Cancer Cell
Article

High TGFβ-Smad Activity Confers Poor Prognosis in Glioma Patients and Promotes Cell Proliferation Depending on the Methylation of the PDGF-B Gene

Alejandra Bruna,[1] Rachel S. Darken,[1] Federico Rojo,[1] Alberto Ocaña,[1] Silvia Peñuelas,[1] Alexandra Arias,[1] Raquel Paris,[3] Avelina Tortosa,[4] Jaume Mora,[3] Jose Baselga,[1] and Joan Seoane[1,2,*]
[1] Medical Oncology Program, Vall d'Hebron University Hospital Research Institute, 08035 Barcelona, Spain
[2] Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain
[3] Developmental Tumor Laboratory, Hospital Sant Joan de Déu, 08950 Esplugues de Llobregat, Spain
[4] Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), Universitat de Barcelona, 08907 Hospitalet de Llobregat, Spain
*Correspondence: jseoane@ir.vhebron.net
DOI 10.1016/j.ccr.2006.11.023

SUMMARY

TGFβ acts as a tumor suppressor in normal epithelial cells and early-stage tumors and becomes an oncogenic factor in advanced tumors. The molecular mechanisms involved in the malignant function of TGFβ are not fully elucidated. We demonstrate that high TGFβ-Smad activity is present in aggressive, highly proliferative gliomas and confers poor prognosis in patients with glioma. We discern the mechanisms and molecular determinants of the TGFβ oncogenic response with a transcriptomic approach and by analyzing primary cultured patient-derived gliomas and human glioma biopsies. The TGFβ-Smad pathway promotes proliferation through the induction of PDGF-B in gliomas with an unmethylated *PDGF-B* gene. The epigenetic regulation of the *PDGF-B* gene dictates whether TGFβ acts as an oncogenic factor inducing PDGF-B and proliferation in human glioma.

INTRODUCTION

Tumors of glial origin, gliomas, are the most frequent primary tumors that arise in the brain. The most malignant form of glioma, glioblastoma multiforme (grade IV), is one of the most aggressive human cancers, with a median survival of less than 1 year. Despite recent advances in cancer biology, this statistic has not changed significantly over the past years (Holland, 2001; Kleihues et al., 2002; Maher et al., 2001; Sanai et al., 2005; Zhu and Parada, 2002).

Among other pathways, the TGFβ pathway has been implicated in glioma (Rich, 2003). TGFβ is a multifunctional cytokine that controls tissue homeostasis and embryonic development. TGFβ binds and activates a membrane receptor serine/threonine kinase complex, which phosphorylates Smad2 and Smad3. Upon phosphorylation, Smads accumulate in the nucleus, form transcriptional complexes with Smad4 and other transcription factors, and regulate transcription (Massague et al., 2005). In addition, TGFβ can signal through Smad-independent pathways (Derynck and Zhang, 2003; Moustakas and Heldin, 2005). TGFβ is a strong inhibitor of proliferation in epithelial cells, astrocytes, and immune cells, and it is considered to be a tumor suppressor factor. Some tumors acquire mutations in elements of the TGFβ pathway in order to escape from the TGFβ cytostatic response (Seoane, 2006). On the other hand, some malignant tumors, including gliomas, selectively lose the capacity of TGFβ to inhibit proliferation maintaining the TGFβ pathway intact (Seoane, 2006). In

SIGNIFICANCE

Glioma is the most common tumor of the brain, and its most malignant form, glioblastoma multiforme, is virtually incurable. Despite recent advances, further study of the molecular mechanisms governing this malignancy is required in order to design successful therapeutic protocols based on rational molecular targeting. Our work demonstrates that the TGFβ-Smad pathway has a crucial role in glioma being a molecular marker of poor prognosis. Furthermore, we identify the mechanisms and the molecular determinants of the oncogenic response to TGFβ in human glioma, showing that the induction of PDGF-B by TGFβ and the epigenetic regulation of the *PDGF-B* gene dictates the TGFβ oncogenic function. This work provides biomarkers for patient stratification in anti-TGFβ therapies and identifies therapeutic targets against this disease.

those cases, TGFβ can act as an oncogenic factor, and it can induce proliferation, angiogenesis, invasion, and metastasis as well as suppress the antitumoral immune response. Thus, TGFβ has a dual role in oncogenesis, and depending on the type and stage of the tumor, it can act as a tumor suppressor or a tumor promoter factor (Derynck et al., 2001; Siegel and Massague, 2003). The oncogenic role of TGFβ has prompted the development of therapeutic strategies based on the inhibition of the TGFβ pathway (Arteaga, 2006; Dumont and Arteaga, 2003; Yingling et al., 2004). The understanding of the mechanisms that mediate the malignant transformation of TGFβ will improve the development of rational and successful therapeutic strategies.

Little is known about the mechanisms involved in the switch of the TGFβ response toward malignancy. In a previous report, we have shown that the loss of the TGFβ antiproliferative response in glioma is in part due to the inability of TGFβ to induce p21Cip1 when the PI3K-AKT pathway is hyperactive and FoxG1 is expressed (Seoane et al., 2004). However, not much is known about the mechanisms responsible for the oncogenic function of TGFβ once the antiproliferative response is lost. PDGF-B is a polypeptide that can dimerize and form homodimers or heterodimers with PDGF-A. PDGF-BB and PDGF-AB are potent mitogens and angiogenic factors that interact with two tyrosine kinase receptors (PDGFRα and PDGFRβ) (Fredriksson et al., 2004). PDGF-B has been clearly implicated in gliomagenesis (Dai et al., 2001; Guo et al., 2003; Ostman, 2004). Our results show that TGFβ promotes glioma cell proliferation through the induction of PDGF-B in tumors with an unmethylated *PDGF-B* gene. The methylation status of the *PDGF-B* gene is what determines the ability of TGFβ to induce PDGF-B and proliferation, and hence an oncogenic response in human glioma.

RESULTS

A Hyperactive Smad Pathway Correlates with High Proliferation and Poor Prognosis in Glioma

In order to analyze the activity of the TGFβ pathway in glioma, we determined the levels of p-Smad2 in a collection of 52 patient-derived biopsies of astrocytomas of different grades obtained from surgical resections (Table 1). We decided to assess the cellular levels of p-Smad2 as an indicator of the TGFβ activity because Smad2 is the substrate of the TGFβ receptor I kinase (TβRI), and hence phosphorylated Smad2 levels correlate with the intensity of the TGFβ signal received by the cell. Immunohistochemical (IHC) analysis of the 52 tumors showed that p-Smad2 was mainly localized in the nucleus and that the intensity of the staining was variable depending on the tumor sample (Figure 1A; Table 1). Total Smad2 levels were not significantly different between tumor samples (Figure 1A; Table 1). We determined the levels of TGFβ family members that can induce Smad2 phosphorylation (TGFβ1, TGFβ2, TGFβ3, Activin A, and Nodal) in 37 samples from our collection of 52 tumors (Table 1; Figure S1 in the Supplemental Data available with this article online). A significant correlation was observed between p-Smad2 levels and TGFβ2 ($p < 0.001$; coef. 0.660; $n = 37$) and TGFβ3 ($p < 0.001$; coef. 0.459; $n = 37$). No correlation was observed between p-Smad2 and the other ligands assessed (Table 1; Figure S1). This indicates that high expression of TGFβ2 and TGFβ3 can account for the increased levels of p-Smad2 observed in tumors. Ki67 levels were also analyzed in adjacent slides in order to determine the proliferation rate of the glioma cells (Figure 1A; Table 1). p-Smad2 and Ki67 levels were plotted against each other, and a statistical analysis using the Spearman's correlation test showed a significant correlation between these parameters ($p < 0.001$) (Figure 1B). These results suggested that TGFβ could play a causal role in the induction of proliferation. Consistently, some reports have already described that TGFβ can be an inducer of cell proliferation in glioma cell lines (Piek et al., 1999; Rich et al., 1999).

We obtained a well-documented medical history of a subgroup of 25 patients with different tumor grades and treatments who were diagnosed after January 2000 and had a complete surgical resection to be able to accurately determine the time to progression (Table 1). The 25 patients were divided into two groups based on the p-Smad2 levels found on their tumor biopsies. Tumors from 13 patients expressed high levels of p-Smad2 (histo-score [H score] higher than the median of p-Smad2 H scores, 110), while 12 patients had a p-Smad2 H score equal to or lower than 110. Progression-free and overall survival curves were estimated by the Kaplan-Meier method and compared with the two-sided log-rank test (Figures 1C and 1D). A significant difference was observed in progression-free ($p = 0.0015$) and overall survival ($p = 0.012$) between patients whose tumors had high levels of p-Smad2 and those whose tumors did not. These data indicate that a hyperactive TGFβ-Smad pathway is a poor prognosis factor.

Gliomas are classified into four grades based on histological criteria set by the World Health Organization (WHO), and glioma grade is a predictor of tumor prognosis (Kleihues et al., 2002). As expected, p-Smad2 levels correlated with glioma grade (Figure S2), suggesting that the TGFβ-Smad pathway has an important role in glioma progression and is a molecular biomarker that could be used as an alternative predictor of disease outcome.

Effect of TGFβ on Glioma Cell Proliferation

In order to study the effect of TGFβ on glioma cell proliferation, we studied a panel of nine different glioma cell lines. The screen was performed taking advantage of an inhibitor of the TβRI, LY2109761 (Eli Lilly). This highly selective compound acts as an ATP-binding competitor. We first determined the dose of LY2109761 needed for the complete inhibition of the TβRI kinase activity. As Smad2 is the substrate of the TβRI kinase, we determined the minimal dose of LY2109761 required for the suppression of p-Smad2 levels. In U87MG and U373MG cell lines, a dose of 2 μM was enough to completely abolish the

