Active CREB1 promotes a malignant TGF-β2 autocrine loop in glioblastoma.

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Abstract

In advanced cancer including glioblastoma, the TGF-β pathway acts as an oncogenic factor. Some tumors exhibit aberrantly high TGF-β activity and the mechanisms underlying this phenomenon are not well understood. We have observed that TGF-β can induce TGF-β2 generating an autocrine loop leading to aberrantly high levels of TGF-β2. We identified CREB1 as the critical mediator of the induction of TGF-β2 by TGF-β. CREB1 binds to the TGF-β2 gene promoter in cooperation with SMAD3 and is required for TGF-β to activate transcription. Moreover, the PI3K-AKT and the RSK pathways regulate the TGF-β2 autocrine loop through CREB1. The levels of CREB1 and active p-CREB1 correlate with TGF-β2 in glioblastoma. In addition, using patient-derived in vivo models of glioblastoma, we found that CREB1 levels determine the expression of TGF-β2. Our results show that CREB1 can be considered a biomarker to stratify patients for anti-TGF-β treatments and a therapeutic target against glioblastoma.

Significance

TGF-β is considered a promising therapeutic target and several clinical trials using TGF-β inhibitors are generating encouraging results. Here, we discerned the molecular mechanisms responsible for the aberrantly high levels of TGF-β2 found in certain tumors and we propose biomarkers to predict the clinical response to anti-TGF-β therapies.
Introduction

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

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

The TGF-β ligands (TGF-β1, TGF-β2, TGF-β3) bind and activate a heterodimeric complex formed by the TβRII and the TβRI and initiate an intracellular signaling cascade through the phosphorylation of the specific receptor-regulated SMADs (R-SMADs) 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, TGF-β is a pleiotropic cytokine and the response to the TGF-β signal depends on the cellular context. The basis of the pleiotropic response to TGF-β resides in part in the fact that SMADs have low affinity for DNA and they cooperate with other transcription factors in order 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 TGF-β in a particular cell (1, 10, 11).

Glioma is the most frequent primary tumor of the brain, and glioblastoma (GBM, a grade-IV glioma) is the most aggressive of human tumors with virtually no efficient therapies (12-15). We and others have recently shown that TGF-β pathway has a key role in GBM. The TGF-β pathway is aberrantly active in some GBM tumors and, moreover, elevated p-SMAD2 (used as a readout of TGF-β activity) correlates with poor prognosis in GBM patients (2, 16). However, not much is known about the molecular mechanisms underlying the abnormally high levels of TGF-β activity. Interestingly, no genomic alterations have been identified in GBM components of the TGF-β pathway that could explain the hyperactivity of TGF-β found in some tumors (1). The stroma (inflammatory cells in particular) is one of the sources of TGF-β (17). Moreover, recent work from our group has shown that the amplification of the deubiquinating enzyme USP15 gene is responsible for the stabilization of the TβRI and hyperactivation of the TGF-β pathway (18). Still, USP15 gene amplification (only present in 2% of GBM) and the stroma-derived TGF-β do not account for the large proportion of GBM tumors that present high TGF-β activity.

Here, we aimed to understand the molecular mechanisms responsible for the increased activity of TGF-β in GBM. We observed that TGF-β can induce the expression of TGF-β2 thus generating
an autocrine loop that leads to the accumulation of TGF-β2 ending in a hyperactivation of the TGF-β signal.

We went on to discern the mechanisms underlying the TGF-β autocrine loop and we identified the cAMP response element-binding protein 1 (CREB1) as a critical mediator and a SMAD cofactor of the induction of TGF-β2 by TGF-β. CREB1 belongs to the CREB/ATF family of transcription factors and is activated by means of phosphorylation in its Ser133 residue by different kinases including AKT and RSK (19-22). CREB factors promote tumorigenesis in many cancers, including non–small-cell lung carcinoma (NSCLC), acute myeloid leukemia (AML), and glioma (19, 23-27). Interestingly, the CREB/ATF family of transcription factors have been shown to regulate and cooperate with SMADs in the transcriptional regulation of multiple genes (28-31).
Results

