGF-β on the CD44high Population and the Oncogenic Potential of GBM Neurospheres

(A) Cells from GBM1 were infected with the indicated lentivirus. Neurospheres were treated with 100 pM TGF-β1 for 3 hr, 2 μM TβRI inhibitor for 8 hr, or left untreated, and the levels of Id1, Id3, p-Smad2, Smad2, and tubulin were determined by immunoblotting.

(B) Cells from GBM1 neurospheres previously infected with the indicated lentivirus were treated with 100 pM TGF-β or 2 μM TβRI inhibitor for 7 days or left untreated. FACS analysis of CD44 levels (left panel) and quantification of the frequency of CD44high cells are shown (right panel). Bars represent the percent of CD44high cells in untreated, TGF-β-treated, and TβRI inhibitor-treated lentiviral-infected neurospheres, as indicated. Data are presented as means.

(C–E) Cells from GBM1 neurospheres previously infected with the indicated lentivirus were treated for 7 days with 2 μM TβRI inhibitor, or left untreated. Subsequently, cells were dissociated, counted, and equal numbers of cells were inoculated in the brain of NOD-SCID mice. (C) Images from the entire mouse brains were obtained by MRI. Arrowheads indicate tumors. (D) Tumor area was quantified ($p = 0.004$ comparing mice inoculated with untreated neurospheres with mice inoculated with neurospheres treated with the TβRI inhibitor; $p = 0.002$ comparing mice inoculated with neurospheres infected with the lentivirus control with mice inoculated with neurospheres infected with the sh2 *ID1/ID3*). (E) Tumor incidence was determined. Data are presented as means ± SD.

(F–H) Cells from GBM1 neurospheres previously infected with the indicated lentivirus were treated during 7 days with 2 μM TβRI inhibitor or left untreated. Subsequently, cells were dissociated, counted, and equal numbers of cells were inoculated in the brain of immunocompromised mice. (F) Images from the entire mouse brains were obtained by MRI. Arrowheads indicate tumors. (G) Tumor area was quantified ($p = 0.04$ comparing mice inoculated with control neurospheres with mice inoculated with Id1 overexpressing neurospheres), and (H) tumor incidence was determined. Data are presented as means ± SD. See also Figure S6.

60% (Figure 8A). Interestingly, Id1 staining did not always overlap with CD44 staining (data not shown). However, the careful analysis of the Id1 staining showed that, in around 20% of the tumors, Id1-expressing cells tend to localize in the proximity of tumor vessels (detected through CD31 staining) and coexpress high levels of CD44 (Figures 8B and 8C). We quantified the localization of Id1+ cells in four different patients, and found that around 70%–90% of the Id1+ cells were located within 100 μm from vessels (Figure S7A). The perivascular reinforcement of Id1 and CD44 staining indicated that the CD44high/Id1high GICs tend to be localized in endothelial niches (Figures 8B and 8C; Figure S7A).

(F) Cells from the indicated GBM neurospheres were left untreated or treated for 10 days with 100 pM TGF-β or 2 μM TβRI inhibitor. CD44 levels were determined by FACS analysis. Right panels show quantification of the percentage of CD44high cells. Data are presented as means. See also Figure S5.

