Canonical BMP2/4 signaling degrades SOX2 in glioblastoma propagating cells.

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ABSTRACT

The effect of Bone Morphogenetic Proteins (BMPs) on glioblastoma propagation cells (GPCs) is being debated [1-5]. We observed that exposure of GPCs to BMP2/4 initiates a rapid proteasomal degradation of the pluripotency factor SOX2 followed by the differentiation of GPCs into glial cells using 5 primary human glioblastoma lines tested. Enforced expression of SOX2 in GPCs antagonized BMP2/4-induced gliogenesis and reduction of proliferation. Both outcomes are consistent with the role of SOX2 in normal neurogenesis [6-8]. Using gene expression analysis, we observed that level of SOX2 in different glioblastoma lines tracks with the levels of oncogenes H/K/N-RAS and OLIG2. Our work provides support for BMP2/4-SOX2 axis in GPCs and points to the proteasome-regulated degradation of SOX2 that initiates glycolytic differentiation similar to that observed during normal development [6, 7]. Our work supports a potential for developing novel therapeutic strategies aimed at differentiation of GPC into non-tumorigenic glia.

Introduction

Glioblastoma represents the most common type of intracranial tumor (50% of total gliomas) and, with an incidence of approximately 10000 new cases each year in the United States [9] and a very poor prognosis (median survival = 12-15 months) [10], it is undoubtedly one of the most dreaded types of brain tumors. The standard therapeutic approach for the treatment of this

disease is radiotherapy in combination with chemotherapeutic Temozolomide treatment. Despite such treatments, glioblastoma are inexorably recurrent, underscoring the dire need for new, more efficient therapies. Glioblastoma is heterogeneous and a growing body of evidence speaks in favor of the existence, within the tumor mass, of a subpopulation of highly undifferentiated stem-like cells that resemble normal neural stem cells (NSCs) in terms of marker expression and differentiation potential [11-13]. Such population, termed Cancer Stem Cells or Tumor Initiating Cells, or Tumor Propagating Cells. Glioblastoma Propagating Cells (GPCs, the term we will use here), efficiently forms tumors in xenograft experiments and seem to be responsible for the radio- and chemo-resistance to standard clinical treatments which inexorably lead to GBM recurrence [14, 15]. Recent large-scale single-cell RNA-seq analyses of freshly isolated glioblastoma tumors revealed trilineage hierarchy centered on cancer progenitors, which appear to be the equivalent of glial progenitors during normal brain development [16]. Remarkably, glioblastoma progenitors were identified as the most proliferating cells within tumor mass driving chemoresistance and tumor growth. RNA velocity analysis revealed conservative cellular trajectories from GPCs to differentiated neuronal, oligo, astrocytic, and mesenchymal cells within all primary tumors analyzed [16]. Such hierarchical organization and naturally occurring differentiation trajectories in human glioblastoma identifies proliferating GPCs as target cells and strongly support the idea of further promoting naturally occurring differentiating of glioblastoma GPCs into non-tumorigenic cells.

Developmental pathways controlling self-renewal and differentiation of normal NSCs may be relevant to GPCs. For instance, the transcription factor SRY (sex-determining region)-box 2 (SOX2), which is required for self-renewal, proliferation and neurogenic differentiation of different type of NSCs [6, 7, 17, 18], is highly expressed *in vivo* in hyperproliferative areas of glioblastoma [19], marks proliferating GPCs *ex vivo* [16], expressed, albeit to variable extent, in cultured GPCs [5, 11] and functions as an oncogene by supporting their proliferation and tumor initiation capability [20]. On the other end, Bone Morphogenetic Proteins (BMPs), a group of cytokines of the TGF- β superfamily implicated in differentiation, apoptosis and proliferation of NSCs according to their developmental stage and microenvironment [21, 22], have been shown to exert a tumor suppressor activity on glioblastoma due to their ability to promote differentiation of GPCs and loss of their tumorigenic potential [1-3]. Despite the identification of such important players, the molecular mechanisms underlying SOX2 and BMP functions in GBM GPCs is poorly understood. Recent work from two independent groups arrived at different conclusions with respect to BMP2/4 effect on glioblastoma TPC. Sachdeva et al., 2019

