Active CREB1 Promotes a Malignant TGFα2 Autocrine Loop in Glioblastoma
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RESEARCH ARTICLE

ABSTRACT
In advanced cancer, including glioblastoma, the TGFβ pathway acts as an oncogenic factor. Some tumors exhibit aberrantly high TGFβ activity, and the mechanisms underlying this phenomenon are not well understood. We have observed that TGFβ can induce TGFα2, generating an autocrine loop leading to aberrantly high levels of TGFα2. We identified cAMP-responsive element–binding protein 1 (CREB1) as the critical mediator of the induction of TGFα2 by TGFβ. CREB1 binds to the TGFB2 gene promoter in cooperation with SMAD3 and is required for TGFβ to activate transcription. Moreover, the PI3K–AKT and RSK pathways regulate the TGFα2 autocrine loop through CREB1. The levels of CREB1 and active phosphorylated CREB1 correlate with TGFα2 in glioblastoma. In addition, using patient-derived in vivo models of glioblastoma, we found that CREB1 levels determine the expression of TGFα2. Our results show that CREB1 can be considered a biomarker to stratify patients for anti-TGFβ treatments and a therapeutic target in glioblastoma.

SIGNIFICANCE: TGFβ is considered a promising therapeutic target, and several clinical trials using TGFβ inhibitors are generating encouraging results. Here, we discerned the molecular mechanisms responsible for the aberrantly high levels of TGFα2 found in certain tumors, and we propose biomarkers to predict the clinical response to anti-TGFβ therapies. Cancer Discov; 4(10): 1230–41. ©2014 AACR.

See related commentary by Wotton, p. 1123.

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INTRODUCTION

Work over the past decade has shown that the TGFβ pathway is a therapeutic target in advanced tumors. Step by step, the oncogenic response to TGFβ is being elucidated, evidencing that TGFβ promotes tumor progression in a complex and pleiotropic manner. A potent immune suppressor, TGFβ at high levels facilitates tumor growth through the inhibition of the anticancer immune response. On the other hand, TGFβ has been shown to promote proliferation, angiogenesis, and invasion through the induction of metalloproteases, to enhance the self-renewal of cancer-initiating cells (CIC), and to mediate metastasis due in part to the induction of an epithelial-to-mesenchymal transition (1–5).

The oncogenic response to TGFβ has prompted the design of therapies based on the blockade of the TGFβ signal (6, 7). Several inhibitors of the TGFβ pathway are being developed in clinical trials, and some patients are benefitting from anti-TGFβ-based therapies (8, 9). However, we are still unable to predict which patients will respond to the inhibition of the TGFβ pathway. The identification of biomarkers of response to treatment is mandatory to stratify the patients who should be treated with inhibitors of TGFβ. Importantly, high TGFβ activity confers poor prognosis and, in this sense, one hypothesis to be considered is that the aberrantly high TGFβ activity found in some tumors might confer a selective advantage to the tumor. Hence, the inhibition of the TGFβ signal in those tumors could lead to a therapeutic effect. It is then crucial to identify and study the characteristics of tumors that show aberrantly high TGFβ activity.

The TGFβ ligands (TGFβ1, TGFβ2, and TGFβ3) bind and activate a heterodimeric complex formed by the TβRII and the TβRI and initiate an intracellular signaling cascade through the phosphorylation (p) of the specific receptor-regulated SMADs (R-SMAD) SMAD2 and SMAD3. The phosphorylation of R-SMADs facilitates their binding to SMAD4, and the SMAD complex shuttles to the nucleus, where it regulates gene expression (10). Importantly, TGFβ is a pleiotropic cytokine, and the response to the TGFβ signal depends on the cellular context. The basis of the pleiotropic response to TGFβ resides, in part, in the fact that SMADs have low affinity for DNA and they cooperate with other transcription factors to bind gene promoters. Hence, the presence or the transcriptional activity of the SMAD cofactors is what determines the specific genes that are activated by TGFβ in a particular cell (1, 10, 11).