Table 1. Characteristics of the Patients

Patient #	Age	Diagnosis	Grade	Surgery	Treatment	Time to progression	Overall survival	Ki67	p-SMAD2	SMAD2	Tβ1	Tβ2	Tβ3	Activin	Nodal	PDGF-B
	years					*days*	*days*	*%*	*HScore*	*HScore*	*%*	*%*	*%*	*%*	*%*	*HScore*
1	31	FA	II	CR	S	1310	1760	5	50	200	2	7	8	0	15	60
2	2	FA	II	CR	S	511	1885	1	40	250						10
3	47	GBM	IV	CR	S+Ct+Rt	536	1275	20	180	240	4	61	19	0	8	90
4	15	AA	III	CR	S+Ct+Rt	1681	1681	15	100	200	8	58	12	0	14	130
5	56	AA	III	PR	S+Ct+Rt+T			10	15							40
6	70	GBM	IV	CR	S			10	200	200						180
7	59	AA	III	PR	S+Ct+Rt			10	120	200	18	63	48	16	20	190
8	47	AA	III	CR	S+Ct+Rt	485	485	15	190	200						110
9	57	GBM	IV		S+Ct+Rt+T			15	200	210	16	64	22	37	13	210
10	71	GBM	IV	CR	S+Rt	365	365	10	250	180	19	96	56	38	53	290
11	68	GBM	IV	CR	S+Ct+Rt	144	365	10	180	250	10	68	32	7	17	120
12	41	AA	III	CR	S+Rt			10	110	230	7	40	20	9	23	250
13	29	FA	II	CR	S+Rt	861	861	5	40	200						60
14	50	GBM	IV	PR	S+Ct+Rt			15	280	210	10	92	37	6	25	270
15	4	PA	I	CR	S	561	561	10	10	230						40
16	48	AA	III	PR	S			15	120	200	9	59	24	22	36	180
17	67	GBM	IV		S			30	150	200	14	94	28	35	26	100
18	10	PA	I	CR	S	477	477	1	40	200						10
19	28	FA	II	CR	S			2	80	180	9	53	43	8	25	80
20	35	AA	III	PR	S+Ct+Rt			10	150	230						180
21	31	FA	II	CR	S+Ct+Rt	1174	1174	15	130	230						150
22	64	GBM	IV	PR	S+Rt			30	280	100						220
23	44	GBM	IV	CR	S+Ct+Rt	193	221	40	280	270	17	84	34	38	48	290
24	16	PA	I	PR	S			2	30	200	22	45	13	35	31	40
25	52	GBM	IV	PR	S+Ct+Rt			10	100	230	11	53	15	22	19	210
26	18	PA	I	CR	S	1171	1171	2	30	200	12	26	3	51	15	40
27	71	GBM	IV	PR	S			15	200	200	22	67	39	37	40	180
28	9	PA	I	CR	S	1765	1765	5	10	250	8	4	9	20	17	40
29	30	FA	II	PR	S+Ct+Rt			15	180	180						110
30	74	GBM	IV	CR	S+Rt	332	422	25	110	280	44	73	30	31	76	180
31	63	GBM	IV	CR	S	60	60	10	200	200	75	83	28	36	74	150
32	46	GBM	IV	PR	S+Rt			10	200	180	100	100	100	53	100	270
33	59	GBM	IV	CR	S+Ct+Rt	292	365	35	110	250	26	34	9	0	42	240
34	1	PA	I	CR	S	849	849	2	100	250	15	28	1	19	37	150
35	16	PA	I	PR	S			10	100	200	50	78	78	46	46	100
36	35	AA	III	PR	S+Ct+Rt+T			20	190							180
37	13	PA	I		S			1	10	230	16	41	4	0	29	80
38	31	FA	II		S			1	30	200	10	40	10	0	24	10
39	31	PA	I	CR	S	377	377	1	70	180	25	43	10	87	33	30
40	18	GBM	IV	CR	S+Ct+Rt	189	485	10	200	250	48	92	18	37	38	90
41	74	GBM	IV	CR	S	60	60	15	210	180	44	59	41	100	27	220
42	70	GBM	IV	CR	S+Ct+Rt			35	280	200	33	63	34	30	32	130
43	18	PA	I	CR	S			2	0	150	15	42	2	8	26	10
44	49	GBM	IV	CR	S+Ct+Rt	333	333	30	130	150	23	39	22	38	45	180
45	70	GBM	IV	CR	S+Ct+Rt	392	480	15	230	150	16	43	3	37	33	230
46	70	GBM	IV	CR	S			10	150	200	14	61	3	39	35	210
47	33	FA	II	CR	S+Rt			2	80	250	36	53	29	74	50	160
48	42	FA	II	PR	S+Rt			5	120	250	33	58	27	45	40	80
49	14	PA	I	CR	S			5	110	200						130
50	5	PA	I	CR	S	113	113	1	0	200	67	64	37	77	44	10
51	35	AA	III		S+Ct+Rt			1	10	150						30
52	35	GBM	IV	CR	S+Ct+Rt	147	395	20	230	200						180

AA, anaplastic astrocytoma; FA, fibrillary astrocytoma; GBM, glioblastoma; PA, pilocytic astrocytoma; CR, complete resection; PR, partial resection; S, surgery; Ct, chemotherapy; Rt, radiotherapy; T, temozolomide.

induction of p-Smad2 by 200 pM TGFβ (Figure S3). Next, we performed a BrdU incorporation assay with the panel of nine glioma cell lines treated with TGFβ and/or 2 μM LY2109761 in order to determine the effect of TGFβ on cell-cycle progression. Glioma cell lines were classified in two groups depending on whether TGFβ induced proliferation (U373MG, A172, C4) or whether TGFβ inhibited or did not have any major effect on proliferation (U87MG, T98G, C3, C52, hs683, U251) (Figure 2A). Previous reports using some of those cell lines agreed with our results (Piek et al., 1999; Rich et al., 1999). In all the cell lines tested, the TβRI inhibitor blocked the TGFβ response and did not have a major effect on proliferation when assayed in isolation (Figure 2A).

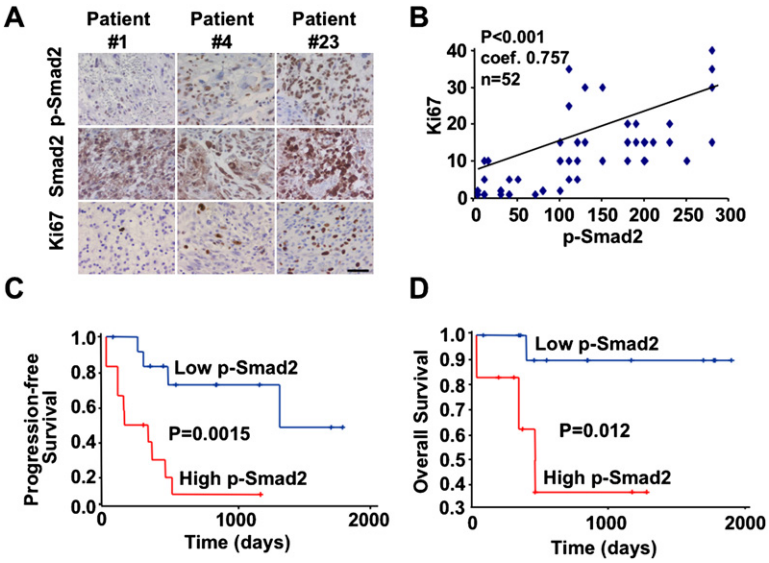

Figure 1. Effect of the TGFβ-Smad Pathway in Human Glioma Proliferation

(A) Immunohistochemical staining of human glioma sections derived from patients 1, 4, and 23 (Table 1) using p-Smad2, Smad2, or Ki67 antibodies. Scale bar, 100 μm.

(B) Correlation between the levels of p-Smad2 and Ki67 (n = 52; p < 0.001, two-tailed Spearman test coefficient: 0.757).

(C and D) Kaplan-Meier estimates of time to progression and overall survival. Differences between patients with high p-Smad2 (H score p-Smad2 >110) and low p-Smad2 (H score p-Smad2 ≤ 110) were highly significant (p = 0.0015 and p = 0.012) by log-rank test in time-to-progression and overall survival curves, respectively.

We decided to focus on two cell lines in which TGFβ had opposed effects, U373MG and U87MG. We performed a BrdU incorporation assay treating the cells with increasing amounts of TGFβ for 20 hr, and we blocked TGFβ signaling with the LY2109761 compound or a neutralizing antibody against TGFβ (Figure 2B). TGFβ increased or decreased BrdU incorporation in a dose-dependent manner in U373MG cells and U87MG cells, respectively, and the effect of TGFβ was blocked by either the TβRI inhibitor or the TGFβ-neutralizing antibody. In addition, cells were treated with TGFβ and/or LY2109761 and counted after 72 hr. TGFβ increased the number of U373MG cells and decreased the number of U87MG cells (Figure S4). The TβRI inhibitor blocked the TGFβ effect on both cell lines and did not affect cell proliferation when tested in isolation (Figure S4).

Mediators of TGFβ-Induced Proliferation

We next decided to discern the mechanism of the induction of proliferation by TGFβ in glioma. TGFβ was able to induce proliferation in three of the nine glioma cell lines tested. Moreover, the induction of proliferation by TGFβ was dependent on the TβRI activity because the TGFβ effect was blocked by LY2109761. In order to identify which TGFβ responses mediate the induction of proliferation, we compared the TGFβ-responsive genes of U373MG cells (where TGFβ induces proliferation) and U87MG cells (where TGFβ inhibits proliferation) (see Figure 2A). U373MG and U87MG cell lines were treated with TGFβ, LY2109761, or a combination of both for 3 hr to detect the TGFβ responses that are rapid and hence more likely to be direct. RNA was extracted from those cells, and a transcriptomic analysis using U133A plus Affymetrix microarrays was performed. We focused on the gene responses regulated by TGFβ that were blocked by the presence of TβRI inhibitor. We obtained 78 and 87 gene responses to TGFβ in U373MG and U87MG cells, respectively, that were dependent on the TβRI activity. Consistent with what has been previously reported (Piek et al., 1999; Rich et al., 1999; Seoane et al., 2004), p21Cip1 and c-Myc were not regulated by TGFβ in U373MG and U87MG cell lines (see Figure 2D). The p15Ink4b gene is deleted in both cell lines (Rich et al., 1999). This indicates that the TGFβ cytostatic response program is lost in U373MG and U87MG cell lines. Comparing the gene responses to TGFβ in U373MG and U87MG, we observed 63 gene responses specific for U373 cells; 15 genes were common between U373MG and U87MG, and 72 genes were specific for U87MG (Figure 2C; Table 2). Interestingly, most of the 15 common TGFβ-responsive genes were previously described to be regulated by TGFβ in epithelial cells (Kang et al., 2003). This fact validated our approach and the transcriptomic analysis. We carefully analyzed the 63 gene responses to TGFβ that were specific for U373MG cells looking for genes that could explain the increase in proliferation observed in TGFβ-treated U373MG cells. Six genes from the U373MG-specific responses were described to be involved in cell proliferation using the Gene Ontology description of molecular functions (Table 2). From those six genes, *PDGF-B* stood out. PDGF-B is a well-known factor that has already been shown to be involved in glioma progression and has been implicated in the TGFβ-proliferative function (Dai et al., 2001; Fredriksson et al., 2004; Ostman, 2004).