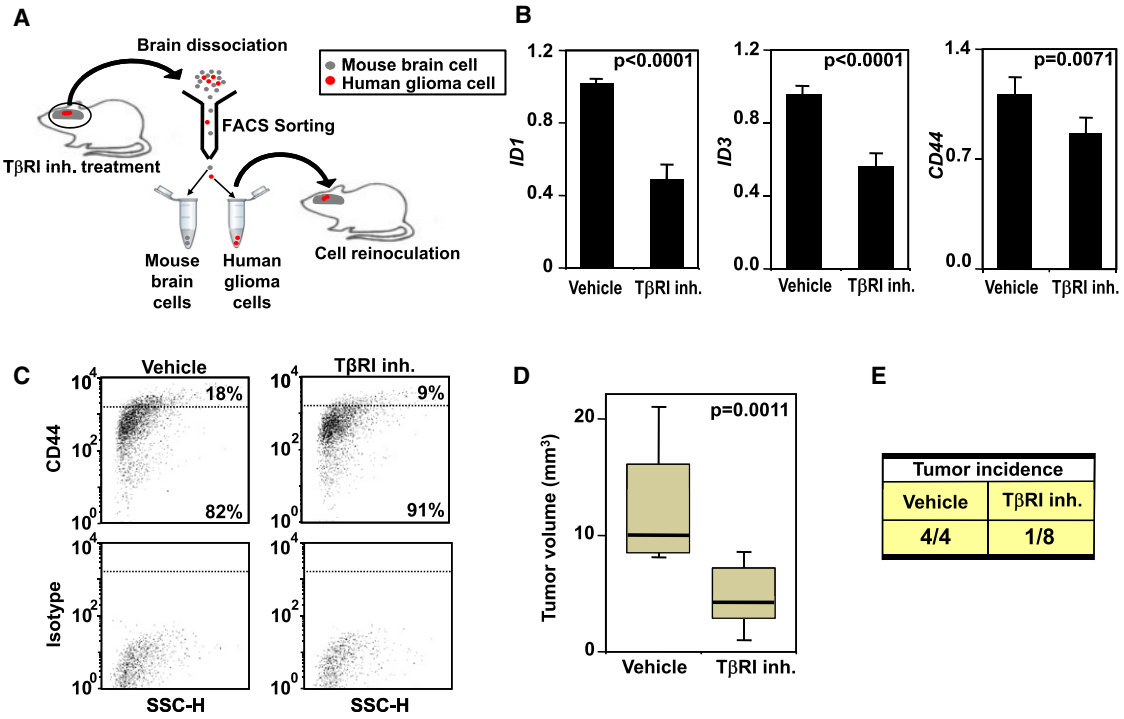

Figure 7. TβRI Inhibitor Decreases Id1, Id3, and CD44 Levels in Tumors and Prevents Tumor Recurrence in Vivo

(A) Scheme showing the experimental procedure.

(B and C) Cells from GBM1 neurospheres were inoculated in the brain of immunocompromised mice. Forty days after surgery, mice were orally treated twice a day with 100 mg/kg of TβRI inhibitor for 10 days. Subsequently, human tumor cells were isolated from the brain of mice treated or untreated with TβRI inhibitor through sorting of human MHC-I-positive cells. (B) *ID1*, *ID3*, and *CD44* transcripts levels were determined by qRT-PCR, and (C) CD44 levels were assessed by FACS. Isotype control is shown. Data are presented as means ± SD.

(D and E) Human tumor cells obtained from mice treated or untreated with the TβRI inhibitor were inoculated in the brain of secondary mice. (D) Thirty days after surgery, images from the entire mouse brains were obtained by MRI, tumor area was quantified (n = 4 mice inoculated with cells from untreated mice; n = 8 mice inoculated with cells from TβRI inhibitor treated mice), and (E) tumor incidence was determined. Data are presented as means ± SD.

If CD44^{high}/Id1^{high} cells are enriched in GICs, patients with high levels of this type of cells should have a worse prognosis, since GICs are responsible for tumor resistance to therapy and tumor relapse. To test this hypothesis, we analyzed the REMBRANDT database and observed that patients with GBM tumors expressing simultaneously high levels of Id1 and high levels of CD44 had a shorter overall survival than the rest of the patients (Figure 8D). This indicates that the combination of high levels of Id1 and CD44 is a poor prognosis factor in GBM, further confirming that CD44^{high}/Id1^{high} cells might have a critical role in tumor progression and relapse. Interestingly, high levels of Id1 in isolation also conferred poor prognosis in patients with GBM, and, moreover, GBM patients with high levels of Id1 and Id3 showed a trend toward a shorter life expectancy, although it was not statistically significant (Figures S7B and S7C).

Treatment of a GBM Patient with the TβRI Inhibitor Decreased the Id1 and CD44 Levels in the Tumor

A phase I-II clinical trial is being conducted in our hospital to test LY2157299, a derivative of the TβRI inhibitor that we used in this study (Calvo-Aller et al., 2008). A salvage surgical resection was indicated for one patient after 2 months on treatment. Since we already had a sample of the same patient's tumor prior to being enrolled in the TβRI inhibitor study, we were able to compare samples before and after treatment with the TβRI inhibitor. Due to the heterogeneity of GBM tumors, randomly selected areas of the same tumor mass were analyzed. Levels of Id1 and CD44 were determined in samples from the tumor prior to and after treatment with the TβRI inhibitor. The levels of Id1 and CD44 were decreased in the samples obtained after treatment with the TβRI inhibitor (Figures S7D and S7E), confirming our results that the TβRI inhibitor targets the CD44^{high}/Id1^{high} cell population in the context of just one human patient.