TGF-β1 and TGF-β2 are highly expressed in GBM and confer poor prognosis

First, we decided to address whether the high activity of the TGF-β pathway present in some GBM tumors was due to the overproduction of the TGF-β ligands. Through the analysis of public data bases (Oncomine and REMBRANDT) we analyzed the mRNA levels of the TGF-β ligands and we observed that the levels of TGF-β1 and TGF-β2 mRNA were higher in GBM than in normal brain tissue while TGF-β3 is expressed at similar levels in GBM and in normal brain (Fig. 1A). Interestingly, high levels of TGF-β1 or TGF-β2 conferred poor prognosis in GBM patients and that was not the case for TGF-β3 (Fig. 1B). Based on these results, we decided to discern the molecular mechanisms involved in the overproduction of TGF-β2 mRNA.

Since the TGF-β2 gene is not targeted for amplification in GBM (data not shown, TCGA), we quickly discarded the possibility that TGF-β2 mRNA overproduction was due to an increase in gene dosage and decided to focus on the transcriptional regulation of TGF-β2. We observed that TGF-β2 mRNA was induced by TGF-β1 in a GBM cell line (LN299), while TGF-β1 and TGF-β3 mRNA levels were not affected by TGF-β1 treatment (Fig. 2A). Moreover, the effect of TGF-β on TGF-β2 was not specific of the ligand since TGF-β1, TGF-β2 and TGF-β3 induced the expression of TGF-β2 (Fig. 2B). As expected the induction of the mRNA levels implied an induction of TGF-β2 protein secretion as observed through ELISA (Fig. 2C). In addition, the induction of TGF-β2 by TGF-β 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 TGF-β ligand induced TGF-β2 secretion and in turn TGF-β2 could induce its own
expression. We decided to evaluate whether this phenomenon was unique for LN299 cells and we analyzed several cell lines (including GBM and non-GBM cell lines) for the presence of the TGF-β2 loop. We observed that TGF-β1 did not induce TGF-β2 in all GBM cell lines tested (i.e. A172), and on the other hand, the TGF-β2 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 TGF-β2 by TGF-β**

We went on to discern the molecular mechanisms involved in the regulation of TGF-β2 by TGF-β and we decided to study the TGF-β2 gene promoter region since 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 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 the TGF-β2 gene transcription. In order to assess this hypothesis, we performed RNA interference experiments using independently designed shRNAs and siRNAs targeting CREB1. Knock-down of CREB1 decreased the induction of TGF-β2 by TGF-β indicating that CREB1 is necessary for the regulation of the TGF-β2 transcription by TGF-β (Fig. 3B,C). To further validate this result, we decided to develop an alternative experimental approach using an endogenous repressor of CREB1 called ICER (32). Overexpression of ICER impaired the ability of TGF-β to induce TGF-β2 (Fig. 3D). These results indicated that CREB1 is required for the TGF-β autocrine loop.

In order to prove that CREB1 binds to the identified CRE in the proximal region of the TGF-β2 promoter, we performed chromatin immunoprecipitation (ChIP) experiments. p-CREB1 and
SMAD2 and/or SMAD3 bound to the proximal region of the TGF-β2 promoter and the interaction was increased in response to TGF-β (Fig. 4A). Moreover, we performed reporter assays using a reporter construct containing the proximal -77/+64 region of the TGF-β2 promoter upstream of the luciferase gene. A reporter with a mutation in the CRE binding site was also engineered. TGF-β mildly but consistently induced the TGF-β2 reporter. However, TGF-β was not able to induce the transcriptional activity of the mutated version of the TGF-β2 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 TGF-β (Fig. 4C). To further assess the role of SMAD2 and SMAD3 in the process, we independently knocked down SMAD2 and SMAD3 using RNA interference. Knockdown of SMAD2 did not affect the induction of TGF-β2 by TGF-β while knockdown of SMAD3 repressed the TGF-β2 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 TGF-β2 promoter and induced the transcriptional activation of the promoter.