We first validated that *PDGF-B* mRNA levels were induced by TGFβ in U373MG and not in U87MG using RT-PCR (Figure 2D). We also validated 13 other genes, including the five remaining gene responses classified as related to cell proliferation by Gene Ontology and *p21Cip1* and *c-Myc* as part of the TGFβ cytostatic response (Figure 2D). We checked whether TGFβ induced the secretion of the PDGF-BB and PDGF-AB ligands using an ELISA assay. Indeed, U373MG cells treated with TGFβ secreted much more PDGF-BB and PDGF-AB compared to U87MG (Figure 2E). The secreted PDGF-BB and PDGF-AB were functional, as we detected an increase in the

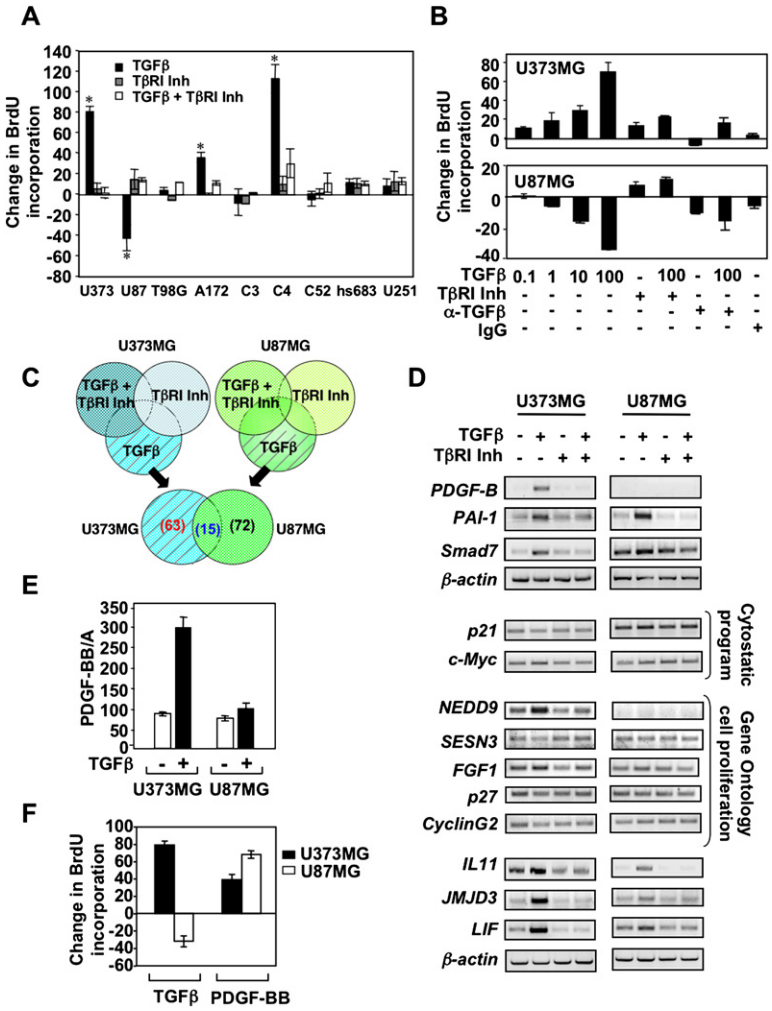

Figure 2. Mediators of the TGFβ Proliferative Response

(A) Cells from the indicated human glioma cell lines were serum deprived and treated with 100 pM TGFβ (black bars), 2 μM TβRI inhibitor (gray bars), or both (white bars) for 20 hr, and a BrdU incorporation assay was performed. Results are represented as the percentage change from control (DMSO-treated cultures). All data are represented as the mean ± SD from three independent experiments. (*$p < 0.05$, **$p < 0.001$).

(B) U373MG and U87MG cells were treated with increasing doses of TGFβ, 2 μM of TβRI inhibitor, 2 μg/ml of TGFβ1-neutralizing antibody, and an IgG control antibody as indicated, and a BrdU incorporation assay was performed. Results are represented as in (A).

(C) Venn diagram representing data obtained from the microarray analysis of gene expression profiles of serum-starved U373MG and U87MG cells treated for 3 hr with 100 pM TGFβ and/or 2 μM LY2109761.

(D) U373MG and U87MG cells were treated with 100 pM TGFβ and/or 2 μM LY2109761 for 3 hr in serum-free media, and the levels of the indicated transcripts were determined by RT-PCR. *β-actin* was determined as a loading control.

(E) Levels of PDGF-BB/AB were determined by an ELISA assay in conditioned media from U373MG and U87MG cells treated or untreated with TGFβ for 72 hr.

(F) U373MG and U87MG cells were treated with 100 pM TGFβ or 20 μM recombinant PDGF-BB in serum-free media. Proliferation was determined by a BrdU incorporation assay 20 hr after treatment. Results are expressed as in (A).

phosphorylation of the PDGF receptor in U373MG treated with TGFβ. Yet, in U87MG, the PDGF receptor remained unphosphorylated upon TGFβ addition (Figure S5). We next confirmed that the PDGF-B pathway was functional in both cell lines, since recombinant PDGF-BB was able to induce proliferation in U373MG and U87MG cells (Figure 2F).

Role of PDGF-B as a Mediator of the Induction of Proliferation by TGFβ

We next assessed whether PDGF-B was also induced by TGFβ in other glioma cell lines. We screened the panel of nine glioma cell lines and found that PDGF-B was induced by TGFβ in four of them, U373MG, A172, C4, and C52 (Figure 3A). Either PDGFRα or PDGFRβ was expressed in all the cell lines tested, indicating that the TGFβ-induced PDGF-B had appropriate receptors available to transduce the signal (Figure 3A). To determine whether the TGFβ pathway was functional, we determined the induction of p-Smad2 levels and the TGFβ target genes *PAI-1* and *Smad7* by TGFβ. Indeed, TGFβ increased p-Smad2 levels and induced either PAI-1 or Smad7 in all the cell lines tested (Figure 3A).

Our data showed a strong relationship between the induction of PDGF-B and the proliferative response to TGFβ (Figure 3A and Figure 2A). In U373MG, A172, and C4, TGFβ induced both proliferation and *PDGF-B* transcription. In C52 cells, TGFβ induced PDGF-B but had no effect on proliferation. Recombinant PDGF-BB was not able to induce proliferation in C52 cells (data not shown), indicating that the PDGF-BB pathway is not functional in those cells.

We decided to assess whether the induction of proliferation by TGFβ was mediated by PDGF-B. For this purpose, we used a neutralizing antibody against PDGF-B to specifically block the effect of the secreted PDGF-BB and PDGF-AB in TGFβ-treated cells. U373MG cells were treated with TGFβ, recombinant PDGF-BB, and/or anti-PDGF-BB for 72 hr, and then an MTT assay was performed to determine the number of viable cells. The neutralizing antibody against PDGF-B decreased both the TGFβ- and the recombinant PDGF-BB-mediated induction of proliferation in U373MG cells, and it did not affect cell proliferation when assayed in isolation (Figure 3B). We used another approach to discern the effect of PDGF-B on TGFβ-treated cells. We generated cell lines derived

Table 2. TGFβ-Responsive Genes in U373MG and U87MG Cells

U373MG specific gene responses

Symbol	Description	Gene Ontology
COL4A1	collagen, type IV, alpha 1	↑ Extracellular Matrix
PCDH9	protocadherin 9	↑ Cell adhesion
DACT1	dapper homolog 1	↑
JMJD3	jumonji domain containing 3	↑
C18orf25	chromosome 18 ORF 25	↑
NKX3-1	NK3 transcription factor related	↑ Transcription
ARK5	AMP-activated protein kinase 5	↑
NEDD9	neural developmentally down-regulated 9	↑ Cell proliferation
FGF1	fibroblast growth factor 1 (acidic)	↑ Cell proliferation
ENC1	ectodermal-neural cortex	↑
LHX6	LIM homeobox 6	↑ Transcription
LIF	leukemia inhibitory factor	↑ Immune response
HLA-DRB1	major histocompatibility complex II DR beta 1	↑
DNM3	dynamin 3	↑
DLG1	DKFZP586B0319 protein	↑
KLF7	Kruppel-like factor 7	↑ Transcription
LEFTY2	left-right determination factor 2	↑ Signaling
MAFK	v-maf oncogene K	↑ Transcription
GLS	glutaminase	↑ Metabolism
SLITRK6	SLIT and NTRK-like family, member 6	↑
KCNJ15	potassium inwardly-rectifying channel J15	↑
ZNF92	zinc finger protein 92 (HTF12)	↑ Transcription
PDGFB	platelet-derived growth factor B	↑ Cell proliferation
C14orf139	chromosome 14 open reading frame 139	↑
TBX3	T-box 3	↑
PRSS35	protease, serine, 35	↓ Proteolysis
CCNG2	cyclin G2	↓ Cell proliferation
TP53INP1	p53 inducible nuclear protein 1	↓ Apoptosis
SEC24D	SEC24 related gene family, member D	↓
OLFM3	olfactomedin 3	↓
BIRC3	baculoviral IAP repeat-containing 3	↓ Apoptosis
CHI3L1	chitinase 3-like 1	↓ Metabolism
GFAP	glial fibrillary acidic protein	↓
FLRT3	fibronectin leucine rich transmembrane 3	↓ Cell adhesion
HNMT	histamine N-methyltransferase	↓
SESN3	Sestrin 3	↓ Cell proliferation
SBLF	stoned B-like factor	↓
CDKN1B	cyclin-dependent kinase inhibitor 1B	↓ Cell proliferation
TXNIP	thioredoxin interacting protein	↓
PI15	protease inhibitor 15	↓
ARRDC3	arrestin domain containing 3	↓
SSPN	Sarcospan	↓
VAMP1	vesicle-associated membrane protein 1	↓
IFIH1	interferon induced with helicase C domain 1	↓ Apoptosis
IFIT2	interferon-induced protein 2	↓ Immune response
BTG2	BTG family, member 2	↓ DNA repair
PNRC1	proline-rich nuclear receptor coactivator 1	↓ Transcription
KBTBD6	kelch repeat and BTB (POZ) domain 6	↓
CALCRL	Calcitonin receptor-like	↓ Signaling
HAS2	Hyaluronan synthase 2	↓
TWIST1	twist homolog 1	↓ Transcription
CEBPD	C/EBPdelta	↓ Transcription
GDA	guanine deaminase	↓ Metabolism
DDIT4	DNA-damage-inducible transcript 4	↓
GATM	glycine amidinotransferase	↓
KLF9	Kruppel-like factor 9	↓ Transcription
KBTBD7	kelch repeat and BTB (POZ) domain 7	↓
ABCA13	ATP-binding cassette, sub-family A 13	↓
CNR1	cannabinoid receptor 1	↓ Signaling
C18orf14	chromosome 18 ORF 14	↓
PKP2	plakophilin 2	↓ Cell adhesion
COL11A1	collagen, type XI, alpha 1	↓ Extracellular Matrix
SEMA3A	semaphorin 3A	↓ Signaling