DISCUSSION

The hypothesis that, within the heterogeneous tumor mass, cells with characteristics of stem cells are responsible for tumor initiation, recurrence, and resistance to therapy is redirecting the therapeutic efforts against cancer (Dick, 2008; Gupta et al., 2009a; Zhou et al., 2009). CICs should be targeted in order to eradicate tumors and, hence, the understanding of the molecular pathways involved in the control of CICs will provide effective therapeutic targets against cancer. With this in mind, we decided to address the mechanisms of action of the TGF-β inhibition in GBM.

The TGF-β pathway has a relevant role in cancer, and several anti-TGF-β compounds are now under clinical development. In

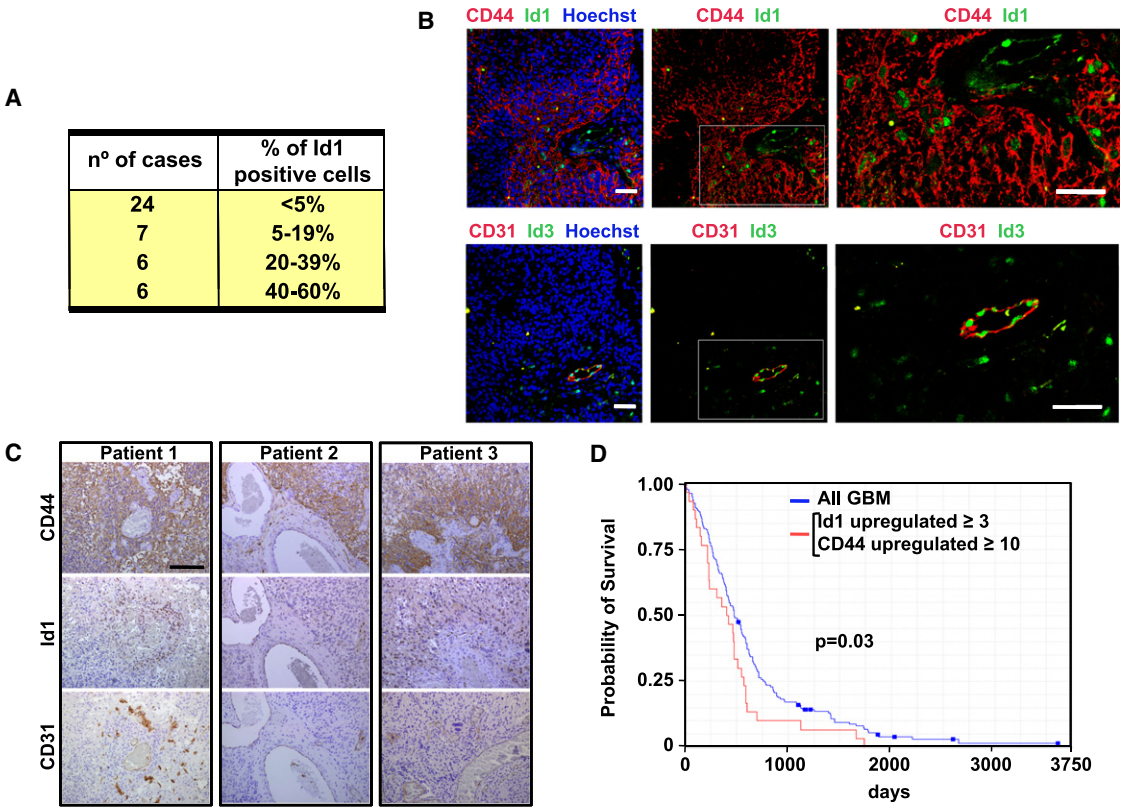

Figure 8. Id1 and CD44 Coexpression Correlates with Overall Survival in GBM Patients

(A) A tissue microarray of 43 different GBM patients was stained with an antibody against Id1 and the frequency of Id1-positive nuclei was calculated.