Glioma is the most frequent primary tumor of the brain, and glioblastoma (GBM, a grade 4 glioma) is the most aggressive of human tumors, with virtually no efficient therapies (12–15). We and others have recently shown that the TGFβ pathway has a key role in GBM. The TGFβ pathway is aberrantly active in some GBM tumors and, moreover, elevated p-SMAD2 (used as a readout of TGFβ activity) correlates with poor prognosis in patients with GBM (2, 16). However,
not much is known about the molecular mechanisms underlying the abnormally high levels of TGFβ activity. Interestingly, no genomic alterations have been identified in GBM components of the TGFβ pathway that could explain the hyperactivity of TGFβ found in some tumors (1). The stroma (inflammatory cells in particular) is one of the sources of TGFβ (17). Moreover, recent work from our group has shown that the amplification of the deubiquitinating enzyme USP15 gene is responsible for the stabilization of the TGFβ derived TGFβ amplification (present in only 2% of GBMs) and stromally of transcription factors has been shown to regulate and glioma (19, 23–27). Interestingly, the CREB/ATF family of transcription factors is activated by means of phosphorylation of its Ser133 residue by different kinases, including AKT and ribosomal S6 kinase (RSK) (19–22). CREB phosphorylation participates in the activation of TGFβ pathway (18). Still, USP15 gene amplification (present in only 2% of GBMs) and stroma-derived TGFβ do not account for the large proportion of GBM tumors that present high TGFβ activity.

Here, we aimed to understand the molecular mechanisms responsible for the increased activity of TGFβ in GBM. We observed that TGFβ can induce the expression of TGFβ2, thus generating an autocrine loop that leads to the accumulation of TGFβ2, ending in a hyperactivation of the TGFβ signal.

We went on to discern the mechanisms underlying the TGFβ autocrine loop and identified the cAMP-responsive element-binding protein 1 (CREB1) as a critical mediator and a SMAD cofactor in the induction of TGFβ2 by TGFβ. CREB1 belongs to the CREB/activating transcription factor (ATF) family of transcription factors and is activated by means of phosphorylation of its Ser133 residue by different kinases, including AKT and ribosomal S6 kinase (RSK) (19–22). CREB factors promote tumorogenesis in many cancers, including non–small cell lung carcinoma, acute myelogenous leukemia, and glioma (19, 23–27). Interestingly, the CREB/ATF family of transcription factors has been shown to regulate and cooperate with SMADs in the transcriptional regulation of multiple genes (28–31).

RESULTS

TGFβ1 and TGFβ2 Are Highly Expressed in GBM and Confer Poor Prognosis

First, we decided to address whether the high activity of the TGFβ pathway present in some GBM tumors was due to the overproduction of the TGFβ ligands. Through the analysis of public databases [Oncomine and Repository for Molecular Brain Neoplasia Data (REMBRANDT)], we analyzed the mRNA levels of the TGFβ ligands, and we observed that the levels of TGFβ1 and TGFβ2 mRNA were higher in GBM than in normal brain tissue, while TGFβ3 was expressed at similar levels in GBM and in normal brain (Fig. 1A). Interestingly, high levels of TGFβ1 or TGFβ2 conferred poor prognosis in patients with GBM, and that was not the case for TGFβ3 (Fig. 1B). On the basis of these results, we decided to discern the molecular mechanisms involved in the overproduction of TGFβ2 mRNA.

Because the TGFβ2 gene is not targeted for amplification in GBM (data not shown, The Cancer Genome Atlas), we quickly discarded the possibility that TGFβ2 mRNA overproduction was due to an increase in gene dosage and decided to focus on the transcriptional regulation of TGFβ2. We observed that TGFβ2 mRNA was induced by TGFβ1 in a GBM cell line (LN229), whereas TGFβ1 and TGFβ3 mRNA levels were not affected by TGFβ1 treatment (Fig. 2A). Moreover, the effect of TGFβ on TGFβ2 was not specific to the ligand because TGFβ1, TGFβ2, and TGFβ3 induced the expression

![Figure 1](image-url) Expressions of TGFβ ligands in GBM. A, expression of TGFβ1, TGFβ2, and TGFβ3 mRNA in healthy brain tissue and GBM was examined in the Oncomine database. B, Kaplan-Meier curves showing the overall survival of patients with TGFβ1, TGFβ2, and TGFβ3 mRNA levels upregulated the indicated fold. Statistical significance was assessed by the log-rank test. Data were obtained from the REMBRANDT program from the National Cancer Institute.
A TGFβ induces TGFβ2 expression in GBM and non-GBM cell lines. A, qRT-PCR of TGFβ1, TGFβ2, and TGFβ3 in LN229 cells treated with TGFβ1 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD. B, qRT-PCR of TGFβ2 in LN229 cells treated with TGFβ1, TGFβ2, and TGFβ3 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. *, P < 0.05, using the Student t test; data, mean ± SD. C, secreted TGFβ2 protein levels determined by ELISA in culture supernatant from LN229 cells treated with TGFβ for 72 hours. ***, P < 0.005, using the Student t test; data, mean ± SD. D, immunoblot analysis and qRT-PCR of TGFβ2 in LN229 cells treated with TGFβ1 and/or the TβRI inhibitor (TβRI inh.) LY-2109761 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. *, P < 0.05, using the Student t test; data, mean ± SD. E, qRT-PCR of TGFβ2 in GBM and non-GBM cell lines treated with TGFβ for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD.