U373MG and U87MG common gene responses

Symbol	Description	Gene Ontology
C14orf31	chromosome 14 ORF 31	↑
FLJ10970	hypothetical protein FLJ10970	↑
GADD45B	growth arrest and DNA-damage-inducible, beta	↑
SKIL	SKI-like	↑ Transcription
IL11	interleukin 11	↑ Signalling
SMAD7	SMAD, mothers against DPP homolog 7	↑ Transcription
IL6	interleukin 6	↑ Signalling
PAI1	plasminogen activator inhibitor type 1	↑
NFATC2	Nuclear factor of activated T-cells	↑ Transcription
SOX4	SRY (sex determining region Y)-box 4	↑ Transcription
COL1A1	collagen, type I, alpha 1	↑ Extracellular matrix
JAG1	Jagged 1	↑ Signalling
TUFT1	tuftelin 1	↑
ID2	inhibitor of DNA binding 2	↓

U87MG specific gene responses

Symbol	Description	Gene Ontology
ADM	adrenomedullin	↑
TSPAN2	tetraspanin 2	↑
CHST3	carbohydrate (chondroitin 6) sulfotransferase 3	↑
NANOS1	nanos homolog 1	↑
DDX6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	↑
ANXA1	Annexin A1	↑
VDR	vitamin D receptor	↑ Signaling
RUNX1	runt-related transcription factor 1	↑ Transcription
EREG	Epiregulin	↑ Signaling
FKHL18	forkhead-like 18	↑ Transcription
KCTD4	potassium channel tetramerisation domain 4	↑
ARID5B	AT rich interactive domain 5B	↑
PTX3	pentraxin-related gene	↑
RGS4	regulator of G-protein signalling 4	↑
PGM2L1	phosphoglucomutase 2-like 1	↑ Metabolism
LRRC15	leucine rich repeat containing 15	↑
ARID2	AT rich interactive domain 2	↑
RHOB	ras homolog gene family, member B	↑
TGFBR3	transforming growth factor, beta receptor III	↑ Signaling
DDIT4L	DNA-damage-inducible transcript 4-like	↑
AMOTL2	angiomotin like 2	↑
PHLDA1	pleckstrin homology-like domain A1	↑
KIF21A	kinesin family member 21A	↑
MBNL1	Muscleblind-like	↑
FST	follistatin	↑ Signaling
LHX8	LIM homeobox 8	↑
FGF18	Fibroblast growth factor 18	↑ Signaling
KLF10	Kruppel-like factor 10	↑ Transcription
GLI2	GLI-Kruppel family member GLI2	↑ Signaling
ADAM19	a disintegrin and metalloproteinase domain 19	↑ Protease
HEY1	hairy/enhancer-of-split related YRPW motif 1	↑ Transcription
KLF5	Kruppel-like factor 5	↑ Transcription
MAP3K4	Mitogen-activated protein kinase kinase kinase 4	↑ Signaling
C15orf29	Chromosome 15 ORF 29	↑
AMIGO2	amphoterin induced gene 2	↑
LIN7A	lin-7 homolog A	↑
HMOX1	heme oxygenase 1	↑
PLAT	plasminogen activator, tissue	↑
ZNF542	zinc finger protein 542	↑ Transcription
CITED2	Cbp/p300-interacting transactivator 2	↑ Transcription
CCRN4L	CCR4 carbon catabolite repression 4-like	↑
AIG1	Androgen-induced 1	↓
TPST1	tyrosylprotein sulfotransferase 1	↓
PPM2C	protein phosphatase 2C	↓
DNAJB5	DnaJ (Hsp40) homolog, B 5	↓
STK38L	serine/threonine kinase 38 like	↓
BCOR	BCL6 co-repressor	↓ Transcription
RRAD	Ras-related associated with diabetes	↓
DLX1	distal-less homeo box 1	↓ Transcription
BCL2L11	BCL2-like 11	↓ Apoptosis
FOXO1A	forkhead box O1A	↓ Transcription
ISG20	interferon stimulated gene 20kDa	↓
TNFAIP6	tumor necrosis factor, alpha-induced protein 6	↓
HS3ST2	heparan sulfate 3-O-sulfotransferase 2	↓
DKK1	dickkopf homolog 1	↓
C20orf112	chromosome 20 open reading frame 112	↓
BMPR2	bone morphogenetic protein receptor, type II	↓ Signaling
SOX9	SRY (sex determining region Y)-box 9	↓ Transcription
PPP1R14C	protein phosphatase 1, regulatory subunit 14C	↓
ID4	inhibitor of DNA binding 4	↓ Transcription
PTHLH	parathyroid hormone-like hormone	↓
MSC	musculin	↓
IL1B	interleukin 1, beta	↓ Signaling
SMAD6	SMAD, mothers against DPP homolog 6	↓ Signaling
TPM1	Tropomyosin 1 (alpha)	↓
GALNTL1	UDP-N-acetyl-alpha-D-galactosamine	↓ Metabolism
C10orf30	Chromosome 10 open reading frame 30	↓
IL1F5	interleukin 1 family, member 5 (delta)	↓ Signaling
SNF1LK	SNF1-like kinase	↓
ULK1	unc-51-like kinase 1	↓
TRIB1	tribbles homolog 1	↓
FAT	FAT tumor suppressor homolog 1	↓

U373MG and U87MG cells were treated with TGFβ and/or the TβRI inhibitor for 3 hr, and then total RNA was subjected to Affymetrix analysis with the U133-A plus microarray. Arrows indicate whether TGFβ induced or repressed gene expression.

from U373MG that constitutively expressed a short-hairpin RNA targeting PDGF-B. The TGFβ-induced *PDGF-B* mRNA as well as PDGF-BB/AB ligands were greatly diminished in two U373MG cell lines that expressed two independent short-hairpin RNAs, U373 sh1-PDGF-B and U373 sh2-PDGF-B (Figures 3C and 3D). When cell proliferation was assayed, the induction of proliferation by TGFβ was partially blocked in the cell lines that expressed sh1-PDGF-B and sh2-PDGF-B (Figure 3E). Altogether, these results indicated that PDGF-B is one of the main mediators of the induction of proliferation by TGFβ in U373MG cells.

STI571 (Gleevec, Novartis) is a potent inhibitor of PDGF kinase receptors as well as of Abl kinase and the c-Kit kinase receptor (Druker, 2004). To complement and corroborate our previous results, we used STI571 as a tool to assess the effect of an inhibition of the PDGF receptor on TGFβ-induced proliferation. In order to determine the dose of STI571 to be used in our experiments, we first performed a dose-response experiment of STI571 in U373MG cells treated or untreated with PDGF-BB. Addition of STI571 in isolation inhibited U373MG cell proliferation in a dose-dependent manner. Moreover, the lowest concentration of STI571 that repressed the induction of proliferation mediated by 20 nM recombinant PDGF-BB was 5 μM (Figure S6). We decided to assess whether 5 μM STI571 could suppress TGFβ-induced proliferation in U373MG cells. We first observed that 5 μM STI571 prevented the phosphorylation of the PDGF receptor in response to TGFβ and to recombinant PDGF-BB. Thus, STI571 inhibited the activation of the PDGF receptor by TGFβ (Figure 3F). STI571 was able to decrease cell proliferation when assayed in isolation despite that in untreated U373MG cells the PDGF receptor is not phosphorylated and hence not active (Figures 3F and 3G). This suggested that STI571 was acting by PDGF receptor-independent mechanisms in untreated U373MG cells. However, STI571 inhibited the TGFβ-mediated induction of

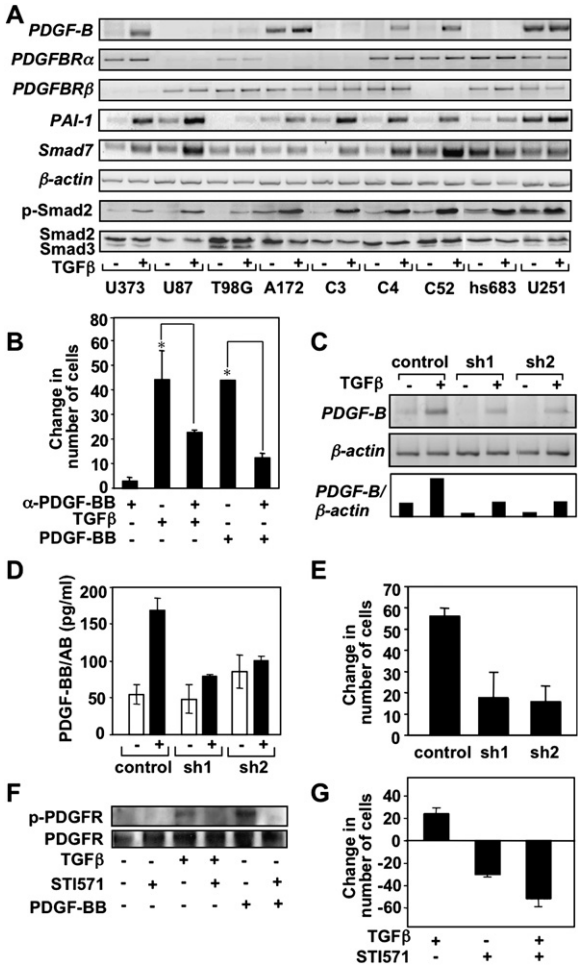

Figure 3. PDGF-B Mediates TGFβ-Induced Proliferation in Glioma Cells

(A) The indicated nine human glioma cells lines were left untreated or treated with 100 pM TGFβ for 3 hr in serum-free media, and the levels of *PDGF-B*, *PDGFRα*, *PDGFRβ*, *PAI-1*, *Smad7*, and *β-actin* mRNA were determined by RT-PCR analysis. p-Smad2 and Smad2 were determined by immunoblotting in cells treated under the same conditions.

(B) Number of viable cells was determined using an MTT assay in U373MG cells after a 72 hr treatment with 1 ng/ml of PDGF-B-neutralizing antibody and/or 100 pM TGFβ or 20 μM recombinant PDGF-BB in 0.2% serum media. Results are represented as the percentage change from control (untreated cells). All data represent the mean ± SD from three experiments (*$p < 0.05$).

(C and D) RNA interference was used to specifically knock down TGFβ-induced PDGF-B in U373MG. Polyclonal cell cultures stably expressing sh1-PDGF-B, sh2-PDGF-B, or a control vector (*control*) were treated with 100 pM TGFβ for 3 hr; RNA was harvested; and *PDGF-B* mRNA levels were determined by RT-PCR analysis. *β-actin* expression was used for normalization. Alternatively, serum-starved cells were treated with 100 pM TGFβ for 72 hr, and levels of PDGF-BB/AB were analyzed by ELISA in the conditioned media.

(E) U373MG-TH (*control*), U373MG-sh1-PDGF-B, and U373MG-sh2-PDGF-B cells were treated with 100 pM TGFβ for 72 hr in 0.2% serum media. Number of viable cells was determined by an MTT assay, and results were expressed as the percentage change from control (DMSO-treated cells). All data represent the mean ± SD from three experiments.