suggested that exposure to BMP2/4 induces glioma stem cell quiescence [4]. Remarkably, pretreatment glioblastoma with BMP4 led to 3-4 times increase in survival upon orthotopic transplantation in mice [4] suggesting a dramatic improvement compared to the current clinical standards. In contrast, Dalmo et al., 2020 concluded that response is heterogenous among 40 primary human glioblastomas analyzed [5]. Invariably, such response in sensitive cells induced SMAD1/5/9 phosphorylation, SMAD4 expression, and consistent downregulation of SOX2 [5]. Our own observations align closely with the work of Dalmo et al., 2020. We observed that exposure of glioblastoma TPCs (5 primary lines) to BMP2/4 resulted in rapid downregulation of SOX2 followed by reduction in Ki67 and induction of GFAP (Fig. 3A). GBM3 line was initially unresponsive. We discovered that GBM3 was lacking expression of BMPR1; transduction with BMPR1 restored the phenomena. We discovered that BMP2/4 initiates proteasomal degradation of SOX2. Enforced expression of SOX2 in glioblastoma TPCs rescued BMP2/4-induced gliogenesis and reduction of proliferation. It is reassuring that both outcomes are consistent with the role of SOX2 in normal neurogenesis previously uncovered by others and our group [6-8]. Further, SOX2 may be required in GSCs to maintain high levels of known oncogenes such as RAS proteins with NRAS being the oncogene more susceptible to loss of SOX2. These results provide support for BMP2/4-SOX2 axis in glioblastoma TPCs and points to the proteasome-regulated degradation of SOX2 that initiates glycolytic differentiation similar to that observed during normal development [6, 7].

Results

Downregulation of SOX2 is mandatorily required for BMP-induced differentiation of GPCs

Low passage cultures of human primary glioblastoma in NSC media (i.e. serum-free media in the presence of bFGF and EGF) robustly enriches for GPCs [11]. We exploited such observation to derive low passage GPC lines from several primary glioblastoma patients. As expected, our cultures expressed high levels of markers common to both NSCs and GPCs such as Nestin and SOX2 (Fig. S1A). We also verified their tumor initiation capability by transplanting luciferase-transduced GPCs into immunodeficient Rag2^{-/-} & common γ -chain ^{-/-} knockout mice. Tumors could be observed after 43 days with as little as 1000 transplanted cells (Fig. S1B). To evaluate the efficacy of BMP treatment, we treated the cells with BMP2 or BMP4 for 3 days. Even in this short differentiation paradigm, BMP2/4 induced downregulation of the pluripotency factor SOX2, loss of proliferation potential (as evaluated by staining for the cycling marker Ki67)

and acquisition of a glial fate (staining for the astrocyte marker GFAP) in 4 out of 5 patient-derived GPCs (Fig. 1A, Fig. S2). For comparison, growth factors withdrawal (No GFs conditions) didn't induce appreciable differentiation (Fig. S2). Notably, TGF β 1, a closely related cytokine belonging to the same superfamily, didn't induce SOX2 downregulation or loss of proliferation and only marginally increased GFAP expression in comparison to growth factor withdrawal (Fig. S2). even when used at concentrations 100-fold higher than its Effective Dosage 50 (ED50). Since neither growth factor withdrawal nor TGF β 1 could reproduce BMP2/4 effect on GPCs, the BMP2/4 effect is specific and likely mediated through BMPR complexes. We assessed the effect of SOX2 knockdown in GPCs and noticed that shRNA against SOX2 was sufficient to mimic BMP2/4 treatment, namely, inducing loss of Ki67 and increase of GFAP expression (Fig. 1B-E). This BMP2/4 differentiation effect was observed even when the cells were cultured in self-renewing conditions (i.e. in the presence of bFGF/EGF), optimized to prevent differentiation [11]. This points to the dominant effect of BMP2/4 signaling over bFGF/EGF signaling and suggests that reducing SOX2 levels is sufficient to mediate GPC differentiation. The ability of BMPs to downregulate SOX2 in such a short time and the fact that loss of SOX2 mimicked BMP treatment prompted us to investigate the link between these two important regulators of GPC biology.