Figure 2. TGFβ induces TGFβ2 expression in GBM and non-GBM cell lines. A, qRT-PCR of TGFβ1, TGFβ2, and TGFβ3 in LN229 cells treated with TGFβ1 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD. B, qRT-PCR of TGFβ2 in LN229 cells treated with TGFβ1, TGFβ2, and TGFβ3 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. *, P < 0.05, using the Student t test; data, mean ± SD. C, secreted TGFβ2 protein levels determined by ELISA in culture supernatant from LN229 cells treated with TGFβ for 72 hours. ***, P < 0.005, using the Student t test; data, mean ± SD. D, immunoblot analysis and qRT-PCR of TGFβ2 in LN229 cells treated with TGFβ1 and/or the TβRI inhibitor (TβRI inh.) LY-2109761 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. *, P < 0.05, using the Student t test; data, mean ± SD. E, qRT-PCR of TGFβ2 in GBM and non-GBM cell lines treated with TGFβ for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD.

of TGFβ2 (Fig. 2B). As expected, the induction of the mRNA levels resulted in an induction of TGFβ2 protein secretion, as observed through ELISA (Fig. 2C). In addition, the induction of TGFβ2 by TGFβ was blunted by the treatment with the TβRI inhibitor LY-2109761 (Fig. 2D). This indicated that in LN299 cells an autocrine loop was generated where any TGFβ ligand induced TGFβ2 secretion and, in turn, TGFβ2 could induce its own expression. We decided to evaluate whether this phenomenon was unique for LN299 cells and analyzed several cell lines (including GBM and non-GBM cell lines) for the presence of the TGFβ loop. We observed that TGFβ1 did not induce TGFβ2 in all GBM cell lines tested (e.g., A172), and, on the other hand, the TGFβ2 loop was also present in some non-GBM cell lines, such as HACAT, MCF-7, SUM-159, and MDA-MB-231 (Fig. 2E).

CREB1 Regulates the Induction of TGFβ2 by TGFβ

We went on to discern the molecular mechanisms involved in the regulation of TGFβ2 by TGFβ and studied the TGFβ2 gene promoter region because our previous data showed that the regulation was at the transcriptional level. Looking for conserved regions among different animal species through sequence alignment, we found two conserved SMAD-binding elements (SBE) surrounding a cAMP response element (CRE) described to support CREB1 binding (Fig. 3A). CREB1 has been shown to interact and cooperate with SMADs in the transcriptional activation of certain genes (30, 31). This suggested that CREB1 cooperates with SMADs to activate TGFβ2 gene transcription. To assess this hypothesis, we performed RNAi experiments using independently designed shRNAs and siRNAs targeting CREB1. Knockdown of CREB1 decreased the induction of TGFβ2 by TGFβ, indicating that CREB1 is necessary for the regulation of TGFβ2 transcription by TGFβ (Fig. 3B and C). To further validate this result, we decided to develop an alternative experimental approach using an endogenous repressor of CREB1 called inducible cAMP early repressor (ICER; ref. 32). Overexpression of ICER impaired the ability...
CREB1 is required for the TGFβ autocrine induction of TGFβ2 by TGFβ. A, nucleotide sequence alignment for 3 species: Homo sapiens (H.s.), Pan troglodytes (P.t.), and Mus musculus (M.m.). The SBEs and CREB1 site (CRE) are indicated relative to the transcription start site. ClustalW sequence alignment for 3 animal species shows the conservation of the binding sites. B, qRT-PCR of TGFβ2 and CREB1 in LN229 cells expressing an siRNA targeting CREB1 treated with TGFβ for 3 hours. GAPDH mRNA levels were used as an internal normalization control. "***", P < 0.005, using the Student t test; data, mean ± SD. C, qRT-PCR of TGFβ2 and CREB1 in LN229 cells expressing an siRNA targeting CREB1 treated with TGFβ for 3 hours. GAPDH mRNA levels were used as an internal normalization control. "***", P < 0.005, using the Student t test; data, mean ± SD. The molecular weights are shown.

