

# Identification of Novel Therapeutic Targets in Glioblastoma

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## ABSTRACT

Glioblastoma is the most common and malignant brain cancer in adults. Current therapy consisting of surgery followed by radiation and temozolomide has a moderate success rate and the tumor reappears. Among the features that a cancer cell must have to survive the therapeutic treatment and reconstitute the tumor is the ability of self-renewal. Therefore, it is vital to identify the molecular mechanisms that regulate this activity.

SOX2 is a transcription factor whose activity has been associated with the maintenance of the undifferentiated state of cancer stem cells in several tissues including the brain. Several groups have detected increased SOX2 levels in biopsies of glioblastoma patients, with the highest levels associated with poor outcome. Therefore, SOX2 silencing might be a novel therapeutic approach to combat cancer and particularly brain tumors. In this review, we will summarize the current knowledge about SOX2 in glioblastoma and recapitulate several strategies, which have recently been described targeting SOX2 in this malignancy.

## SOX2

SOX (*sex-determining region Y (SRY)-box*) factors are a family of transcriptional regulators that carry out important functions during embryonic development and are key components for the maintenance of the stem cells in adult tissues. This family of transcription factors is characterized by a conserved high mobility group (**HMG**) DNA-binding domain and is composed of 20 members divided into 8 groups (from A to H), based on their HMG sequence identity [1]. Members within a subfamily conserve at least 80% identity in their HMG-domain, in addition to sharing other well-conserved regions. Moreover, members from the same group might have overlapping expression patterns, share biochemical properties, and perform synergistic or redundant functions. In contrast, members from different subgroups usually develop different functions [2].

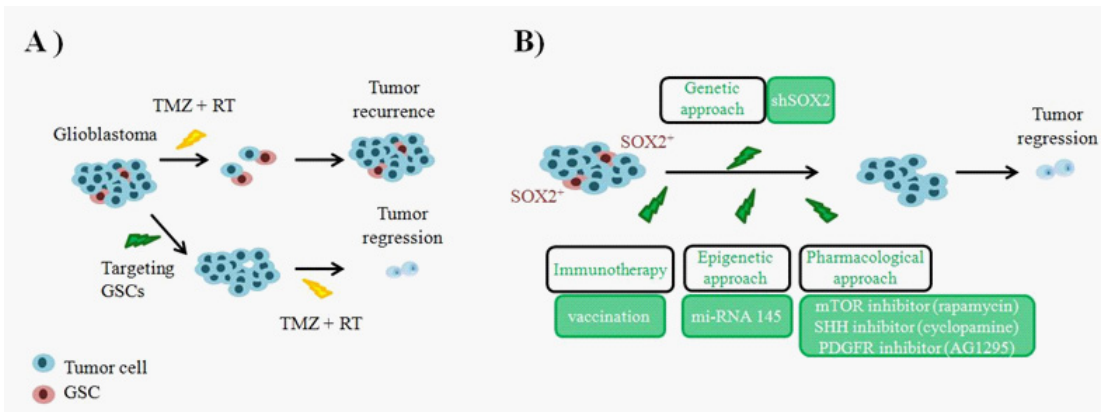
SOX2 is a member of the SOXB1 group (together with SOX1 and SOX3), which is required for the maintenance of the embryo before implantation. SOX2 has a role in cell fate and maintenance of the progenitor's identity during embryogenesis. It is also important for tissue homeostasis and regeneration by maintaining stem cell activity in several compartments, particularly in the central nervous system (**CNS**), in adults [3]. During recent years several studies have demonstrated the impact of SOX2 deregulation in a wide variety of human diseases. A heterozygous inactivation of SOX2 causes syndromic microphthalmia-3 (**MCOPS3**), a genetic disease characterized by anophthalmia, microphthalmia, mild hypopituitarism and sometimes learning difficulties, convulsions, motor dysfunctions and growth problems [4]. On the contrary, SOX2 upregulation has been linked to the development and maintenance of several types of cancers [3,5-7].