To understand if gliogenic differentiation and loss of GPC proliferation potential is direct consequence of SOX2 ablation downstream of BMP2/4, we overexpressed exogenous SOX2 under the control of a strong promoter (human phosphoglycerate kinase - PGK) and then treated GPCs with BMP2/4. Remarkably, enforces level of SOX2 antagonized BMP2/4 effect (Figure S3) preventing the loss of proliferation and astrocytic differentiation (Fig. 2A-B, Fig. S4A-B). These results suggest that the loss of SOX2 is a primary event downstream of BMP2/4 and it is required to mediate BMP2/4 effects on GPCs.

BMPR1 and SMAD phosphorylation is required for SOX2 degradation

Out of the five patient-derived GPC lines assayed for BMP activity, one (GBM3) didn't downregulate SOX2 in response to BMP2/3 treatment (Figures 1A,4A). Similarly, GBM3 didn't exhibit loss of proliferation (Fig. 1A), differentiation (Fig. 1A) and activation of BMP downstream effectors pSMAD1,5,8 (Fig. 3B-C). Of note, this line was still sensitive to SOX2 knockdown because a 40-50% downregulation of SOX2 using shRNA (Fig S6A, B, E) was sufficient to reduced proliferation (Fig. S6C-F). To understand this naturally occurring resistance to BMP2/4, we profiled the expression of known BMP receptors by qPCR. In contrast to other BMP-

responsive lines, GBM3 clearly lacked the expression of BMPR1A (Fig. 3D). We introduced BMPR1A into GBM3 by electroporation and assessed responsiveness to BMP4 b. Remarkably, the exogenous expression of BMPR1A was sufficient to re-establish a BMP2/4 sensitive and downregulation of SOX2 (figure 3E-F). These results strongly suggest that BMPR1A mediates the observed SOX2 downregulation at least in GBM3 line.

The canonical pathway downstream of BMPs results in activation through phosphorylation of SMAD1,5,8 which in turn bind to the co-activator SMAD4 and activate specific transcriptional networks [23]. To understand if canonical SMAD signaling is required to mediate SOX2 degradation in GBM GPCs, we knocked-down the co-activator SMAD4 in BMP responsive GPCs. Downregulation of SMAD4 (Fig. 3G) impaired the ability of BMP4 to downregulate SOX2 (Fig. 3H-I) suggesting that a canonical SMAD signaling is required for such effect.

BMP2/4 induces SOX2 protein degradation through the proteasome system

In order to gain mechanistic insights into the process of BMP-mediated downregulation of SOX2, we analyzed the kinetics of SOX2 protein and mRNA levels during BMP4 treatment. By western blot, SOX2 protein level downregulation was evident as early as 6 hours of treatment in concomitance with an increase in GFAP expression (Fig. 4A). In stark contrast, qPCR analysis did not reveal any downregulation of SOX2 mRNA at such early time point while still revealing GFAP mRNA increase (Fig. 4B). The decrease in SOX2 mRNA became evident only at later time points (data not shown). These data suggest that an early event of BMP signaling in GPCs is represented by SOX2 protein degradation without downregulation of the transcript. The major pathways responsible for protein degradation are the ubiquitin proteasome (UPS) and the autophagy-lysosome systems. Contrary to autophagy which functions mainly in the cytoplasm, UPS is ubiquitous and can mediate the degradation of nuclear factors [24, 25]. Considering the above, we tested whether the proteasome system is responsible for BMP-mediated SOX2 degradation. We treated GBM GPCs with BMPs and blocked the UPS with the proteasome inhibitors MG132 (Fig. 4C-E) and Bortezomib (Fig. S5). As assessed by Western blot (Fig. 4C) and immunofluorescence (Fig. 4D, Fig. S5), pharmacological inhibition of the proteasome with both compounds was able to block BMP mediated SOX2 degradation without affecting BMP signaling (i.e. SMAD1,5,8 phosphorylation, Fig. 4C). These results suggest that BMP signaling requires a functional UPS system downstream of SMAD1,5,8 in order to actively degrade SOX2 protein. Taken together, these observations suggest that BMP-mediated degradation of SOX2

requires a functional canonical BMPR1A-SMAD activation pathway which ultimately results in proteasomal degradation of SOX2.