To prove that CREB1 binds to the identified CRE in the proximal region of the TGFβ2 promoter, we performed chromatin immunoprecipitation (ChIP) experiments. p-CREB1 and SMAD2 and/or SMAD3 bound to the proximal region of the TGFβ2 promoter, and the interaction was increased in response to TGFβ (Fig. 4A). Moreover, we performed reporter assays using a reporter construct containing the proximal −77/+64 region of the TGFβ2 promoter upstream of the luciferase gene. A reporter with a mutation in the CRE-binding site was also engineered. TGFβ mildly but consistently induced the TGFβ2 reporter. However, TGFβ was not able to induce the transcriptional activity of the mutated version of the TGFβ2 reporter (Fig. 4B). We decided to immunoprecipitate endogenous p-CREB1 and, interestingly, found that endogenous SMAD3, but not SMAD2, interacted with p-CREB1 in response to TGFβ (Fig. 4C). To further assess the role of SMAD2 and SMAD3 in the process, we independently knocked down SMAD2 and SMAD3 using RNAi. Knockdown of SMAD2 did not affect the induction of TGFβ2 by TGFβ, whereas knockdown of SMAD3 repressed the TGFβ2 autocrine loop (Fig. 4D). Together, our results showed that p-CREB1 formed a complex with SMAD3 to bind the CRE and the SBEs at the proximal region of the TGFβ2 promoter and induced the transcriptional activation of the promoter.

PI3K and RSK Regulate the TGFβ2 Autocrine Loop through CREB1

CREB1 transcriptional activity is dependent on CREB1 phosphorylation at Ser133 by many different serine/threonine protein kinases, including AKT and p90 RSK (19–22). Phosphorylation at Ser133 is required for CREB1 binding to the transcriptional coactivator CREB1-binding protein, enabling the CREB1-dependent activation of transcription (33, 34). We reasoned that if CREB1 transcriptional activity...
is required for the TGFβ2 autocrine loop, the signaling pathways that regulate CREB1 activity should also affect the induction of TGFβ2 by TGFβ. To validate this hypothesis, we performed a pharmacologic approach using selective inhibitors of the PI3K-AKT pathway (LY-294002) and RSK (BI-D1870). As expected, CREB1 phosphorylation was reduced upon treatment with PI3K and RSK inhibitors, indicating that CREB1 activity was regulated by the PI3K-AKT and the RSK pathways (Fig. 5A and B). In cells treated with PI3K or RSK inhibitors, TGFβ was not able to induce TGFβ2 mRNA at the same level as in control cells (Fig. 5A and B). Moreover, both PI3K and RSK inhibitors impaired TGFβ2 protein secretion induced by TGFβ as measured by ELISA (Fig. 5C and D). Interestingly, the effect of the inhibitor was more pronounced on the secretion of TGFβ2 than on the mRNA level of TGFβ2, possibly indicating a putative regulation of the TGFβ2 loop at the level of protein translation. Further work is required to validate this hypothesis.

**TGFβ2 Expression Correlates with CREB1 Protein Levels in Patient Tumors**

We then hypothesized that if CREB1 is required for the TGFβ2 autocrine loop, tumors with high levels of TGFβ2 might present elevated levels of CREB1 and p-CREB1. Through the analysis of the REMBRANDT database, we observed a positive correlation between TGFβ2 and CREB1 mRNA levels in human GBM samples (Fig. 6A). Interestingly, there were no tumors with high TGFβ2 and low CREB1 (see the upper left quadrant of Fig. 6A), showing the relevance of the CREB1 transcription factor for the expression of TGFβ2. The same analysis was performed in the case of TGFβ1, and no correlation was observed between TGFβ1 and CREB1 levels (Fig. 6B). We then addressed whether TGFβ2 protein levels correlated with the levels of phosphorylated CREB1 by performing an IHC analysis of a collection of GBM samples. As expected, a positive correlation between TGFβ2 and p-CREB1 staining was observed (Fig. 6C). Examples of high and low TGFβ2, p-CREB1–expressing tumors are shown. Interestingly, using the same collection of samples, we determined that p-CREB1 also correlated with p-SMAD2 levels (Supplementary Fig. 1). Again using the REMBRANDT database, we observed that the patient population with tumors expressing high TGFβ2 and CREB1 had a significantly shorter overall survival (OS) than the rest of the patients, indicating that combined upregulation of p-CREB1 and TGFβ2 confers poor prognosis in patients with GBM (Fig. 6D).
Figure 5. PI3K and RSK regulate the TGFβ-mediated induction of TGFβ2 through CREB1. A, immunoblot analysis and qRT-PCR of TGFβ2 in LN229 cells treated with TGFβ for 3 hours and the PI3K inhibitor (inh) LY-294002 for 24 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD. B, immunoblot analysis and qRT-PCR of TGFβ2 in LN229 cells treated with increasing amounts of the RSK inhibitor BI-D1870 for 24 hours and TGFβ for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***, P < 0.005, using the Student t test; data, mean ± SD. C, secreted TGFβ2 protein levels determined by ELISA in LN229 cells treated with TGFβ for 48 hours and the PI3K inhibitor for 72 hours. *, P < 0.05, using the Student t test; data, mean ± SD. D, secreted TGFβ2 protein levels determined by ELISA in LN229 cells treated with the RSK inhibitor BI-D1870 for 72 hours and TGFβ for 48 hours. *, P < 0.05, using the Student t test; data, mean ± SD.