**PI3K and RSK regulate the TGF-β2 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 ribosomal S6 kinase (RSK) (19-22). Phosphorylation at Ser133 is required for CREB1 binding to the transcriptional co-activator CREB1-binding protein (CBP) enabling the CREB1-dependent activation of transcription (33, 34). We reasoned that if CREB1 transcriptional activity is required for the TGF-β2 autocrine loop, the signaling pathways that regulate CREB1 activity should also impact on the induction of TGF-β2 by TGF-β. To validate this hypothesis, we performed a
pharmacological approach using selective inhibitors of the PI3K-AKT pathway (LY-294002) and RSK (BI-D1870). As expected, CREB1 phosphorylation was reduced upon treatment with the PI3K inhibitor and the RSK inhibitor indicating that CREB1 activity was regulated by the PI3K-AKT and the RSK pathways (Fig. 5A, B). In cells treated with the PI3K or RSK inhibitors, TGF-β was not able to induce TGF-β2 mRNA at the same level as in control cells (Fig. 5A, B). Moreover, both PI3K or RSK inhibitors impaired TGF-β2 protein secretion induced by TGF-β as measured by ELISA (Fig. 5C, D). Interestingly, the effect of the inhibitor was more pronounced on the secretion of TGF-β2 than on the mRNA level of TGF-β2 possibly indicating a putative regulation of the TGF-β loop at the level of protein translation. Further work is required to validate this hypothesis.

**TGF-β2 expression correlates with CREB1 protein levels in patient tumors**

We then hypothesized that if CREB1 is required for the TGF-β2 autocrine loop, tumors with high levels of TGF-β2 might present elevated levels of CREB1 and p-CREB1. Through the analysis of the REMBRANDT database, we observed a positive correlation between TGF-β2 and CREB1 mRNA levels in human GBM samples (Fig. 6A). Interestingly, there were no tumors with high TGF-β2 and low CREB1 (see the upper left quadrant of Fig. 6A) showing the relevance of the CREB1 transcription factor on the expression of TGF-β2. The same analysis was performed in the case of TGF-β1 and no correlation was observed between TGF-β1 and CREB1 levels (Fig. 6B). We went on to address whether TGF-β2 protein levels correlated with the levels of phosphorylated CREB1 performing an immunohistochemistry analysis of a collection of GBM samples. As expected, a positive correlation between TGF-β2 and p-CREB1
staining was observed (Fig. 6C). Examples of a high and a low TGF-β2, 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). Using again the REMBRANDT database, we observed that the patient population with tumors expressing high TGF-β2 and CREB1 had a significantly shorter overall survival than the rest of the patients indicating that combined upregulation of p-CREB1 and TGF-β2 confers poor prognosis in GBM patients (Fig. 6D).

**CREB1 regulates the levels of TGF-β2 in patient-derived xenograft models**

We then decided to address the relevance of the CREB1-dependent TGF-β2 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 brain of immunocompromised mice (Fig. 7A). The tumors generated in mice reproduce the same histopathological characteristics and oncogenic mutations as the tumor of the patient (4). We identified two patients that showed elevated expression levels of CREB1 and TGF-β2 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 TGF-β2 (Fig. 7B), further demonstrating that CREB1 protein levels are crucial for the expression of high levels of TGF-β2 in GBM.

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

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

Work from our lab and others has shown that tumors present diverse levels of TGF-β activity. In particular in GBM, we observed that some tumors show aberrantly high TGF-β activity and this correlated with poor prognosis (16). The presence of an extremely high TGF-β activity in the most aggressive tumors suggests that TGF-β may confer a selective advantage to the tumor and hence the inhibition of the TGF-β pathway might exhibit an anti-tumoral effect. But, why are there tumors with such a high TGF-β activity? Interestingly, the answer does not reside in the presence of activating genomic alterations of components of the TGF-β pathway as it happens in many other oncogenic pathways. In fact, in glioblastoma, the components of the TGF-β signal are seldom mutated and no gene amplifications of the TGF-β ligands nor hyperactive mutations in the TGF-β receptors have been described (1).