(F) U373MG cells were pretreated with 5 μM STI571 for 2 hr and treated with either 100 pM TGFβ or 20 μM of recombinant PDGF-BB for a further 3 hr. Equal amounts of protein were subjected to immunoprecipitation using an anti-PDGFR antibody and immunoblotting using antibodies against phospho-tyrosine (upper panel) and PDGFR (lower panel).

(G) U373MG cells were treated with 100 pM TGFβ, 5 μM STI571, and 20 μM of recombinant PDGF-BB in 0.2% serum media for 72 hr as indicated, and cells were counted. Results are expressed as the percentage change from control (untreated cells). All data represent the mean ± SD from three experiments.

proliferation due to its effect on the PDGF receptor (see Figure 3F), and moreover, the combination of TGFβ and STI571 inhibited proliferation to a greater extent than STI571 in isolation. This suggested that, in the presence of STI571, TGFβ had an antiproliferative response in U373MG cells (Figure 3G). In order to identify the mechanism that mediates this antiproliferative effect, we analyzed the regulation of genes involved in cell-cycle arrest (*p21Cip1*, *p27Kip1*, and *c-Myc*) as well as *Smad7* and *PAI-1*. Interestingly, an increase in p21Cip1 levels was detected in cells treated with TGFβ and STI571 and could explain the antiproliferative effect of the combination of TGFβ and STI571 (Figure S7).

Mechanisms of the Differential Activation of PDGF-B in Response to TGFβ

Our results showed that TGFβ promotes proliferation via the induction of PDGF-B expression in certain glioma cells. Still, we did not know why TGFβ was able to induce PDGF-B in some glioma cells but not in others. In order to address this issue, we decided to discern the molecular mechanisms involved in the activation of *PDGF-B* transcription by TGFβ. First, we assessed whether the induction of PDGF-B by TGFβ in U373MG was direct or required new protein synthesis. TGFβ induced PDGF-B in the presence of cycloheximide, an inhibitor of RNA translation, indicating that the transactivation of *PDGF-B* by TGFβ did not require the synthesis of other factors (Figure 4A). Other reports have shown that Smads can bind and transactivate the proximal region of the *PDGF-B* promoter in endothelial cells and macrophages (Taylor and Khachigian, 2000; Chow et al., 2005). To determine whether Smads were also involved in the induction of PDGF-B in glioma cells, we knocked down Smad2, Smad3, Smad2 and -3, and Smad4 using RNA interference and determined whether PDGF-B induction by TGFβ was affected. The induction of PAI-1 by TGFβ was also analyzed as an experimental control. Indeed, Smad2, Smad3, and Smad4 were required for PDGF-B induction by TGFβ, since PDGF-B levels were decreased when any of the Smads were downregulated in the presence of TGFβ (Figure 4B). We also performed a chromatin immunoprecipitation assay (ChIP) and observed that endogenous Smad2 or -3 bound the proximal *PDGF-B* promoter in response to TGFβ in U373MG cells but not in U87MG (Figure 4C). Smads did not bind to a distal promoter region of the *PDGF-B* gene (Figure 4C). The fact that Smads did not interact with the *PDGF-B* promoter in U87MG could explain why PDGF-B was not induced by TGFβ in those cells.

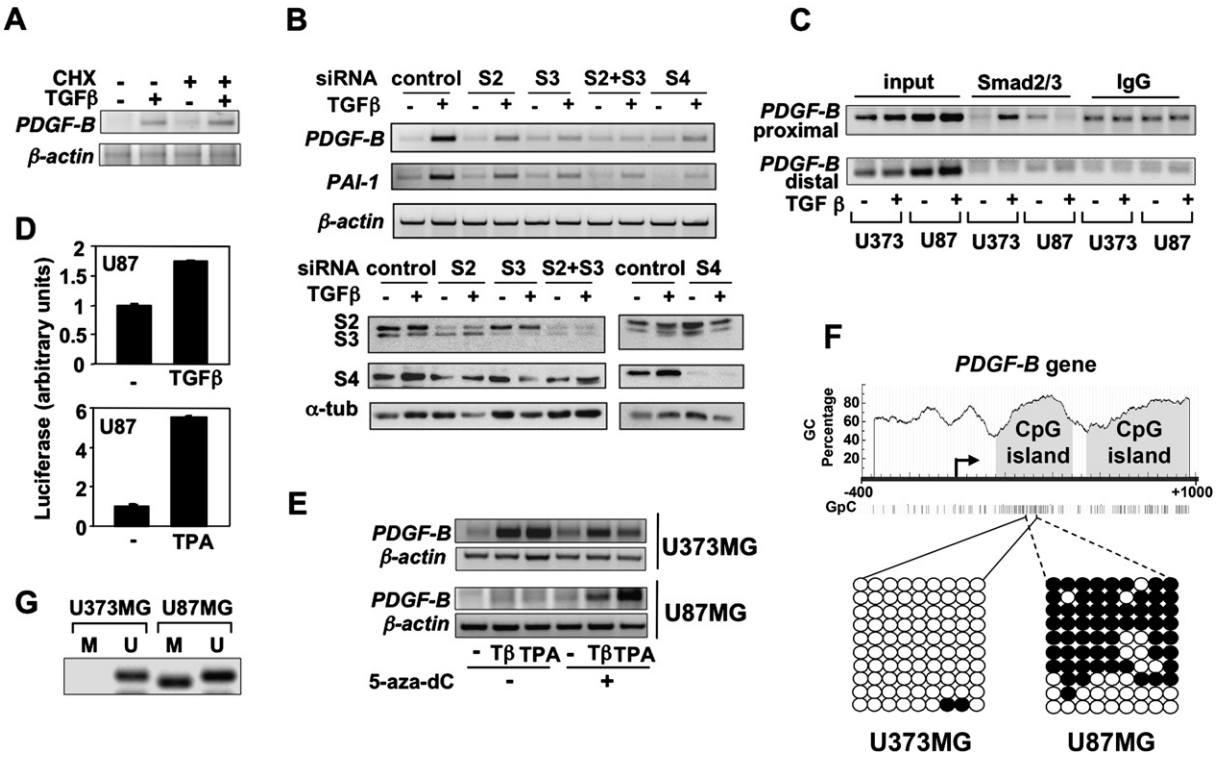

Figure 4. Methylation of the *PDGF-B* Gene Blocks the Induction of PDGF-B by TGFβ

(A) U373MG cells were treated for 16 hr with cycloheximide (*CHX*) and then treated with 100 pM TGFβ for a further 3 hr. mRNA levels of *PDGF-B* and *β-actin* were determined by RT-PCR analysis.

(B) RT-PCR analysis to determine *PDGF-B*, *PAI-1*, and *β-actin* levels, and immunoblotting using specific antibodies against Smad2/3, Smad4, or α-tubulin were performed in U373MG cells treated with 100 pM TGFβ for 3 hr after siRNA-mediated knockdown of Smad2, Smad3, Smad2, and Smad3 or Smad4 as indicated.

(C) U373MG and U87MG cells were treated with 100 pM TGFβ for 3 hr, and ChIP assays were performed with the indicated antibodies and the indicated PCR primers.

(D) U87MG cells were transfected with the −396/+84 *PDGF-B* luciferase reporter, treated with 100 pM TGFβ or 0.2 μM TPA for 20 hr and analyzed for luciferase activity.

(E) U373MG and U87MG were left untreated or treated with 50 μM 5-aza-dC for 3 days and then treated with 100 pM TGFβ or 0.2 μM TPA for 3 hr. *PDGF-B* and *β-actin* mRNA levels were determined by RT-PCR analysis.

(F) Distribution of CpG islands in the −400/+1000 PDGF-B promoter region. Arrow represents the transcription start site. Determination of the methylation status of CpG sites by sequencing of bisulfite-modified DNA. Circles represent the CpG sites present in the +342/+372 PDGF-B promoter region (methylated black circles, unmethylated open circles).

(G) Methylation status of the PDGF-B gene in U373MG and U87MG cells determined by methylation-specific PCR assay. *M*, methylated; *U*, unmethylated.

Strikingly, TGFβ was able to transactivate the −396/+84 *PDGF-B* luciferase reporter in U87MG cells (Figure 4D). Moreover, TPA, a well-known inducer of PDGF-B in endothelial cells (Jin et al., 1993), activated the *PDGF-B* luciferase reporter more than 5-fold (Figure 4D). Interestingly, both TPA and TGFβ did not induce endogenous *PDGF-B* transcription in U87MG cells (Figure 4E). The fact that TGFβ and TPA induced the *PDGF-B* reporter but not the endogenous *PDGF-B* gene in U87MG cells suggested that there was a regulation of the *PDGF-B* gene at the level of the chromatin structure. Thus, we hypothesized that the *PDGF-B* promoter was silenced by methylation in U87MG cells and not in U373MG. Indeed, using a bioinformatic approach, we observed that the 5′UTR of the *PDGF-B* gene contained two CpG islands (see Figure 4F). Inhibition of methylation with an inhibitor of DNA methyltransferases, 5-aza-2′-deoxycytidine (5-aza-dC), facilitated the induction of endogenous PDGF-B by TGFβ and TPA in U87MG, indicating that DNA methylation could be blocking the TGFβ-mediated induction of PDGF-B (Figure 4E). Consistently, Smad2/3 bound the proximal PDGF-B promoter in response to TGFβ in U87MG cells treated with 5-aza-dC (Figure S8). In addition, U87MG cells treated with the 5-aza-dC lost the partial antiproliferative effect of TGFβ in a dose-dependent manner, indicating that the rescue of the induction of PDGF-B could antagonize the antiproliferative response to TGFβ (Figure S9). Sequencing of bisulfite-modified DNA showed that one of the CpG islands of the 5′UTR of *PDGF-B* was highly methylated in U87MG compared to U373MG (Figure 4F). In addition, we developed a *m*ethylation-specific *P*CR (MSP) assay and confirmed the bisulfite