Figure 6. TGFβ2 correlates with CREB1 expression in GBM patient tumors. A and B, graphs showing the correlation between CREB1 and TGFβ1 (B) or TGFβ2 (A) mRNA levels in patient GBM tumor samples. Data obtained from the REMBRANDT database. A Spearman test was used, and the correlation coefficient (ρ) and the two-tailed P value are shown. C, graph showing the correlation between p-CREB1 and TGFβ2 protein levels in tissue microarrays (TMA) from patient GBM samples. Not all spots were evaluable in all stainings. A Spearman test was used, and the correlation coefficient (ρ) and the two-tailed significance are shown. Representative images from the TMAs are shown; scale bar, 50 μm. D, Kaplan–Meier curves showing the OS of patients with TGFβ2 mRNA levels upregulated ≥3-fold and CREB1 mRNA levels upregulated ≥2-fold. Statistical significance was assessed by the log-rank test. Data obtained from the REMBRANDT database.
CREB1 Regulates the Levels of TGFβ2 in Patient-Derived Xenograft Models

We then decided to address the relevance of the CREB1-dependent TGFβ2 autocrine loop in patients. To this end, we used a patient-derived xenograft (PDX) model based on the orthotopic inoculation using stereotaxis of freshly obtained patient-derived tumor cells in the brains of immunocompromised mice (Fig. 7A). The tumors generated in mice reproduce the same histopathologic characteristics and oncogenic mutations as the tumor of the patient (4). We identified two patients who showed elevated expression levels of CREB1 and TGFβ2 in their tumors. Neurospheres from both patients were infected with lentivirus targeting CREB1, and cells were inoculated in mice. Tumors originated from the cells expressing the shRNA targeting CREB1, expressed lower levels of CREB1 and, importantly, lower levels of TGFβ2 (Fig. 7B), further demonstrating that CREB1 protein levels are crucial for the expression of high levels of TGFβ2 in GBM.

Interestingly, mice inoculated with neurospheres expressing shRNAs targeting CREB1 generated smaller and fewer tumors than control neurospheres (Supplementary Fig. 2). Moreover, the OS of these mice was longer than that of control mice (Fig. 7C). This is in agreement with our previous work in which we showed that TGFβ regulates the ability of neurospheres to initiate tumors (3, 4) and, moreover, the two selected models respond to TGFβ receptor inhibitors (unpublished observations), indicating that in those two tumors the regulation of the TGFβ activity is relevant for cancer progression.

DISCUSSION

The oncogenic function of the TGFβ pathway is progressively being elucidated. In advanced tumors, TGFβ can induce proliferation, invasion, angiogenesis, and immunosuppression (1, 2). In addition, TGFβ can increase the self-renewal capacity of a cell population with stem-cell characteristics called CICs (3–5). Because of its role in oncogenesis, TGFβ is considered a therapeutic target, and several clinical trials using anti-TGFβ agents are now under clinical development (6, 7). However, one of the crucial questions still remaining is how to predict in which tumors TGFβ acts as an oncogenic factor and, thus, which patients should be treated with anti-TGFβ compounds.

Work from our laboratory and others has shown that tumors present diverse levels of TGFβ activity. In particular in GBM, we observed that some tumors show aberrantly high TGFβ activity and this correlated with poor prognosis (16). The presence of extremely high TGFβ activity in the most-aggressive tumors suggests that TGFβ may confer a selective advantage to the tumor and, hence, the inhibition of the TGFβ pathway might exhibit an antitumoral effect. But why are there tumors with such high TGFβ activity? Interestingly, the answer does not reside in the presence of activating genomic alterations of components of the TGFβ pathway as it happens in many other oncogenic pathways. In fact, in GBM, the components of the TGFβ signal are seldom mutated, and no gene amplifications of the TGFβ ligands

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nor hyperactive mutations in the TGFβ receptors have been described (1).