SOX2 in GBM-GPCs controls critical known oncogenes

Despite SOX2 being considered a critical oncogene in glioblastoma, the molecular mechanisms underlying its functions in this context are poorly understood. We knocked down SOX2 and profiled the expression of known oncogenes with established critical roles in glioblastoma such as OLIG2 [26] and RAS [27] genes. We focused on such oncogenes because, despite being critical regulators of GPCs biology, similar to SOX2, these oncogenes display low rates of somatic mutation and therefore their pathological levels could be instead achieved through regulation of transcription (Fig. S7A). We observed that knock-down of SOX2 reduced the levels of OLIG2, KRAS, NRAS, and HRAS in all the tested GPC lines while MELK, a marker of GPCs, was downregulated in 3 out of 4 lines (Fig. 5A). We next correlated the levels of SOX2 downregulation achieved in different lines with the observed reduced levels of specific oncogenes. This analysis revealed that NRAS was the oncogene with the most linear correlation with SOX2 (Pearson correlation coefficient = 0.9206, Fig. 5B). To begin testing whether SOX2 directly regulates NRAS expression, we conducted chromatin immunoprecipitation-qPCR experiments. We observed a robust binding of SOX2 at genomic elements upstream of NRAS (Fig. 5C). Notably, an analysis of 542 Glioblastoma from the TCGA database revealed that SOX2 and NRAS were significantly overexpressed in cancer versus normal brain tissue, suggesting that this oncogenic pathway has a broad relevance to glioblastoma biology (Fig. S7B). Remarkably, enforced expression of exogenous SOX2 maintains high levels of NRAS and OLIG2 proteins even when GPCs were cultured in the presence of BMP4 (Fig. 5D-E) suggesting a direct and dominant effect of SOX2 on NRAS and OLIG2 expression.

Overall, our results suggest that the BMP-SOX2 axis regulates a network of important players in glioblastoma biology and that manipulation of SOX2 levels can be exploited to mitigate multiple oncogenic signals in a wide range of Glioblastoma.

DISCUSSION

A subpopulation of cancer cells with stem-like characteristics have been identified so far in several cancers such as breast, colon, lung and brain [12, 16, 28-30]. These cells have high tumorigenic capacity, resemble tissue-specific stem cells and seem to be resistant to standard chemo- and radiotherapy [14, 15]. Contrary to the idea of quiescent glioblastoma stem cells that

resist therapy [4], single cell analysis of freshly dissociated tumors revealed glioblastoma propagating cell are highly proliferative [16]. Critically, RNA velocity analysis revealed conservative differentiation trajectories from GPCs to neuronal, oligo, astrocytic, and mesenchymal cells within all primary tumors analyzed [16]. Such universally present differentiation observed in freshly isolated human glioblastoma samples strongly support the idea of further promoting naturally occurring differentiating of glioblastoma GPCs into non-tumorigenic cells.

Supporting the similarities between normal neural stem cells and GPCs, we observed that genes involved in regulation of neural stem cells during development also play a critical role in GPCs. Critically, SOX2, a well-known pluripotency gene, one of 4 Yamanaka factors [31], and key regulator of NSC and embryonic stem cells biology in general [6, 7, 17, 18, 32], has been shown function as an oncogene in several GPCs from different types of tumors [20, 33-35]. BMPs are important morphogens/developmental regulators with a broad effect on normal NSCs which seem to be dependent on the developmental stage and environmental milieu [22, 36]. Here we have documented that BMP2/4 antagonize SOX2 in GPCs by inducing a rapid degradation of SOX2 protein. This effect resemble BMP4 morphogenic activity on normal neuroepithelial cells of the dorsal part of the neural tube where, in order to acquire a migratory neural crest fate, NSCs must downregulate SOX2 [6]. The ability of BMP to downregulate on SOX2 in GPCs requires both a functional proteasome system and a canonical SMAD signaling. Of note, proteasome inhibition has been proposed as a possible strategy to cancer and gliomas in particular [37], but their advance is hampered by generalized toxicity issues, likely due to a universal requirement for proteasomal degradation for organismal homeostasis. In the case of GPCs, blocking proteasome activity could prevent SOX2 degradation and differentiation of GPCs within the tumor mass. This observation might explain, at least in part, why several proteasome inhibitors failed to show efficacy in clinical trials in glioma patients [38, 39]. In mouse ES cells precise level of Sox2 protein is control by a balanced methylation and phosphorylation switch [40]. Namely, Set7 monomethyl at K119 inhibits Sox2 transcriptional activity and induces Sox2 ubiquitination and degradation. The E3 ligase WWP2 specifically interacts with K119-monomethylated Sox2 through its HECT domain to promote Sox2 ubiquitination and degradation. In contrast, AKT1 phosphorylates Sox2 at T118 and stabilizes Sox2 by antagonizing K119me by Set7 and vice versa [40]. Interestingly, competitive binding of E3 ligases TRIM26 and WWP2 was reporter to control ubiquitin proteasomal degradation of

SOX2 protein in glioblastoma [41]. It is likely that BMP2/4 signaling tip the balance towards increased degradation of SOX2 through WWP2-dependent mechanism.