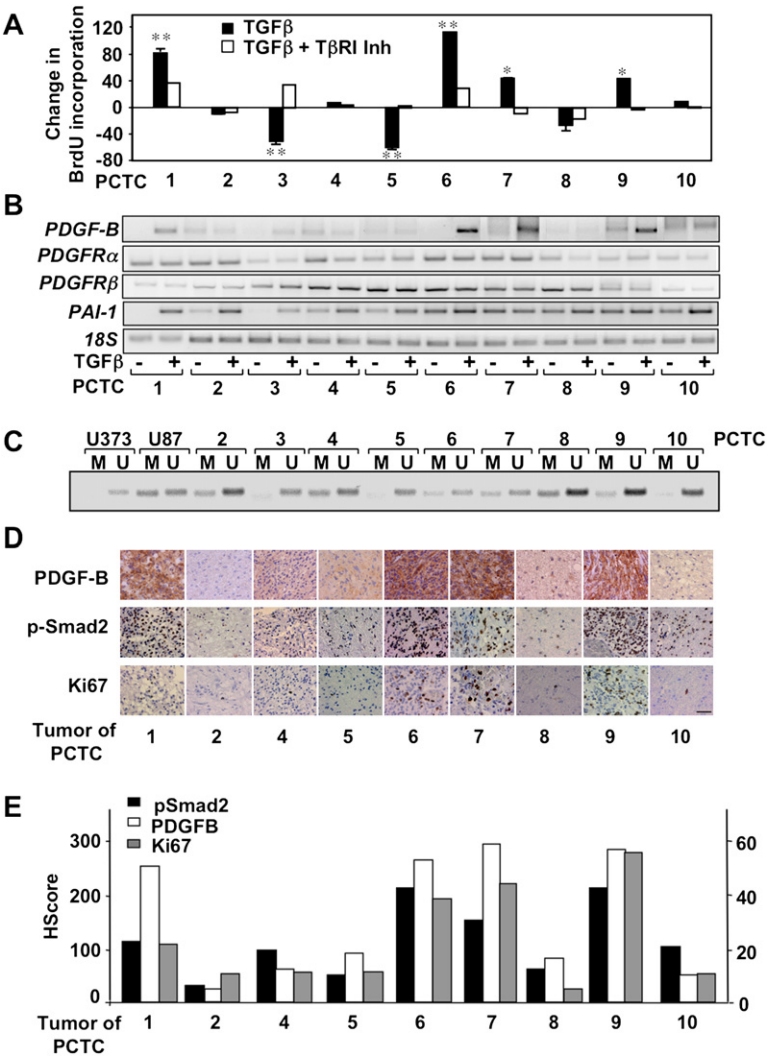

Figure 5. TGFβ Proliferative Response and PDGF-B Induction in Primary Cultured Tumor Biopsies

(A) Serum-deprived primary cultured tumor cells (PCTC 1–10) were treated with 100 pM TGFβ alone or in combination with 2 μM TβRI inhibitor for 20 hr. BrdU incorporation was measured and is expressed as the percentage change from control (DMSO-treated cultures). All data are represented as the mean ± SD of triplicate experiments. (*$p < 0.05$, **$p < 0.001$).

(B) PCTCs were left untreated or treated with 100 pM TGFβ for 3 hr in serum-free media and subjected to RT-PCR analysis to detect the mRNA levels of the indicated genes.

(C) Methylation status of the *PDGF-B* gene in PCTCs 2-10 determined by methylation-specific PCR assay.

(D) PDGF-B, p-Smad2, and Ki67 levels were determined by IHC in the nine human gliomas from which we obtained PCTCs. Scale bar, 100 μm.

(E) Quantification of PDGF-B, p-Smad2, and Ki67 levels determined in (D).

sequencing results (Figure 4G). Altogether, these results showed that methylation of the *PDGFB* gene prevents the induction of PDGFB by TGFβ in U87MG cells.

The TGFβ Proliferative Response, PDGF-B Induction, and Methylation of the *PDGF-B* Gene in Patient-Derived Gliomas

Established cell lines have been in culture for a long time adapting to grow in artificial conditions and diverging from the original tumor cells (Lee et al., 2006). We decided to analyze the TGFβ proliferative response in cells from human gliomas grown in culture for a very short period of time, hence, with characteristics similar to the cells present in the tumor mass (Lee et al., 2006). Therefore, we extended our studies to primary cultured tumor cells (PCTCs) from patient-derived glioma biopsies. Tumor cells from ten different gliomas of diverse grades were seeded less than half an hour after tumor resection, and in less than three to five passages we obtained enough cells to perform a cell proliferation assay. Cells were left untreated or treated with TGFβ or with TGFβ plus the TβRI inhibitor, and a BrdU incorporation assay was performed. TGFβ induced proliferation in four PCTCs, decreased proliferation in two PCTCs, and had no major effect on the rest of the PCTCs (Figure 5A). In all cases, the TβRI inhibitor blocked the TGFβ effect, indicating that the effect of TGFβ on proliferation was dependent on the TβRI activity. In addition, the levels of *PDGF-B*, *PDGFRα/β*, and *PAI-1* transcripts were analyzed by RT-PCR. PDGFRα or -β was expressed in all PCTCs. Moreover, PAI-1 was induced by TGFβ, indicating that the TGFβ pathway was functional in the PCTCs (Figure 5B). We observed that only four PCTCs induced PDGF-B in response to TGFβ. Interestingly, those four PCTCs were the same ones in which TGFβ induced proliferation (Figures 5A and 5B). Our results showed that the capacity of TGFβ to induce proliferation coincided with its ability to induce PDGF-B both in PCTCs as well as in glioma cell lines. Moreover, we analyzed the methylation status of the *PDGF-B* gene of PCTCs 2–10 using the MSP assay and bisulfite-treated DNA sequencing in order to assess whether methylation was responsible for the lack of the PDGF-B induction by

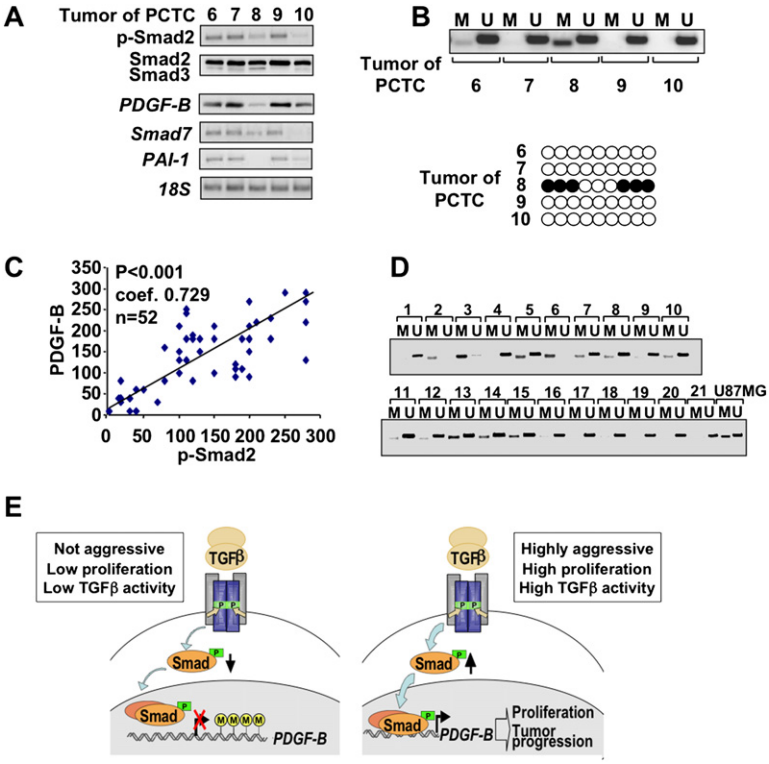

Figure 6. The Methylation Status of the PDGF-B Gene Accounts for the Lack of PDGF-B Expression in Human Glioma

(A) p-Smad2 and Smad2/3 protein levels and *PDGF-B*, *Smad7*, *PAI-1*, and *18S* mRNA levels were determined in the human gliomas from which we obtained PCTC 6–10 using immunoblotting and RT-PCR analysis, respectively.

(B) Methylation status of the *PDGF-B* gene in tumors from PCTCs 6–10 determined by methylation-specific PCR assay and sequencing of bisulfite-modified DNA. (Black circles represent CpG sites found methylated in more than a 50% of ten sequenced clones.)

(C) Correlation between the levels of p-Smad2 and PDGF-B ($n = 52$; $p < 0.001$, two-tailed Spearman test coefficient: 0.729).

(D) Methylation status of the 21 tumors expressing low levels of PDGF-B (H score < 100) determined by methylation-specific PCR assay of paraffin sections. *M*, methylated; *U*, unmethylated.

(E) In gliomas that are not aggressive and have low levels of proliferation, the induction of PDGF-B by TGFβ is impaired due to methylation of the *PDGF-B* promoter, and the TGFβ-Smad pathway is poorly active. On the other hand, in highly aggressive and proliferative gliomas, the *PDGF-B* gene is not methylated, PDGF-B is induced by TGFβ, and the TGFβ-Smad pathway is hyperactive.

TGFβ. Three (PCTCs 2, 4, and 8) of the six PCTCs in which TGFβ did not induce PDGF-B had a methylated gene indicating that the lack of TGFβ-mediated *PDGF-B* transactivation is due to epigenetic silencing (Figure 5C; Figure S10). Still other unknown mechanisms besides methylation are preventing the induction of PDGF-B by TGFβ in PCTCs 3, 5, and 10. As expected, none of the PCTCs in which TGFβ induced PDGF-B had a methylated *PDGF-B* gene. Moreover, neither normal human neuroprogenitors, human fetal astrocytes, nor human fetal neurons had a methylated *PDGF-B* gene (Figure S10).

Next, we decided to analyze the tumor samples that generated the PCTCs. The levels of p-Smad2, PDGF-B, and Ki67 were analyzed by IHC in paraffin sections of the tumors that generated the PCTCs except for PCTC 3 due to lack of sample. As expected from our results in Figure 1, p-Smad2 levels correlated with Ki67 levels. Interestingly, PDGF-B levels also correlated with p-Smad2 levels. Moreover, high levels of p-Smad2, PDGF-B, and Ki67 were detected in those tumors in which TGFβ induced cell proliferation and not in the others (Figures 5A, 5D, and 5E). We then determined the p-Smad2 and total Smad2 levels of the tumors that generated PCTCs 6, 7, 8, 9, and 10 by immunoblotting and observed that in the tumors that generated the PCTCs in which TGFβ was not inducing proliferation (PCTCs 8 and 10), p-Smad2 levels were low, corroborating and validating the IHC result (Figure 6A). In addition, *PDGF-B*, *Smad7*, and *PAI-1* RNA levels were analyzed by RT-PCR, and the expression of all three TGFβ target genes was also low in those tumors that generated PCTCs 8 and 10 (Figure 6A). In order to address whether the epigenetic regulation of the *PDGF-B* gene was involved in the lack of PDGF-B expression, we determined the methylation status of the *PDGF-B* gene sequencing of bisulfite-modified DNA and MSP assay in the tumor samples. The tumor that expressed the lowest levels of PDGF-B and had low p-Smad2 levels (tumor of PCTC 8) had its promoter methylated (Figure 6B). This agrees with the results of the *PDGF-B* methylation status of PCTC 8 (see Figure 5C and Figure S10).