To discern the molecular mechanisms underlying the increased TGFβ activity in GBM, we specifically focused on TGFβ2. We observed that TGFβ induced TGFβ2 and that in turn TGFβ2 induced its own expression. This generated a self-feeding autocrine loop, leading to extremely high levels of TGFβ2. However, the autocrine loop was not present in all the cell lines tested, and hence the TGFβ2 response to TGFβ1 depended on the cellular context. This is a typical characteristic of the TGFβ signaling pathway. The specific responses to TGFβ are determined by the characteristics of the cell that receives the signal. Frequently, the pleiotropic response to TGFβ is determined by the SMAD transcription complex. SMADs have low affinity for DNA, and they cooperate with other transcription factors to bind to specific gene promoters. Thus, the presence of a certain cofactor is what determines the transcriptional response to TGFβ (10, 11). In the case of the induction of TGFβ2 by TGFβ1, we identified CREB1 as the SMAD cofactor. CREB1 interacts with SMAD3, not SMAD2, binds to the TGFβ2 promoter, and is required for the TGFβ1-dependent induction of TGFβ2. Still further studies are required to understand why CREB1 is expressed in certain tumors and not others. Most likely, the state of differentiation of the tumor cell is what determines the expression of CREB1.

We corroborated our data by analyzing human tumors. A correlation between CREB1 or p-CREB1 and TGFβ2 was observed and, more importantly, no tumor expressing low levels of CREB1 presented high TGFβ2. This was not the case for TGFβ1. Importantly, patients with tumors expressing high levels of CREB1 and TGFβ2 had a shorter OS than the rest of the patients. Moreover, CREB1 transcriptional activity is induced upon phosphorylation by many different protein kinases that in turn are regulated by oncopgenic signaling pathways, such as the PI3K–AKT and the RSK pathways (19, 21, 22). This implies that to establish a TGFβ autocrine loop, the tumor cell has to express CREB1 and, at the same time, exhibit an active PI3K or RSK pathway to acquire a high levels of phosphorylated CREB1 (Fig. 7D). In this sense, we observed that p-CREB1 correlated with TGFβ2 in patients with GBM. Interestingly, although functional direct cross-talk between the PI3K and TGFβ pathways has been described in breast cancer (35), we did not observe a strong modulation of TGFβ activity by the PI3K–AKT pathway and vice versa, indicating that direct PI3K-TGFβ cross-talk is not present in our GBM cells.

To functionally validate the relevance of CREB1 in the regulation of TGFβ2 expression and its autocrine loop, we decided to use in vivo models that recapitulate the human tumor as faithfully as possible. Instead of using established cell lines that are adapted to grow in vitro and, hence, diverge from real tumors, we decided to use PDXs in which freshly obtained GBM samples are stereotactically inoculated in the brain of immunocompromised mice (4). Through loss-of-function studies in two PDX models in which TGFβ2 and CREB1 were highly expressed, we demonstrated that CREB1 expression was required for TGFβ2 expression and, hence, showed that our proposed mechanism of regulation of TGFβ2 levels is relevant in vivo in patient-derived models.

The TGFβ pathway is considered a therapeutic target in GBM, and TGFβ inhibitors are currently under clinical development, showing promising results (8, 9). Right now, it is crucial to be able to predict which patients may benefit from the inhibition of the TGFβ pathway. Biomarkers to stratify patients to be treated with inhibitors of TGFβ are needed. It is reasonable to hypothesize that tumors with high TGFβ activity might have better chances to respond to TGFβ inhibition, because high TGFβ activity confers poor prognosis and provides a selective advantage to the tumor. Our work sheds light on the molecular mechanisms underlying the aberrantly high levels of TGFβ2 found in tumors. The transcription factor CREB1 is crucial for the generation of a malignant autocrine TGFβ2 loop, and hence CREB1 expression/phosphorylation levels might be considered a biomarker of response to anti-TGFβ treatments. Moreover, because CREB1 has been described as a putative therapeutic target (36, 37), our results suggest that compounds against CREB1 could be effective anticancer agents due to their effect on the oncogenic levels of TGFβ2.

### METHODS

#### Plasmids

The CREB1 short hairpin sequence used was 5′-GAGAGAG GTCCGTCATAATG-3′, and the lentiviral knockdown sequences targeting CREB1 were (i) 5′-ACCACAAAAGTACGTTCA-3′ and (ii) 5′-TGAACGTTCATTTGTTGGT-3′ (Open Biosystems). Knockdown of CREB1, SMAD2, and SMAD3 by siRNA was performed by transfection of SmartPool siRNA (Dharmacon). Silencer Negative Control No. 1 siRNA (Dharmacon) was used as negative control. The TGFβ2 promoter constructs comprise a genomic DNA fragment spanning bases −77 to +63 of TGFβ2 (relative to the transcriptional start codon) cloned into pGL3-basic vector with or without a CREB1-binding site mutation (CGTCAC to TGGCAC; ref. 38).