In order to discern the molecular mechanisms underlying the increased TGF-β activity in GBM, we specifically focused on TGF-β2. We observed that TGF-β induced TGF-β2 and that in turn
TGF-β2 induced its own expression. This generated a self-feeding autocrine loop leading to extremely high levels of TGF-β2. However, the autocrine loop was not present in all the cell lines tested and hence the TGF-β2 response to TGF-β depended on the cellular context. This is a typical characteristic of the TGF-β signaling pathway. The specific responses to TGF-β are determined by the characteristics of the cell that receives the signal. Frequently, the pleiotropic response to TGF-β 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. Then the presence of a certain co-factor is what determines the transcriptional response to TGF-β (10, 11). In the case of the induction of TGF-β2 by TGF-β, we identified CREB1 as the SMAD cofactor. CREB1 interacts with SMAD3, not SMAD2, binds the TGF-β2 promoter and is required for the TGF-β-dependent induction of TGF-β2. Still further studies are required in order 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 analyzing human tumors. A correlation between CREB1 or p-CREB1 and TGF-β2 was observed and more importantly no tumor expressing low levels of CREB1 presented high TGF-β2. This was not the case for TGF-β1. Importantly, patients with tumors expressing high levels of CREB1 and TGF-β2 had a shorter overall survival 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 oncogenic signaling pathways such as the PI3K-AKT and the RSK pathways (19, 21, 22). This implies that in order to establish a TGF-β2 autocrine loop the tumor cell has to express CREB1 and, at the same time, exhibit an active PI3K or RSK pathways in order to acquire a high level of phosphorylated CREB1 (Fig. 7D). In this
sense, we observed that p-CREB1 correlated with TGF-β2 in GBM patients. Interestingly, although a functional direct cross-talk between the PI3K and TGF-β pathways has been described in breast cancer (35), we did not observe a strong modulation of the TGF-β activity by the PI3K/AKT pathway and vice versa, indicating that the PI3K-TGF-β direct cross-talk is not present in our GBM cells.

To functionally validate the relevance of CREB1 in the regulation of the TGF-β2 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 where 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 TGF-β2 and CREB1 were highly expressed, we demonstrated that CREB1 expression was required for TGF-β2 expression and hence we showed that our proposed mechanism of regulation of TGF-β2 levels is relevant in vivo in patient-derived models.

The TGF-β pathway is considered a therapeutic target in GBM and, nowadays, TGF-β inhibitors are under clinical development showing appealing results (8, 9). It is right now crucial to be able to predict which patients may benefit from the inhibition of the TGF-β pathway. Biomarkers to stratify patients to be treated with inhibitors of TGF-β are needed. It is reasonable to hypothesize that tumors with high TGF-β activity might have better chances to respond to TGF-β inhibition since high TGF-β 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 TGF-β2 found in tumors. The transcription factor CREB1 is crucial for the generation
of a malignant autocrine TGF-β2 loop and hence CREB1 expression/phosphorylation levels might be considered a biomarker of response to anti-TGF-β treatments. Moreover, since CREB1 has been described as a putative therapeutic target (36, 37), our results suggest that compounds against CREB1 could be effective anti-cancer agents due to their effect on the oncogenic levels of TGF-β2.
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Materials and methods

Plasmids

The CREB1 short hairpin sequence used was: 5’- GAGAGAGGTCCGTCTAATG-3’ and the lentiviral knockdown sequences targeting CREB1 used were: (1) 5’-ACCAACAAATGACAGTTCA-3’; (2) 5’-TGAACTGTCATTTGTTGGT-3’ (Open Biosystems). Knock down 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 TGF-β2 promoter constructs comprise a genomic DNA fragment spanning bases -77 to +63 of TGF-β2 (relative to the transcriptional start codon) cloned into pGL3-basic vector with or without a CREB1 binding site mutation (CGTCAC to TGGCAC) (38).

Antibodies and reagents

Specific antibodies against p-SMAD2, SMAD2, p-AKT, AKT, p-CREB1 and CREB1 (Cell Signaling); TGF-β2 and CREM (Santa Cruz Biotechnology); Tubulin (Sigma) were used for immunoblot and immunohistochemistry. Antibodies against SMAD2/3 (Upstate), ChIPAb+™ Phospho-CREB1 (Ser133) (Millipore) and rabbit IgG (Upstate) were used for ChIP and immunoprecipitation.