(B) CD44/Id1 and CD31/Id3 double immunofluorescence of samples from GBM patients. Magnification of the indicated areas stained with Id1/CD44 and Id3/CD31 are shown in the right panels. Nuclei were counterstained with Hoechst. Scale bar, 50 μm.

(C) CD44, Id1, and CD31 immunohistochemistry of samples from GBM patients. Scale bar, 100 μm.

(D) Kaplan-Meier curves showing that the overall survival of patients with both *ID1* mRNA levels upregulated ≥3-fold and *CD44* mRNA levels upregulated ≥10-fold is significantly lower than the rest of the patients ($p = 0.03$) by log-rank test. Data obtained from the Repository for Molecular Brain Neoplasia Data (REMBRANDT) program form the National Cancer Institute. See also Figure S7.

glioma, TGF-β can act as an oncogenic factor, and is considered a therapeutic target. Indeed, here we used a model of GBM based on the inoculation of patient-derived GICs in NOD-SCID mice reproducing the original tumor from the patient. Using this preclinical model, we observed that treatment with TGF-β inhibitors prevented GBM growth. In order to study the effect of TGF-β inhibitors on GBM, we decided to pursue an unbiased approach to identify all the common gene responses to the TβRI inhibitor in patient-derived tumor cells. *ID1* and *ID3* were present among the genes regulated by the TβRI inhibitor. Id proteins have been shown to be involved in cell differentiation (Perk et al., 2005), and, recently, Id1 has been described to be highly expressed in neural stem cells (Nam and Benezra, 2009). For this reason, we hypothesized that the TβRI inhibitor could have an effect on GICs through the regulation of Id1 and Id3 proteins. Indeed, Id1 and Id3 are expressed in patient-derived neurospheres, and treatment of neurospheres with the TβRI inhibitor decreased Id1 and Id3 expression.

In GBM neurospheres, TGF-β induces *ID1* transcription through a TGF-β-responsive element located at the same promoter region previously described to support the repression of *ID1* transcription by TGF-β in epithelial cells (Kang et al., 2003). Thus, TGF-β promotes two transcriptional opposed responses in epithelial cells and GBM neurospheres through the same promoter region. This antagonistic context-dependent response to TGF-β depends on the activity of the TNF-α pathway. TNF-α induces the transcriptional repressor ATF3 that, in epithelial cells, is recruited to the Id1 promoter by an activated Smad complex (Kang et al., 2003). In GBM neurospheres, in contrast to epithelial cells, ATF3 is not expressed, and the Smad complex acts as a transcriptional activator of the Id1 promoter. When GBM neurospheres are treated with TNF-α, TGF-β loses the ability to induce Id1. Hence, the dual response of Id1 to TGF-β is controlled by the crosstalk of TGF-β and TNF-α through, most likely, ATF3.

We observed that the expression of Id1 in patient-derived neurospheres was heterogeneous, with some cells expressing high levels of nuclear Id1. Interestingly, cells expressing high levels of Id1 coexpressed high levels of CD44. Several reports have shown that CICs of different tumor types (i.e., breast, colon, and prostate) are characterized by the expression of high levels of CD44 (Visvader and Lindeman, 2008). However, in glioma, CD44 has not been described as a marker to identify GICs. We observed that the CD44high/Id1high population was enriched for

GICs and generated tumors reproducing the heterogeneous cell population of the original tumor from the patient. Of note, Id1 has recently been shown to be expressed in the B1 type adult neural stem cells (Nam and Benezra, 2009), suggesting a parallelism between GICs and normal neural stem cells. Remarkably, TGF-β regulated the CD44^{high}/Id1^{high} population. The effect of TGF-β and the TβRI inhibitor on the CD44^{high}/Id1^{high} compartment took several days, and was dependent on protein synthesis, indicating that TGF-β controlled the cell differentiation status of the CD44^{high}/Id1^{high} population through, most likely, epigenetic regulation.