#### Antibodies and Reagents

Specific antibodies against p-SMAD2, SMAD2, p-AKT, AKT, p-CREB1, CREB1 (Cell Signaling Technology), TGFβ2, CREM (Santa Cruz Biotechnology), and Tubulin (Sigma) were used for immunoblot analysis and IHC. Antibodies against SMAD2/3 (Upstate), ChIPa1 Phospho-CREB1 (Ser133; Millipore), and rabbit IgG (Upstate) were used for ChIP and IP.

The treatments used were as follows: TFGF1, TFGF2, TFGF3 (100 pmol/L; R&D Systems), PI3K inhibitor LY-294002 (10 μmol/L; Merck Millipore), TβRI inhibitor LY-2109761 (Tocris), and RSK inhibitor BI-D1870 (5 μmol/L; Axon Medchem BV).

#### Cell Culture

GBM neurospheres were generated as described previously (4, 39). Briefly, tumor samples were processed within 30 minutes after surgical resection. minced pieces of human GBM samples were digested with collagenase 1 (200 U/ml; Sigma) and DNase 1 (500 U/ml; Sigma) in PBS for 2 hours at 37°C with constant vigorous agitation. The single-cell suspension was filtered through a 70-mm cell strainer (BD Falcon) and washed with PBS. Finally, cells were resuspended and subsequently cultured in neurosphere medium. 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 FGF2; PeproTech). Human GBM specimens were obtained from the Vall d’Hebron Hospital (Barcelona, Spain). The clinical protocol was approved by the Vall d’Hebron Institutional Review Board, with informed consent obtained from all subjects. The
293T, A172, U373, U251, and LN229 cell lines were obtained from the ATCC and cultured in DMEM supplemented with 10% FBS. Cell lines were passaged for less than 6 months following resuscitation and were not authenticated. Transient transfections were carried out using either the calcium phosphate transfection method or Lipofectamine 2000 (Invitrogen). Lysates were collected 72 hours after transfection.

**RNA Extraction, Retrotranscription, and Quantitative Real-Time PCR**

Cells were seeded in 60-mm plates at 70% confluence. After the described treatments, cultured cells were disrupted in lysis buffer from the RNeasy Mini Kit (Qiagen), and mRNA was purified following the manufacturer’s instructions. mRNA was quantified, and 300 to 800 ng of mRNA from each sample was retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the product indications. After cDNA synthesis, quantitative real-time PCR (qRT-PCR) was performed. All qRT-PCR was performed using TaqMan probes from Applied Biosystems, according to the manufacturer’s recommendations. Reactions were carried out in an ABI 7900 sequence detector (PerkinElmer) and results were expressed as fold change calculated by the C_{\text{t}} method relative to the control sample. GAPDH and POU5F1 were used as internal normalization controls.

**Western Blot Analysis**

Cells were lysed in RIPA buffer supplemented with protease inhibitors (Roche). Whole-cell extracts were quantified using the BCA Protein Assay Kit (Pierce) and were then separated on 7% to 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk and probed with specific antibodies. Blots were then incubated with a horseradish peroxidase-linked second antibody and developed with chemiluminescence.

**ELISA**

For the quantitative determination of TGFβ2 protein levels secreted to the media, we used the Human TGFβ2 Quantikine ELISA Kit (R&D Systems) following the manufacturer’s specifications. Supernatant from serum-starved cells was collected 72 hours after the start of the indicated treatments.

**siRNA Transfection**

LN229 cells were seeded and transfected with 100 pmol/L of siRNAs against SMAD2 or SMAD3 using Lipofectamine 2000 (Invitrogen). Silencer Negative Control No. 1 siRNA was used as a negative control.

**Retroviral Infections**

The 293T Phoenix-Ampho cells were transfected using the calcium phosphate transfection method with the retroviral vectors pLNCX2, pLNCX2-ICER, pReveroSuper, and pReveroSuper-shCREB1. After 16 hours, medium was replaced with recombinant retrovirus was harvested for an additional 24 hours. For infection, medium containing recombinant lentivirus was added to previously dissociated neurospheres. Polybrene (Sigma) was added at a concentration of 8 μg/mL. Following 16 hours of incubation, the neurospheres were washed and incubated in fresh neurosphere medium. The 293T cells were incubated in fresh neurosphere medium containing sodium butyrate (5 mmol/L) for 24 hours, and a second round of neurosphere infection was repeated as previously described.