One of our lines (GBM3) lacked expression of BMPR1A and re-introduction of this particular BMP receptor was sufficient to re-establish sensitivity to BMP2/4 induced degradation of SOX2. Curiously, a BMP2/4-resistant glioblastoma line lacking BMPR1B expression but expressing BMPR1A was previously reported by Lee and colleagues [1]. This suggest that different receptors may mediate BMP activity in different glioblastoma lines and that different patient-specific genetic alterations might explain resistance to BMP2/4.

Despites SOX2 being known to play a critical role in maintaining tumorigenicity of glioblastoma, the molecular mechanisms underlying this effect still poorly understood. Here we linked levels of SOX2 to the expression of several other oncogenes that share with SOX2 two main characteristics: 1) low rate of mutations in Glioblastoma and 2) a reported critical role in regulation of glioblastoma tumorigenicity. Such oncogenes (OLIG2 and RAS family genes) robustly responded to SOX2 knock-down across different GPC lines by reducing levels of expression. In particular, the connection between SOX2 and NRAS seems clinically relevant. In fact, analysis of tumors from The Cancer Genome Atlas showed robust overexpression of both oncogenic proteins across 541 glioblastoma, thus suggesting that these two oncogenes might have a general relevance in glioblastoma despites the known heterogeneity of this tumor [42, 43]. Concluding, here we identified a BMP-SOX2 axis in glioblastoma multiforme that pave the road to the development of potentially useful therapeutic treatments for the modulation of SOX2 oncogenic signaling in glioblastoma. Future experiments will focus on genes and network interaction downstream of SOX2 and NRAS as well as investigating whether proteasomal degradation of SOX2 is specific to BMP2/4 or common multiple glycolytic signals.

EXPERIMENTAL PROCEDURES

Cell Culture

All primary glioblastoma cell lines were established in the laboratory of Dr. Kornblum at UCLA under IRB#11-000432 Detection of Neural Stem Cells in Adult Brain Tumors.

Monolayer cultures of patient-derived GPCs cells were propagated onto Matrigel-coated plates (BD Biosciences, final concentration = 1:30, overnight coating at 4°C) using base medium (1:1 ratio of DMEM/F12 Glutamax/Neurobasal medium [Gibco], 2% B27 supplement without vitamin A [Gibco], 10% BIT 9500 [StemCell Technologies], and 1 mM glutamine [Gibco]) supplemented with 20ng/ml EGF (Chemicon), 20ng/ml bFGF, 5 µg/ml insulin (Sigma) and 5 mM nicotinamide

(Sigma)). glioblastoma-GPCs were split enzymatically with Accutase (Chemicon) approximately once a week at a 1:3 ratio. For BMP/TGF β 1 treatments (all from R&D), BMP2 was used at 200ng/ml (corresponding to 1X its Effective Dosage 50 -ED50, as reported by the manufacturer), BMP4 was used at 100ng/ml (3.3X its ED50) and TGF β 1 was used up to 20 ng/ml (100X its ED50).

Immunocytochemistry

Cells were rinsed with PBS and fixed in 4% paraformaldehyde (PFA)/PBS for 10 min at room temperature. After blocking with PBSAT (3% BSA and 0.5% Triton X-100 diluted in PBS) for 1h at room temperature, cells were incubated overnight at 4°C with the primary antibodies diluted in PBSAT. Antibodies: mouse anti-SOX2 (R&D, MAB2018), rabbit anti-GFAP (Millipore AB5804), rabbit anti-KI67 (Vector, VP-RM04), rabbit anti-OLIG2 (Millipore AB9610), mouse anti-NRAS (Santa Cruz, sc-31), pSMAD1,5,8 (Cell Signaling, 9511) The appropriate fluorochrome-conjugated secondary antibody was used with every primary antibody at a 1:1000 dilution. Nuclear co-staining was performed by incubating cells with DAPI nuclear dye.