Taking into account that TGF-β regulated Id1 and CD44 expression, and that the Id1 regulation was prior to CD44, we hypothesized a causal relationship between the two effects. Indeed, Id1 and Id3 knockdown and Id1 overexpression decreased and increased, respectively, the CD44 ^{high} cell population. In addition, Id1 overexpression blunted the effect of the TβRI inhibitor on the CD44^{high} compartment. Thus, TGF-β and the TβRI inhibitor regulate the CD44^{high}/Id1^{high} GIC population in tumors through the regulation of Id1 and Id3, and, moreover, the decrease of Id1 is required for the effect of the TβRI inhibitor on GICs.

Our previous work and that of others (Ikushima et al., 2009; Penuelas et al., 2009) has shown that TGF-β is involved in the control of GICs through the induction of LIF, Sox2, and Sox4. Our data show that LIF, Sox2, Sox4, Id1, and Id3 are elements of a TGF-β gene program that controls and is required for the maintenance of the CD44^{high}/Id1^{high} cell population. Interestingly, Id1 and Id3 control the expression of LIF and Sox2 and Sox4, indicating that Id1 and Id3 could be the master regulators of the TGF-β-GIC gene program.

In many cases, the lack of success with GBM treatment is due to GIC-dependent tumor relapse. In order to assess the effect of TβRI inhibitors in GBM recurrence, we treated mice bearing patient-derived tumors with the anti-TGF-β compound, and determined how the treatment was affecting the CD44^{high}/Id1^{high} compartment and the capacity of tumor cells to reinitiate a tumor in another mouse recipient. In this way, we evaluated the ability of tumor cells to relapse after treatment with the TβRI inhibitor. Remarkably, cells isolated from tumors treated with the TβRI inhibitor presented a decreased CD44^{high}/Id1^{high} compartment, and generated less and smaller tumors than cells isolated from tumors treated with vehicle, indicating that anti-TGF-β treatments could be effective in preventing tumor recurrence through the elimination of GICs.

TGF-β has been previously shown to be involved in CD44^{high} CICs. The TGF-β-mediated induction of an EMT in neoplastic mammary epithelial cell populations has been described to result in the enrichment of a CD44 ^{high}/CD24^{low} population (Gupta et al., 2009b; Mani et al., 2008). The parallelism in the regulation of CICs observed in GBM and breast cancer is a concept to be studied further in order to assess whether similar mechanisms are involved in both tumor types.

In human GBM tumors, the frequency of tumoral Id1-positive cells is diverse. Notably, a perivascular reinforcement of CD44 and Id1 staining was observed in a subgroup of tumors, indicating that the CD44^{high}/Id1^{high} GICs are present in an endothelial niche, as has been described previously for CICs. Since GICs are responsible for tumor initiation, propagation, and resistance to therapy, tumors with a high frequency of CD44^{high}/Id1^{high} cells should be associated with a poor prognosis. Indeed, high Id1 and high CD44 expression in human GBM inversely correlates with survival.

We were able to confirm our results in the context of one human patient treated with the TβRI inhibitor. Since a phase I-II trial is being conducted in our hospital, we were able to obtain tumor specimens from the same patient before and after treatment with the TβRI inhibitor. Treatment with the TβRI inhibitor repressed the expression of Id1 and CD44 in the tumor of the patient, confirming that the inhibition of the TGF-β pathway targets the CD44^{high}/Id1^{high} cell population.

The characteristics that define GICs make these cells the critical therapeutic target against cancer, and treatments that do not target GICs will eventually result in tumor relapse. Our work shows that the TGF-β-Id1-CD44 axis regulates GICs, and that anti-TGF-β compounds could decrease the GIC population by inducing the transdifferentiation of the CD44^{high}/Id1^{high} population into a CD44^{low}/Id1^{low} population. Interestingly, several therapeutic anti-TGF-β compounds are now being tested in clinical trials (Seoane, 2008). Our results suggest that those compounds could be effective against GICs and GBM, providing hope in this dismal disease.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Cell Cultures

PCTCs and GBM neurospheres were generated as described previously (Bruna et al., 2007; Günther et al., 2008). Briefly, tumor samples were processed within 30 min after surgical resection. Minced pieces of human GBM 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). 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 GBM specimens were obtained from the Vall d'Hebron Hospital. The clinical protocol was approved by the Vall d'Hebron institutional review board, with informed consent obtained from all subjects.