**Luciferase Assays**

Cells were transfected with different TGFβ2 promoter reporter constructs and pRL-TK Renilla luciferase plasmid (Promega) using Lipofectamine 2000. After a 16-hour incubation at 37°C, cells were treated with TGFβ for a further 32 hours. Luciferase counts were measured using a Sirius Luminometer (Berthold).

The firefly luciferase activity was normalized with renilla luciferase activity. Data are represented as relative activity (compared with basal promoter activity) and are expressed as the mean ± SD of triplicates from a representative experiment.

**Tissue Microarrays and Immunohistochemical Staining**

For tissue microarray (TMA) generation, three 0.6-mm cores were taken from separate areas, and each one was arrayed into recipient blocks in a 1-mm–spaced grid.

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and hydrated. Antigen retrieval was performed using pH 6 Citrate Antigen Retrieval Solution (DAKO). Peroxidase blocking was done with 3% H_2O_2 at room temperature for 10 minutes. For TGFβ2, slides were incubated with a blocking solution (10% normal goat serum, 2% BSA) for 1 hour at room temperature. TGFβ2 antibody (Santa Cruz Biotechnology; sc-90) was used at a 1:500 dilution, p-CREB1 (Cell Signaling Technology; 9198) was used at 1:100 dilution, and p-SMAD2 (Cell Signaling Technology; 3108) was used at 1:250 dilution. A human-specific anti-Nestin antibody was used at 1:200 dilution, to determine tumor area. As a detection system, EnVision FLEX” (DAKO) was used following the manufacturer’s instructions and developed with freshly prepared 0.05% 3′,3-diaminobenzidine tetrahydrochloride. Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. Positive and negative controls were performed in each run. The quantification of the staining was expressed as H score. The H score was determined by the formula: 3 × percentage of strong staining + 2 × percentage of moderate staining + percentage of weak staining, giving a range of 0 to 300 for the H scores.

**Chromatin Immunoprecipitation**

LN229 cells were grown to 70% confluence, serum starved for 16 hours, and cultured in the presence or absence of TGFβ for 1 hour. Cells were trypsinized and crosslinked in 1% formaldehyde for 10 minutes at room temperature. Crosslinking was quenched with a glycine solution (0.125 mol/L) for 5 minutes in formaldehyde, and cells were washed twice with PBS. Pelleted cells were lysed in 1 mL ChIP buffer (1 volume of SDS buffer with 0.5 volumes of Triton buffer), and sonicated in a Bioruptor (Diagenode). Soluble material was quantified by Bradford assays, and p-CREB1 and SMAD2/3 were immunoprecipitated from 1,000 μg of protein. Antibodies were incubated overnight with the chromatin in a 500 μL volume. Immunocomplexes were recovered with 30 μL of a protein A/G bead slurry. Immunoprecipitated material was washed three times with a low-salt buffer and once with a high-salt buffer. DNA complexes were decrosslinked in 100 μL decrosslink buffer (1% SDS and 100 mmol/L NaHCO_3 at 65°C for 3 hours, and DNA was then eluted in 100 μL of water using a PCR purification kit (Qagen). DNA (2 μL) was used for each qRT-PCR.
reaction with SYBR green (Roche). Antibodies against SMAD2/3 (Upstate), ChIPAb Phospho-CREB1 (Ser133; Millipore), and rabbit IgG (Upstate) were used for ChIP. The proximal TGFB2 promoter primer set used spans −84 to −18 relative to the transcriptional start site.

**Immunoprecipitation**

LN229 cells were grown to 70% confluence, serum starved for 16 hours, and cultured in the presence or absence of TGFB1 for 1 hour. Cells were washed with cold PBS and lysed with RIPA. The protein lysate was precleared and incubated overnight at 4°C on a rotator. The supernatant was removed, and protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated for 1 hour at 4°C on a rotator. Samples were washed five to six times with IP buffer and resolved by SDS-PAGE, as described previously.

**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 mice 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 neurologic symptoms or a significant loss of weight.

**Statistical Analyses**

Student t tests were performed for statistical analyses. Data in all graphs are represented as means ± SD of biologic triplicates. A Spearman correlation test was used to analyze the relationships between CREB1, p-CREB1, and TGFB2.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Development of methodology: L. Rodón, M. del Mar Inda, A. Sala-Hojman
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Gonzàlez-Juncà, A. Sala-Hojman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Gonzàlez-Juncà, M. del Mar Inda, A. Sala-Hojman, E. Martinez-Sáez, J. Seoane
Writing, review, and/or revision of the manuscript: L. Rodón, A. Gonzàlez-Juncà, M. del Mar Inda, A. Sala-Hojman, E. Martinez-Sáez, J. Seoane
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Sala-Hojman
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TGFβ2 Autocrine Loop

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