Quantitative PCR.

Total RNA was extracted using the RNeasy kit (Qiagen) and 1 μ g total RNA was reverse transcribed using the Quantitect kit (Qiagen), according to the manufacturer's instructions. 2 μ l of purified cDNA diluted 1:10 was used as a template. qPCR was performed with SYBRGreen master mix (Invitrogen) according to the manufacturer's recommendations. HPRT or GAPDH were used for normalization. Data were analyzed using the $\Delta(\Delta CT)$ method. Primers used are listed in Table S1.

ChIP and qPCR

ChIP was performed using the Ez-ChIP kit (Millipore) according to the manufacturer's recommendations with the following modifications: 1·10⁶ cells equivalents were used for each immunoprecipitation, cells were sonicated to yield chromatin fragments of 100-1000 bp, 5-10 μ g of immunoprecipitating antibodies were employed in each ChIP. Antibodies were: normal rabbit IgG (Millipore, PP64; used a non-specific control) and rabbit anti-SOX2 (Millipore, AB5603). To evaluate factor-specific enrichment at different promoter sites, qPCR was performed using the purified chromatin as a template. Amplification was performed with site specific primers

designed to flank the genomic region of interest. For a primer list see Table S1. qPCR data were normalized to the values obtained with the non-specific antibody (normal rabbit IgG).

Lentivirus-mediated shRNA and overexpression

For shRNA experiments, lentivectors expressing doxycyclin-inducible human SOX2 shRNA or scrambled (SCR) shRNA were described before [6]. GPCs were infected with lentiviral particles to obtain lines stably carrying either SOX2 or SCR inducible shRNA. Alternatively, for short terms experiments, GPCs were acutely infected with the lentiviruses and shRNA was induced no earlier than 3 days post-infection. For SOX2 overexpression, human SOX2 coding sequence was amplified from cDNA obtained from hES-derived neural stem cells and cloned into a lentivector under control of the PGK promoter. SOX2 CDS was inserted upstream of a IRES-GFP sequence. Primers, vectors and details about the cloning strategy are available upon request. All lentivectors were packaged into lentiviral particles at the Viral Vectors Facility at the Sanford-Burnham Medical Research Institute (La Jolla, CA).

Electroporation

BMPRI1A coding sequence was inserted downstream of PGK promoter into a PGK-IRES-GFP vector. A PGK-GFP plasmid was used as a control. The vectors were electroporated into GBM3 with the Neon transfection system (Life Technologies) according to the manufacturer instructions. In details, $1 \cdot 10^5$ cells were electroporated with $1.5 \mu\text{g}$ of plasmid using 3 pulses of 10ms at 1400V.

Knock-down studies with siRNAs

Control and pooled SMAD4 siRNAs were from Dharmacon. siRNAs Were delivered with the RNAiMAX kit according to the manufacturer instructions. In details, $6 \cdot 10^4$ cells were transfected using $0.5 \mu\text{l}$ of RNAiMAX reagent and $4 \mu\text{l}$ of $0.5 \mu\text{M}$ siRNAs.

Digital image analysis

For each given marker, pictures of cells cultured under different experimental conditions were taken with the same exposure time and contrast/brightness parameters. When amount of fluorescence was evaluated, the software ImageJ was used (<http://imagej.nih.gov/ij/>). A minimum of 3 random fields containing at least 100 cells was analyzed for each condition.

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Figure 4: A canonical BMP signaling mediates SOX2 degradation

A) Immunostaining for SOX2 in GBM3 shows lack of responsiveness to BMP4 after 3 days of treatment (*i.e.* SOX2 levels are unaffected). No GFs = growth factors withdrawal.

B-C) Immunostaining (B) and quantification (C) of pSMAD1,5,8 in response to BMP4 treatment in 104A (BMP-responsive) and GBM3 (BMP-resistant) GPC lines. D) qPCR quantification of the reported BMP receptors in BMP responsive (104A,PBT24,GBM2) and unresponsive (GBM3) lines. Note that GBM3 lacks expression of BMPR1A. E-F) Immunostaining (E) and quantification (F) of SOX2 staining in GBM3 GPCs electroporated with GFP control or SOX2-IRES-GFP vectors and cultured in the presence or the absence of BMP4 for 48 hours. Note that re-introducing BMPR1A in GBM3 re-establish sensitivity to BMP treatment in this line. Arrows point to GFP-expressing electroporated cells. G) qPCR quantification of SMAD4 mRNA in GBM2 cells transfected with control (siCTRL) or SMAD4 (siSMAD4) siRNAs, 3 days after transfection.