Plasmids and Reagents

TGF-β1 (R&D Systems), TNF-α (Roche), TβRI inhibitor LY2109761 (Eli Lilly), SB431542 (Tocris), and cycloheximide (Sigma) were used at the indicated concentrations. Specific antibodies against p-Smad2, Smad2 (Cell Signaling), α-Tubulin (Sigma), Id1 (C20; Santa Cruz Biotechnology), Id3 (Biocheck), and lamin A/C and ATF3 (Santa Cruz Biotechnology) were used for immunoblotting. Antibodies against IgG (Upstate) and Smad4 (Hata et al., 2000) were used for ChIP. The following antibodies were used for the detection of proteins: anti-Id1 and anti-Id3 (both from BioCheck); anti-CD44 (Ab-4; Neomarkers); anti-CD31 (clone JC70A [DAKO] and clone 1A10 [Invitrogen]); and anti-SSEA-1 (clone MMA; Abcam).

Where indicated, a nuclear protein extraction was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce), according to the manufacturer's recommendations.

Lentiviral constructs expressing Id1 (cDNA kindly provided by Dr. Francesc Ventura) or short hairpins targeting *ID1*, *ID3*, *LIF*, *SOX2*, and *SOX4* (Open Biosystems, Thermo Scientific) were produced and packaged as previously described (Zufferey et al., 1997). Neurospheres were dissociated in growth media, mixed with virus, and plated. Polybrene (Sigma) was added at a concentration of 8 μg/ml. Cells were incubated with virus for 12 hr, washed with PBS, and incubated in fresh media as previously described (Zufferey et al., 1997).

Neurosphere-Forming and Self-Renewal Assay

For the neurosphere-forming essay, equal numbers of cells were seeded at low cell density (4 cells/μl) in wells of a 96 well plate. Cells were treated with the indicated compounds, and the total number of newly formed neurospheres was counted after 7 days in culture.

For the self-renewal assay, neurospheres were treated with the indicated compounds during 7 days, and then cells were dissociated, counted, and equal numbers of cells were replated at low cell density (4 cells/μl) without treatment in wells of a 96 well plate. The total number of newly formed neurospheres was counted after 7 days in culture, and the percentage of neurosphere-forming cells was determined (Lee et al., 2008; Reynolds and Weiss, 1996).

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 cells were 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. MRI analysis was performed in mice injected intraperitoneally with gadolinium diethylenetriamine penta-acetic acid at a dose of 0.25 mmol gadolinium/kg body weight. T1W MRI images were acquired in a 9.4 T vertical bore magnet interfaced to an AVANCE 400 system (Bruker) with a spin-echo sequence, as described previously (Penuelas et al., 2009).

Analysis of the CD44-Positive Population by Flow Cytometry

Neurospheres were dissociated and individual cells were incubated for 15 min in blocking solution containing 10 μg/ml human IgG, followed by anti-CD44 antibody or the control IgG2b isotype, both FITC conjugated (BD Pharmingen). Cells were incubated for 20 min on ice protected from light, washed in PBS, and stained with propidium iodide (Sigma) to discriminate dying cells. Cells were then analyzed by flow cytometry (FACSCalibur; Beckton Dickinson) or sorted (MoFlo; DAKO) after staining with CD44-FITC.

Isolation of Human Cells from Orthotopic Xenografts in Mouse Brains

Brains from mice inoculated with neurospheres were dissociated and stained with the pan-MHC-I-specific monoclonal antibody (mAb) HP-1F7 (Santa Cruz Biotechnology), followed by secondary PE-conjugated mAb (Dako Cytomation) for subsequent cell sorting of human MHC-I-positive cells (MoFlo-DAKO). Cells obtained were washed and immediately used in subsequent experiments.

Microarray Expression Analysis

RNA was assayed on the Affymetrix microarray platform with the Human Genome U133 Plus 2.0 GeneChips and analyzed with Genespring GX v7.3 (Agilent). The microarray data have been deposited in the Gene Expression Omnibus (GEO)/NCBI public database (accession no. GSE23935).

Statistical Analysis

Student's t test and ANOVA were performed for statistical analysis. Data in graphs are presented as means ± SD.

ACCESSION NUMBERS

Coordinates have been deposited in the Gene Expression Omnibus (GEO)/NCBI public database with accession code GSE23935.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.ccr.2010.10.023.

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