The treatments used were as follows: TGF-β1, TGF-β2, TGF-β3 (100 pM; R&D), PI3K inhibitor LY-294002 (10 μM; Merck Millipore), TβRI inhibitor LY-2109761 (Tocris); RSK inhibitor BI-D1870 (5 μM; 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 I (200 U/ml ;Sigma) and DNase I (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. The clinical protocol was approved by the Vall d’Hebron Institutional Review Board, with informed consent obtained from all subjects. 293T, A172, U373, U251 and LN229 were obtained from the American Type Culture Collection 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 48-72 hours post-transfection.

RNA extraction, retrotranscription and Quantitative Real Time PCR

Cells were seeded in 60 mm plates at 70% confluency. After the described treatments, cultured cells were disrupted in lysis buffer from RNeasy Mini Kit (Qiagen) and mRNA was purified following manufacturer’s instructions. mRNA was quantified and 300-800 ng of mRNA from each sample were retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied biosystems) following the product indications. After cDNA synthesis, qRT-PCR was
performed. All qRT-PCR were performed using Taqman probes from Applied Biosystems, according to the manufacturer's recommendations. Reactions were carried out in an ABI 7900 sequence detector (Perkin Elmer) and results were expressed as fold change calculated by the Ct method relative to the control sample. GAPDH and POLR2A were used as internal normalization controls.

**Western Blot**

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% - 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 an HRP-linked second antibody and developed with chemiluminescence.

**ELISA**

For the quantitative determination of TGF-β2 protein levels secreted to the media, we used the Human TGF-β2 Quantikine ELISA kit (R&D systems) following 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 pM of siRNAs against SMAD2 or SMAD3 using Lipofectamine 2000 (Invitrogen). Silencer® Negative Control No. 1 siRNA was used as negative control.
Retroviral infections

293T Phoenix-Ampho cells were transfected using the calcium phosphate transfection method with the retroviral vectors pLNCX2, pLNCX2-ICER, pRetroSuper, pRetroSuper-shCREB1. After 16 hours, medium was replaced and recombinant retrovirus was harvested for additional 24 hours. For infection, medium containing recombinant retrovirus was added to LN229 cells. Polybrene (Sigma) was added at a concentration of 8 μg/ml. Phoenix-Ampho cells were incubated in fresh medium for additional 8 hours; after this time, the LN229 cells were washed and the medium containing recombinant retrovirus from Phoenix-Ampho cells was added. This process of infection was repeated two more times every 8 hours. Two days later, antibiotic selection was added to the medium (puromycin (1μg/ml); neomycin (0.5 μg/ml); higromycin (100 μg/ml)).

Lentiviral infections

293T cells were transfected using the calcium phosphate transfection method with pMD2.G enveloping plasmid, psPAX.2 packaging plasmid and either pGIPZ or pGIPZ-shCREB1. After 16 hours, medium was replaced by neurosphere medium with 5mM Sodium Butyrate and recombinant lentivirus was harvested for 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. 293T cells were incubated in fresh neurosphere medium containing Sodium Butyrate (5mM) for 24 hours and a second round of neurosphere infection was repeated as previously described.
Luciferase assays

Cells were transfected with different TGF-β2 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 TGF-β 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 +/- s.d. of triplicates from a representative experiment.