H-I) Immunostaining (H) and quantification (I) of SOX2 levels in control (siCTRL) and SMAD4 (siSMAD4) GBM2 cells, cultured for 24h in the presence or the absence of BMP4. Note that in such BMP-responsive line, knock-down of SMAD4 abolishes BMP-mediated SOX2 degradation. Error bars = standard error; **p<0.005. Scale bars = 50 μ m

Figure 5: SOX2 controls oncogenes with ascertain critical role in Glioblastoma

A) qPCR for SOX2, OLIG2, MELK and RAS oncogenes in the reported GBM GPCs lines transduced with control (shCTRL) or SOX2 (shSOX2) inducible lentiviruses. SOX2 was downregulated for 4 days. B) Across lines correlation between the levels of SOX2 knock-down achieved in a particular GPC line and the resulting decrease in the reported oncogenes. Linear correlation coefficient (R^2) is also shown. Note that NRAS displays the strongest linear dependency on SOX2 levels. Scale bars = 50 μ m. C) Chip-qPCR showing SOX2 binding at genomic elements upstream of NRAS gene in GBM2 and PBT24 GPC lines. Numbers are relative to the transcription starting site. D) Immunostaining for NRAS and OLIG2 in PBT24 GPCs transduced with either pgk-GFP or pgk-SOX2-IRES-GFP lentiviruses and cultured in the presence or the absence of BMP4. for 3 days. E) Quantification of NRAS and OLIG2 fluorescence in the conditions reported in (D). Error bars = standard error; **p < 0.005.

Figure S1: Human patient-derived GLIOBLASTOMA GPCs

A) immunostaining for the neural stem cell markers SOX2 and NESTIN in 5 patient-derived glioblastoma GPCs. Scale bar = $50\mu\text{m}$ B) Xenografts of PBT24 GPCs transduced with luciferase lentivirus showing tumors 42 days after transplantation in NSG immunodeficient mice. Number of transplanted cells is reported in the pictures. Cells were stereo tactically injected in the right hippocampus.

Table S1

qPCR:

Gene	Sequence F	Sequence R
<i>GAPDH</i>	GAAGGTGAAGGTCGGAGTC	ATGGGATTTCCATTGATGAC
<i>HPRT</i>	TGGAGTCCTATTGACATCGCCAGT	AACAACAATCCGCCCAAAGGGAAC
<i>HRAS</i>	GAGGAGCGATGACGGAATATAAG	GTTCTGGATCAGCTGGATGG
<i>KRAS</i>	AGGCAAGAGTGCCTTGACGATACA	ACTGGTCCCTCATTGCACTGTACT
<i>MELK</i>	cgggcttaaacgaactgggtt	cctcacgggtacatatcttct
<i>NRAS</i>	CAGTGCCATGAGAGACCAATAC	TCTGCTCCCTGTAGAGGTTAAT
<i>OLIG2</i>	TAAGCTGTTTGCTCACGTGACTGC	CTACAAAGCCCAGTTTGCAACGCA
<i>SMAD4</i>	TCCAGCATCCACCAAGTAATC	GCAGTGCTGGTAGCATTAGA
<i>SOX2</i>	CACAACTCGGAGATCAGCAA	CGGGGCCGGTATTATAATC

ChIP-qPCR primers:

Promoter/regulatory elements	Sequence F	Sequence R	Site
NRAS	ACACTAGCACCTAGCGCTTTC	CCCGCTACGTAATCAGTCG	0
NRAS	CGGTTTTATCTTCCCCCTTG	CGCCATTAACAGAAAAGTGG	-114
NRAS	TTCATGCAAATGAGGAGTGC	CAGTGATCTAAGCAGCAGCAAG	-500
NRAS	TTCAGCCCCCTAACCTAATG	AGTAAGCACTCCTGACCCATTC	-1514
NRAS	AAGTTGGGACCTTGAGTTGG	AGCAAGCACTTGCAAAGGAG	-2000