We finally analyzed the levels of PDGF-B in the collection of 52 astrocytomas by IHC and checked whether high levels of PDGF-B correlated with high levels of p-Smad2. Indeed, that was the case, and a strong and significant correlation ($p < 0.001$) was observed between PDGF-B levels and p-Smad2 levels (Figure 6C). Moreover, we asked whether methylation of the *PDGF-B* gene was responsible for the lack of expression of PDGF-B in the collection of astrocytoma samples. We analyzed those astrocytomas with low expression of PDGF-B (H score < 100), 21 tumors, using the MSP assay and found that around 50% of them (ten tumors) had a highly methylated *PDGF-B* gene (Figure 6D). Interestingly, only one of the ten tumors with a methylated *PDGF-B* gene had high levels of p-Smad2, indicating that there is not a selective pressure during tumor progression to acquire a hyperactive TGFβ-Smad pathway when the *PDGF-B* gene is methylated.

In summary, tumors with low TGFβ-Smad activity and low PDGF-B levels were the ones that generated PCTCs in which TGFβ did not induce proliferation, and vice versa,

tumors with an activated Smad pathway and high PDGF-B levels generated PCTCs in which TGFβ induced PDGF-B and proliferation. Thus, when TGFβ acts as a proliferative factor by inducing PDGF-B, it favors glioma progression and provides a selective advantage for tumor progression, and the tumor tends to acquire a hyperactive TGFβ-Smad pathway. In addition, the methylation of the *PDGF-B* gene accounts for the inability of TGFβ to induce *PDGF-B* transcription and hence proliferation in a large proportion of human gliomas, and tumors do not acquire a hyperactive, oncogenic TGFβ-Smad pathway when the *PDGF-B* gene is methylated.

DISCUSSION

Recent advances in the understanding of the molecular mechanisms that govern oncogenesis have provided meaningful progress in the treatment of many common human cancers. Still, much more has to be done in order to improve present therapeutic approaches. Malignant glioma is one of the most aggressive human cancers, and treatment strategies for this disease have only increased survival slightly (Holland, 2001; Maher et al., 2001; Zhu and Parada, 2002). Among several other pathways, TGFβ has been implicated in glioma (Rich, 2003). In normal epithelial cells, TGFβ is a potent inhibitor of proliferation, and it has been considered a tumor suppressor. During tumor progression, however, the TGFβ antiproliferative function is lost, and in certain cases TGFβ becomes an oncogenic factor inducing cell proliferation, invasion, angiogenesis, and immune suppression (Derynck et al., 2001; Siegel and Massague, 2003). Recently, we and others have begun to unveil the mechanisms through which TGFβ loses its antiproliferative response in glioma (Seoane, 2006). However, not much is known about how TGFβ promotes tumorigenesis.

We have focused our work on the study of the oncogenic role of TGFβ in glioma. We found that a high p-Smad2 level is a poor prognostic marker, supporting that TGFβ is acting as an oncogenic factor in glioma and has an important role in glioma progression. Moreover, this indicates that p-Smad2 is a molecular biomarker of disease outcome in glioma. The significant correlation observed between p-Smad2 levels and the levels of TGFβ2 and TGFβ3 in human tumors indicates that high expression of these two ligands can be responsible for the increased activity of the TGFβ-Smad pathway. In addition, the fact that there is a good correlation between p-Smad2 levels and Ki-67 in human glioma suggested that TGFβ is involved in human glioma cell proliferation. Indeed, we found that TGFβ induces proliferation in some glioma cell lines as it was reported (Piek et al., 1999; Rich et al., 1999). Comparing the TGFβ gene responses of two cell lines (U373MG and U87MG) where TGFβ has opposed effects on proliferation, we found 63 genes that could be involved in TGFβ-mediated induction of proliferation. Among those genes, we focused on PDGF-B due to its known role in carcinogenesis. Indeed, PDGF-B was induced whenever TGFβ activated proliferation. Moreover, blockade of the PDGF-B function (using neutralizing antibodies, RNA interference, or inhibitors of the PDGF receptor) prevented TGFβ-dependent induction of proliferation. Moreover, in primary cultured tumor cells, PDGF-B was induced whenever TGFβ induced proliferation. Consistently, in a collection of 52 gliomas, we found that high p-Smad2 levels strongly correlated with high levels of PDGF-B. These results strongly suggested that PDGF-B is a mediator of the proliferative response to TGFβ in glioma.

TGFβ induced PDGF-B in some gliomas but not in others. In order to assess what determined the induction of PDGF-B by TGFβ, we pursued the study of the molecular mechanisms involved in the PDGF-B transcriptional activation by TGFβ. RNA interference and chromatin immunoprecipitation experiments demonstrated that *PDGF-B* transactivation is mediated by an activated Smad complex that binds to the proximal promoter of *PDGF-B*. Interestingly, Smad2 or -3 did not bind to the proximal promoter of *PDGF-B* in U87MG in response to TGFβ, even though TGFβ activated transcription of a reporter construct containing the proximal region of the *PDGF-B* promoter. This inconsistency could be explained by the chromatin structure of the endogenous *PDGF-B* gene and, more specifically, could be due to the epigenetic regulation of the *PDGF-B* gene. Treatment with methyltransferase inhibitors and sequencing of bisulfite-modified DNA demonstrated that the 5′UTR of the *PDGF-B* gene had methylated CpG islands in U87MG cells. Moreover, around 50% of human gliomas with low PDGF-B expression have a methylated *PDGF-B* gene, indicating that epigenetic silencing accounts for the lack of PDGF-B induction by TGFβ and therefore for the inability of TGFβ to induce glioma cell proliferation in a large proportion human gliomas. Several years ago, PDGF was suggested to be involved in the response to TGFβ of three hyperdiploid glioma cell lines (Jennings et al., 1997), although other studies failed to find this link (Piek et al., 1999; Rich et al., 1999). Our results demonstrate that the proliferative response to TGFβ in human glioma is mediated by the induction of PDGF-B and is dictated by the methylation status of the *PDGF-B* gene.

In many cases, cancer progression is favored by hypermethylation of the promoter of tumor suppressors. Our results showed that lack of methylation of a specific gene facilitates TGFβ-induced proliferation and oncogenesis. To date there is only one other similar and recently published observation. Hypomethylation of the *Pax2* gene facilitates tamoxifen-induced endometrial carcinogenesis showing, as in our case, that hypomethylation of a specific gene can be involved in tumor progression (Wu et al., 2005). In neuroprogenitor cells, the *PDGF-B* promoter is not methylated. This suggests that the presence of an unmethylated *PDGF-B* gene in certain tumors is a characteristic that resembles the neuroprogenitor state and that methylation of the *PDGF-B* gene observed in some tumors might be due to a process of cell differentiation or to an aberrant epigenetic regulation. This is an ongoing subject of study.

The involvement of the Smad-dependent or Smad-independent pathways in TGFβ-promoted oncogenesis

has been a subject of debate (Derynck and Zhang, 2003; Dumont and Arteaga, 2003). Our present work argues that a Smad-dependent signaling through the induction of PDGF-B has a proliferative and oncogenic role in glioma. High levels of p-Smad2 are present in highly proliferative and aggressive tumors when TGFβ is able to induce PDGF-B and proliferation. On the other hand, those tumors where TGFβ is not able to induce PDGF-B and proliferation, mostly due to methylation of the *PDGF-B* gene, do not have high levels of TGFβ activity and tend not to be aggressive. Moreover, tumors with a methylated *PDGF-B* gene do not present a hyperactive TGFβ-Smad pathway (Figure 6E). This might be due to the fact that the induction of PDGF-B by TGFβ favors glioma progression, providing a selective advantage to the tumor cell, and allowing the tumor to acquire a hyperactive TGFβ-Smad pathway. In contrast, tumors with a methylated *PDGF-B* gene do not tend to acquire a hyperactive TGFβ-Smad pathway, since TGFβ is then unable to act as an oncogenic factor. In addition, the fact that PDGF-B mediates the TGFβ proliferative response suggests that blocking PDGF-B function, for example with PDGF receptor inhibitors such as STI571, might prevent the TGFβ oncogenic function.

Due to its oncogenic role, the TGFβ pathway is being evaluated as a therapeutic target (Arteaga, 2006; Dumont and Arteaga, 2003; Yingling et al., 2004). The potential role of TGFβ inhibitors on angiogenesis, immune surveillance, and EMT, in addition to their ability to block PDGF-B induction and thus proliferation, suggests a promising therapeutic benefit of such compounds. The dual and complex role of TGFβ in oncogenesis presents a unique challenge that has to be addressed to be able to select the patient population that may benefit from an anti-TGFβ therapy. The understanding of the exact mechanisms involved in the malignant transformation of TGFβ will improve patient stratification and the development of successful therapeutic strategies as well as provide therapeutic targets to restore normal TGFβ function.

EXPERIMENTAL PROCEDURES

Cell Lines and Primary Cultured Tumor Cells

U87MG, U373MG, A172, and T98G were obtained from American Type Culture Collection. hs683 and U251 were obtained from D. Bigner. C3, C4, and C52 were obtained from J. Cowell. All cell lines were cultured in DMEM with 10% fetal bovine serum (FBS). Fresh brain tumor tissues obtained from both Vall d'Hebron and Sant Joan de Déu Hospitals were collected and processed within 30 min after resection. The clinical protocol was approved by the Vall d'Hebron IRB (CEIC) with informed consent obtained from all subjects. The primary cultured tumor cells were obtained after mechanical dissociation according to the technique previously described (van Beusechem et al., 2002). Briefly, tumor tissue was cut into pieces of ~1–5 mm^3 and plated in a 60 mm^2 tissue culture dish with DMEM with 10% FBS and antibiotics. Additionally and in parallel, minced pieces of tumor were incubated with 200 U/ml collagenase I (Sigma) and 500 U/ml DNaseI (Sigma) in PBS during 1 hr at 37°C with vigorous constant agitation as previously described (Joshi et al., 2000). The single-cell suspension was filtered through a 70 μm cell strainer (BD Falcon), washed with PBS, and suspended in DMEM-10% FBS. Cell cultures were subsequently split 1:2 when confluent and experiments were done before passage 3–5.

Microarray Expression Analysis

RNA was harvested from U373MG and U87MG cell lines treated as indicated. Three independent experiments were performed. Five micrograms of extracted total RNA was used to generate biotinylated complementary RNA (cRNA) following the standard Affymetrix GeneChip protocol. Each sample was hybridized with an Affymetrix Human Genome U133A microarray at the Vall d'Hebron Research Institute Genomics facility. CEL files were imported into the ArrayAssist package (Stratagene) and preprocessed using the RMA (robust multiarray analysis) algorithm with the default parameters. Genes were filtered according to the following criterion: AbsFC with respect to their respective control experiments $\geq$ 2. Genes complying with these criteria were then used for later study. The microarray data have been submitted to the European Bioinformatics Institute (EBI) public database (accession number E-MEXP-903).