## GLIOBLASTOMA

Tumors of the CNS form a heterogeneous group of diseases that comprise less than 2% of the total number of cancer cases. Every year in the world, approximately 350,000 people are diagnosed with gliomas, making it the most common primary brain tumor [8]. According to clinical and histopathological characteristics, WHO classified them into four grades of malignancy: pilocytic astrocytoma (grade I); diffuse astrocytoma (grade II); anaplastic astrocytoma (grade III), and glioblastoma multiforme (GBM, grade IV). GBM is the most common, malignant and lethal glioma subtype in adults, accounting for 12-15% of all brain tumors and about 50% of gliomas. The incidence ranges from 1 to 5 cases per 100,000 people per year, with an average patient survival of around 15 months [8]. This prognosis identifies this type of tumor as one of the most aggressive and fatal cancers overall. According to the clinical presentation, there are two main GBM subtypes; primary or *de novo* GBM and secondary GBM. Primary tumors, the most common form, typically appear in older patients without any prior clinical or histological evidence of a lower grade precursor lesion and they have an aggressive clinical course. Secondary tumors are more frequent in younger people and they progress from a previous lower grade glioma with a less aggressive clinical course [9]. Genetic and transcriptomic expression studies have allowed a more detailed molecular classification identifying 4 GBM subtypes: (i) classical,

with *EGFR* amplification and over expression, *CDKN2A* and *PTEN* deletion, *NES* over expression and activation of NOTCH and SHH signaling pathways; (ii) mesenchymal, with loss of *NF1*, *TP53* and *PTEN*, over expression of *MET*, *CHI3L1*, *CD44* and *MERTK*, and activation of the TNF and NF- $\kappa$ B pathways; (iii) proneural, with *PDGFR* amplification, loss or mutation of *IDH1*, *PIK3K*, *TP53*, *CDKN2A* and *PTEN*, and activation of HIF, PI3K and PDGFR pathways; and (iv) neural, with *EGFR* amplification and over expression, and expression of neuronal markers such as *NEFL*, *SYT1* and/or *GBRA1* [10]. Different subtypes are associated with variable prognosis and response to therapy, and this heterogeneity and this therapy resistance are likely the main characteristics responsible for the glioblastoma patients' dismal outcome.

The cancer stem cell (**CSC**) theory postulates a hierarchically organized system in opposition to the stochastic model of tumor growth. The CSC model suggests that only a small group of cells have quiescence and self-renewal capacity within the tumor bulk, and that those are responsible for tumor maintenance and recurrence [11]. Nowadays there is a lot of evidence that supports the existence of CSC in GBM, called glioma stem cells (**GSC**), and their relevance in the etiology of GBM. Several groups have been able to isolate GSCs from patient derived tumors and multiple experimental data have shown that these cells are responsible for glioblastoma initiation and maintenance [12,13] as well as for recurrence and chemoresistance [14-16]. Moreover, there are different explanations for their origin. One of them proposes that neural stem cells (**NSCs**) undergo malignant transformation while retaining stem cell features. Indeed, inactivation of *TP53*, *INK4a/ARF* locus, *PTEN* and *NF1* tumor suppressors or activation of EGFR/PDGFR/PI3K oncogenic pathways in NSCs induces high grade gliomas [17]. Similarly, transient amplifying progenitors have also been shown as GSCs and the cell of origin of GBM [18]. Another theory claims that more mature or differentiated cells are reprogrammed and form GSCs and high grade gliomas. Indeed, several mutations in astrocytes, oligodendrocyte progenitors or in neurons are sufficient to confer stem cell properties during neoplastic transformation [19,20]. Therefore, in order to establish efficient treatments that can induce a long-lasting clinical response in GBM, it is important to develop strategies that can specifically target GSCs (Figure 1A). CSCs, including GSCs, achieve self-renewal through asymmetric division, in which one daughter cell retains the self-renewal ability, and the other is directed to differentiation. Moreover, heterogeneous tumor cell populations and their respective cell division mode have been shown to confer differential sensitivity to therapy in brain tumors [21]. Therefore, modulation of asymmetric and symmetric division of GSCs may provide novel strategies for targeting differentially the GSC and the bulk tumor mass. Several drugs and approaches have been postulated [22] to directly target the GSC population and/or the molecular mechanisms underlying their regulation. In this review we focused our attention on targeting the SOX2 gene.



**Figure 1:** Significance of glioma stem cells (GSCs) and SOX2 targeting in the recurrence of glioblastoma.

A) Glioblastoma is a heterogeneous tumor composed of GSCs (in red, chemo- and radiotherapy resistant) and of differentiated tumor cells (in blue, chemo- and radiotherapy responsive). GSCs need to be targeted before current standard approaches to achieve tumor regression. TMZ: temozolomide; RT: radiotherapy. B) In order to avoid tumor recurrence, genetic, epigenetic and pharmacological approaches targeting GSCs expressing high levels of SOX2 have been postulated.