Tissue microarrays and immunohistochemical staining

For tissue microarray 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% H2O2 at room temperature for 10 minutes. For TGF-β2, slides were incubated with a blocking solution (10% normal goat serum, 2% BSA) for 1 hour at room temperature. TGF-β2 antibody (Santa Cruz sc-90) was used at a 1:500 dilution, p-CREB1 (Cell Signaling 9198) was used at a 1:100 dilution, and p-SMAD2 (Cell Signaling 3108) was used at a 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+ was used (DAKO) 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: $3 \times$ percentage of strong staining $+ 2 \times$ percentage of moderate staining $+ \text{percentage of weak staining}$, 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 TGF-β 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 M) 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 1000 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 de-crosslinked in 100 μl decrosslink buffer (1% SDS and 100 mM NaHCO$_3$) at 65°C for 3 hours, and DNA was then eluted in 100 μl of water using a PCR purification kit (Qiagen). 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 $TGF-\beta 2$ promoter primer set utilized 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 TGF-β1 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 in IP Buffer (0.25 M NaCl, 0.1% NP40, and 50 mM Hepes pH7.3) with the p-CREB1 antibody (Millipore) or normal rabbit IgG. Then, 30 µl of protein A/G PLUS-Agarose (Santa Cruz) were 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 EU and national directives. The cells were stereotactically inoculated into the corpus striatum of the right brain hemisphere (1 mm anterior and 1.8 mm lateral to the bregma; 2.5 mm intraparenchymal) of 9 week-old NOD-SCID mice (Charles River Laboratories). Mice were euthanized when they presented neurological symptoms or a significant loss of weight.

Statistical analyses

Student's t tests were performed for statistical analyses. Data in all graphs are represented as means ± s.d of biological triplicates. A Spearman correlation test was used to analyze the relationships between CREB1, p-CREB1 and TGF-β2.
References


Figure legends

1. **Expression of TGF-β ligands in GBM.** (a) Expression of TGF-β1, TGF-β2 and TGF-β3 mRNA in healthy brain tissue and GBM was examined in the Oncomine database. (b) Kaplan-Meier curves showing the overall survival of patients with TGF-β1, TGF-β2 and TGF-β3 mRNA levels upregulated the indicated fold. Statistical significance was assessed by log-rank test. Data obtained from the Repository for Molecular Brain Neoplasia Data (REMBRANDT) program from the National Cancer Institute.

2. **TGF-β induces TGF-β2 expression in GBM and non-GBM cell lines.** (a) qRT-PCR of TGF-β1, TGF-β2 and TGF-β3 in LN229 cells treated with TGF-β1 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P < 0.005 using Student’s t test. Data are represented as mean ± s.d. (b) qRT-PCR of TGF-β2 in LN229 cells treated with TGF-β1, TGF-β2 and TGF-β3 for 3 hours. GAPDH mRNA levels were used as an internal normalization control. *P < 0.05 using Student’s t test. Data are represented as mean ± s.d. (c) Secreted TGF-β2 protein levels determined by ELISA in culture supernatant from LN229 cells treated with TGF-β for 72 hours. ***P < 0.005, using Student’s t test. Data are represented as mean ± s.d. (d) Immunoblot and qRT-PCR of TGF-β2 in LN229 cells treated with TGF-β1 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 Student’s t test. Data are represented as mean ± s.d. (e) qRT-PCR of TGF-β2 in GBM and non-GBM cell lines treated with TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P < 0.005 using Student’s t test. Data are represented as mean ± s.d.
3. **CREB1 regulates the autocrine induction of TGF-β2 by TGF-β.** (a) Nucleotide sequence of the proximal region of the TGF-β2 promoter. The SMAD binding elements (SBE) and CREB1 site (CRE) are indicated relative to the transcription start site. ClustalW sequence alignment is shown for 3 animal species (Homo sapiens (H.s), Pan troglodytes (P.t.) and Mus musculus (M.m.)) showing the conservation of the binding sites. (b) qRT-PCR of TGF-β2 and CREB1 in LN229 cells expressing a shRNA targeting CREB1 treated with TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P<0.005 using Student’s t test. Data are represented as mean ± s.d. (c) qRT-PCR of TGF-β2 and CREB1 in LN229 cells expressing a siRNA targeting CREB1 treated with TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P<0.005 using Student’s t test. Data are represented as mean ± s.d. (d) Immunoblot and qRT-PCR of TGF-β2 in LN229 cells expressing ICER treated with TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P<0.005 using Student’s t test. Data are represented as mean ± s.d. The molecular weights are shown.