Immunohistochemistry

Tumor biopsies were obtained from patients with histological diagnosis of glioblastoma, anaplastic astrocytoma, fibrillary astrocytoma, and pilocytic astrocytoma according to the World Health Organization (Kleihues et al., 2002). Informed consent was obtained prospectively, and tissue collection was approved by each Institutional Review Board. Fresh tumor samples were collected from primary tumors under surgery. Samples were fixed immediately after removal in a 10% buffered formalin solution for a maximum of 48 hr at room temperature before being dehydrated and paraffin embedded under vacuum. Areas of representative tumor, away from necrotic foci, were identified on a hematoxylin-eosin-stained section, three 0.6 mm cores were taken from separate areas, and each one was arrayed into recipient blocks in a 1 mm-spaced grid. The following antibodies were used for the detection of proteins: anti-PDGF-B (H55 rabbit polyclonal antibody, Santa Cruz Biotech) (Lou et al., 2004; Toda et al., 1999), anti-Ki67 (clone MIB1, Dako), and anti-p-Smad2 (clone 138D4, Cell Signaling Tech) (Kang et al., 2005). The specificity of the staining with anti-PDGF-B and anti-p-Smad2 antibodies was controlled using other antibodies, preincubating the antibody with the antigen, and performing immunoblotting of glioma extracts (Figure S11). To score a tumor cell as positive, cytoplasmic staining was required for PDGF-B, and nuclear staining was required for Ki67 and p-Smad2. For quantitative analysis of PDGF-B, p-Smad2, and Smad2, the percentage of stained tumor cells and intensity of staining were evaluated in representative high-power fields (×400) on tissue sections using optical microscopy. The result was expressed as a H score ranged 0–300 and calculated as the percentage of weakly stained cells plus the percentage of moderately stained cells multiplied by two plus the percentage of strongly stained cells multiplied by three. For Ki67, the percentage of tumor-stained cells was calculated in representative microscopic fields. Scoring was performed blind to clinical data and was used for statistical analysis.

Statistical Analysis

Overall and progression-free survival curves were estimated by the Kaplan-Meier method and compared with the use of the two-sided log-rank test. Time of tumor recurrence was established from the time of surgery to the date when recurrence was detected. Overall survival was measured from the date of surgical resection to the last follow-up visit or death. Patients without tumor recurrence or alive at the end of follow-up were censored. A Spearman correlation test was used to analyze relationships between the following pairs of parameters: TGFβ ligands and p-Smad2, Ki67 and p-Smad2, and PDGF-B and p-Smad2. An ANOVA test was used to analyze tumor grade and p-Smad2. Data in bar graphs are expressed as the mean ± SD.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and eleven supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/11/2/147/DC1/.

ACKNOWLEDGMENTS

We thank Jonathan M. Yingling from Lilly Research Laboratories for helpful discussion and for providing LY2109761 and Serena Di Cosimo for helpful discussion. We are indebted to D. Park, W.E. Fahl, and D. Trono for reagents. We also thank Fátima Núñez and the Genomic Core Facility of the Vall d'Hebron Hospital Research Institute; Santiago Ramón y Cajal, José Jiménez, Arancha Ortega, and the Pathology Department; and Juan Sahuquillo, Carlos Domínguez, M[a] Antonia Poca, and the Neurosurgery Department of the Vall d'Hebron Hospital. A.B. is an Asociación Española Contra el Cáncer fellow, and R.S.D. is an EMBO fellow. This work has been supported by the Spanish Ministry of Health grant FIS(PI040766), and European Comission Marie Curie International Reintegration grant (MIRG-CT-2005-017121).

Received: June 22, 2006
Revised: October 11, 2006
Accepted: November 16, 2006
Published: February 12, 2007

REFERENCES

Arteaga, C.L. (2006). Inhibition of TGFβ signaling in cancer therapy. Curr. Opin. Genet. Dev. *16*, 30–37.

Chow, E.K., O'Connell, R.M., Schilling, S., Wang, X.F., Fu, X.Y., and Cheng, G. (2005). TLR agonists regulate PDGF-B production and cell proliferation through TGF-β/type I IFN crosstalk. EMBO J. *24*, 4071–4081.

Dai, C., Celestino, J.C., Okada, Y., Louis, D.N., Fuller, G.N., and Holland, E.C. (2001). PDGF autocrine stimulation dedifferentiates cultured astrocytes and induces oligodendrogliomas and oligoastrocytomas from neural progenitors and astrocytes in vivo. Genes Dev. *15*, 1913–1925.

Derynck, R., and Zhang, Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature *425*, 577–584.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-β signaling in tumor suppression and cancer progression. Nat. Genet. *29*, 117–129.

Druker, B.J. (2004). Imatinib as a paradigm of targeted therapies. Adv. Cancer Res. *91*, 1–30.

Dumont, N., and Arteaga, C.L. (2003). Targeting the TGFβ signaling network in human neoplasia. Cancer Cell *3*, 531–536.

Fredriksson, L., Li, H., and Eriksson, U. (2004). The PDGF family: Four gene products form five dimeric isoforms. Cytokine Growth Factor Rev. *15*, 197–204.

Guo, P., Hu, B., Gu, W., Xu, L., Wang, D., Huang, H.J., Cavenee, W.K., and Cheng, S.Y. (2003). Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. Am. J. Pathol. *162*, 1083–1093.

Holland, E.C. (2001). Gliomagenesis: Genetic alterations and mouse models. Nat. Rev. Genet. *2*, 120–129.

Jennings, M.T., Hart, C.E., Commers, P.A., Whitlock, J.A., Martincic, D., Maciunas, R.J., Moots, P.L., and Shehab, T.M. (1997). Transforming growth factor beta as a potential tumor progression factor among hyperdiploid glioblastoma cultures: Evidence for the role of platelet-derived growth factor. J. Neurooncol. *31*, 233–254.

Jin, H.M., Brady, M.L., and Fahl, W.E. (1993). Identification and characterization of an essential, activating regulatory element of the human SIS/PDGFB promoter in human megakaryocytes. Proc. Natl. Acad. Sci. USA *90*, 7563–7567.

Joshi, B.H., Plautz, G.E., and Puri, R.K. (2000). Interleukin-13 receptor alpha chain: A novel tumor-associated transmembrane protein in primary explants of human malignant gliomas. Cancer Res. *60*, 1168–1172.

Kang, Y., Chen, C.R., and Massague, J. (2003). A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol. Cell *11*, 915–926.

Kang, Y., He, W., Tulley, S., Gupta, G.P., Serganova, I., Chen, C.R., Manova-Todorova, K., Blasberg, R., Gerald, W.L., and Massague, J. (2005). Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. Proc. Natl. Acad. Sci. USA *102*, 13909–13914.

Kleihues, P., Louis, D.N., Scheithauer, B.W., Rorke, L.B., Reifenberger, G., Burger, P.C., and Cavenee, W.K. (2002). The WHO classification of tumors of the nervous system. J. Neuropathol. Exp. Neurol. *61*, 215–225.

Lee, J., Kotliarova, S., Kotliarov, Y., Li, A., Su, Q., Donin, N.M., Pastorino, S., Purow, B.W., Christopher, N., Zhang, W., et al. (2006). Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell *9*, 391–403.

Lou, S.M., Li, Y.M., Wang, K.M., Cai, W.M., and Weng, H.L. (2004). Expression of platelet-derived growth factor-BB in liver tissues of patients with chronic hepatitis B. World J. Gastroenterol. *10*, 385–388.

Maher, E.A., Furnari, F.B., Bachoo, R.M., Rowitch, D.H., Louis, D.N., Cavenee, W.K., and DePinho, R.A. (2001). Malignant glioma: Genetics and biology of a grave matter. Genes Dev. *15*, 1311–1333.

Massague, J., Seoane, J., and Wotton, D. (2005). Smad transcription factors. Genes Dev. *19*, 2783–2810.

Moustakas, A., and Heldin, C.H. (2005). Non-Smad TGF-β signals. J. Cell Sci. *118*, 3573–3584.

Ostman, A. (2004). PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. Cytokine Growth Factor Rev. *15*, 275–286.

Piek, E., Westermark, U., Kastemar, M., Heldin, C.H., van Zoelen, E.J., Nister, M., and Ten Dijke, P. (1999). Expression of transforming-growth-factor (TGF)-beta receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF-β1. Int. J. Cancer *80*, 756–763.

Rich, J.N. (2003). The role of transforming growth factor-beta in primary brain tumors. Front. Biosci. *8*, e245–e260.

Rich, J.N., Zhang, M., Datto, M.B., Bigner, D.D., and Wang, X.F. (1999). Transforming growth factor-beta-mediated p15(INK4B) induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines. J. Biol. Chem. *274*, 35053–35058.

Sanai, N., Alvarez-Buylla, A., and Berger, M.S. (2005). Neural stem cells and the origin of gliomas. N. Engl. J. Med. *353*, 811–822.

Seoane, J. (2006). Escaping from the TGFβ anti-proliferative control. Carcinogenesis *27*, 2148–2156.

Seoane, J., Le, H.V., Shen, L., Anderson, S.A., and Massague, J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell *117*, 211–223.

Siegel, P.M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer. Nat. Rev. Cancer *3*, 807–821.

Taylor, L.M., and Khachigian, L.M. (2000). Induction of platelet-derived growth factor B-chain expression by transforming growth factor-beta involves transactivation by Smads. J. Biol. Chem. *275*, 16709–16716.

Toda, S., Nishimura, T., Yamada, S., Koike, N., Yonemitsu, N., Watanabe, K., Matsumura, S., Gartner, R., and Sugihara, H. (1999). Immunohistochemical expression of growth factors in subacute thyroiditis and their effects on thyroid folliculogenesis and angiogenesis in collagen gel matrix culture. J. Pathol. *188*, 415–422.

van Beusechem, V.W., Grill, J., Mastenbroek, D.C., Wickham, T.J., Roelvink, P.W., Haisma, H.J., Lamfers, M.L., Dirven, C.M., Pinedo, H.M., and Gerritsen, W.R. (2002). Efficient and selective gene transfer into primary human brain tumors by using single-chain antibody-targeted adenoviral vectors with native tropism abolished. J. Virol. *76*, 2753–2762.

Wu, H., Chen, Y., Liang, J., Shi, B., Wu, G., Zhang, Y., Wang, D., Li, R., Yi, X., Zhang, H., et al. (2005). Hypomethylation-linked activation of PAX2 mediates tamoxifen-stimulated endometrial carcinogenesis. Nature *438*, 981–987.

Yingling, J.M., Blanchard, K.L., and Sawyer, J.S. (2004). Development of TGF-β signalling inhibitors for cancer therapy. Nat. Rev. Drug Discov. *3*, 1011–1022.

Zhu, Y., and Parada, L.F. (2002). The molecular and genetic basis of neurological tumours. Nat. Rev. Cancer *2*, 616–626.

Accession Numbers

The microarray data have been submitted to the European Bioinformatics Institute (EBI) public database (accession number E-MEXP-903).