4. **CREB1 binds at the proximal region of the TGF-β2 promoter and induces its transcriptional activation.** (a) ChIP of p-CREB1 and SMAD2/3 in LN229 cells treated with TGF-β for 1 hour. A qRT-PCR is shown with specific primers for the TGF-β2 promoter. Fold enrichment is shown relative to IgG. ***P<0.005, *P<0.05 using Student’s t test. Data are represented as mean ± s.d. (b) Luciferase assay in LN229 transfected with the (-77/+64) wild type or (-77/+64) mutCRE TGF-β2 luciferase reporter constructs and treated with TGF-β for 32 hours. *P < 0.05 using Student’s t test. Data are represented as mean ± s.d. (c) Immunoprecipitation of p-CREB1 in LN229 cells treated with TGF-β for 1 hour and immunoblot analysis of SMAD2/3. Asterisk indicates an unrelated band. Arrow head indicates SMAD3 band.
(d) qRT-PCR of TGF-β2 and immunoblot of the indicated proteins in LN229 cells expressing a siRNA targeting SMAD2 or SMAD3 treated with TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P<0.005 using Student’s t test. Data are represented as mean ± s.d.

5. PI3K and RSK regulate the TGF-β-mediated induction of TGF-β2 through CREB1. (a) Immunoblot and qRT-PCR of TGF-β2 in LN229 cells treated with TGF-β for 3 hours and the PI3K inhibitor LY-294002 for 24 hours. GAPDH mRNA levels were used as an internal normalization control. ***P < 0.005 using Student’s t test. Data are represented as mean ± s.d. (b) Immunoblot and qRT-PCR of TGF-β2 in LN229 cells treated with increasing amounts of the RSK inhibitor BI-D1870 for 24 hours and TGF-β for 3 hours. GAPDH mRNA levels were used as an internal normalization control. ***P < 0.005 using Student’s t test. Data are represented as mean ± s.d. (c) Secreted TGF-β2 protein levels determined by ELISA in LN229 cells treated with TGF-β for 48 hours and the PI3K inhibitor for 72 hours. *P < 0.05 using Student’s t test. Data are represented as mean ± s.d. (d) Secreted TGF-β2 protein levels determined by ELISA in LN229 cells treated with the RSK inhibitor BI-D1870 for 72 hours and TGF-β for 48 hours. *P < 0.05 using Student’s t test. Data are represented as mean ± s.d.
6. **TGF-β2 correlates with CREB1 expression in GBM patient tumors.** (a,b) Graphs showing the correlation between *CREB1* and *TGF-β1* (b) or *TGF-β2* (a) mRNA levels in patient GBM tumor samples. Data obtained from the REMBRANDT database. A Spearmann’s 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 TGF-β2 protein levels in tissue microarrays from patient GBM samples. Not all spots were evaluable in all stainings. A Spearmann’s test was used, and the correlation coefficient (ρ) and the two-tailed significance are shown. Representative images from the tissue microarrays are shown. Scale bar, 50 μm (d) Kaplan-Meier curves showing the overall survival of patients with *TGF-β2* mRNA levels upregulated ≥3-fold and *CREB1* mRNA levels upregulated ≥2-fold. Statistical significance was assessed by log-rank test. Data obtained from the REMBRANDT database.

7. **CREB1 regulates TGF-β2 expression in patient-derived xenograft models.** (a) Scheme showing the experimental procedure. (b) Immunohistochemistry of p-CREB1 and TGF-β2 from mouse tumors 60 days after inoculation with neurospheres expressing shRNAs targeting CREB1 and control shRNAs. Scale bar, 50 μm (c) Kaplan Meyer survival curves from mice in (b). (d) The TGF-β2 malignant autocrine loop. In GBM, TGF-β collaborates with the PI3K and RSK pathways through a CREB1-SMAD3 transcriptional complex to induce TGF-β2 expression. This leads to the generation of an autocrine loop and accumulation of TGF-β2 in the tumor, hyperactivation of TGF-β and tumor progression.
Figure 2 Rodon et al.
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Active CREB1 promotes a malignant TGF-β2 autocrine loop in glioblastoma

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