## SOX2 ACTIVITY AND GBM

Several studies have identified an over expression of SOX2 in GBM patient samples. It was first found elevated in 90% of human biopsies studied at the mRNA and protein level in 2007. This research also showed that SOX2 expression was restricted to the nucleus [23]. Since then, over expression of SOX2 (with varying percentages of positive cases) was observed in several different and independent cohorts [16,24-27]. Importantly, high levels of SOX2 have been associated with tumor aggressiveness and worse prognosis [28,29]. Moreover, several groups, including ours, identified SOX2 enrichment in the undifferentiated GSC populations and demonstrated that SOX2 possesses an important role in the maintenance of GSCs. Indeed, downregulation of SOX2 through RNA interference strategies in GSCs impairs proliferation and their ability to form tumors *in vivo* [25,30-32]. Additionally, silencing of SOX2 leads to reduced migration and invasion capabilities [25,33], while it increases senescence and produces an arrest of the cell cycle in G0/G1 [16,34]. The impact of SOX2 in glioblastoma cells has been further substantiated with over expression studies. Indeed, ectopic elevation of SOX2 increases the capacity of invasion and migration [25], in addition to cell proliferation and self-renewal activity in conventional glioma cell lines [16]. In agreement with this last function, SOX2 is one of the transcription factors, together with POU3F2, OLIG2 and SALL2, that is sufficient to reprogram differentiated glioma cells into induced GSCs, similar to GSCs obtained from human biopsies [35]. Altogether these data show that glioma cells have a dependence on SOX2 to maintain their tumorigenic activity with GSCs displaying high levels of SOX2. They also demonstrate that SOX2 possesses an important role in the maintenance of GSCs.

In regards to a putative role of SOX2 controlling cell division modes, a recent work showed that the inhibition of the FACT chaperone complex in GSCs promotes their asymmetrical division in a process that involves SOX2 downregulation [36]. In line with these results, other authors found that the knockdown of HMGA1, a chromatin structure regulator, induces an asymmetric division together with a decrease in SOX2 expression both in GSCs and in colon CSCs. [37,38]. These works suggest that SOX2 action in the maintenance of undifferentiated GSCs could rely on effects promoting symmetrical in addition to the expected asymmetrical division. Importantly, these results support a hierarchical model of glioma cells controlled by *SOX2* expression, which brings up the idea to target SOX2 or to find downstream targetable genes as a strategy to eliminate GSCs and subsequently the tumor.

## UPSTREAM REGULATION OF SOX2

The regulation of SOX2 is a complex network of transcriptional, post-transcriptional and post-translational regulators (Table 1). Some of these regulators are altered in GBM and lead to the over expression of SOX2. Four main signaling pathways are involved in SOX2 expression, including TGF- $\beta$ , SHH, EGFR and FGFR. All these signaling pathways are aberrantly activated in GBM, which leads to the maintenance of the tumor at least in part through SOX2 factor over expression. The inhibition of TGF- $\beta$  signaling decreases the tumorigenicity of GSCs by the suppression of SOX2 activity [31]. SOX2 function is also mediated by other members of the SOX family such as SOX4, acting downstream of the TGF- $\beta$  signaling pathway, and forming a complex with OCT4 at the SOX2 promoter [31,39]. SHH pathway is initiated with the binding of SHH ligand to PTCH receptor, causing the activation of SMO. Active SMO will activate GLI1/2, which then translocates into the nucleus and activates SOX2. The regulation of SOX2 by SHH occurs in neural and brain stem cells [40,41] and the pharmacological inhibition of these pathways silences SOX2 expression and impairs glioma cells' tumorigenic activity [16]. The FGFR pathway regulates SOX2 expression through two main signaling cascades, (i) MEK/ERK and (ii) PI3K/AKT/mTOR, two signaling pathways activated in GBM, and whose suppression leads to the inhibition of tumorigenesis and self-renewal of GSCs [42]. The MEK/ERK pathway regulates the expression of SOX2 through the final phosphorylation of ERK, which translocates into the nucleus and activates the transcription of SOX2. The PI3K/AKT/mTOR pathway regulates positively the expression of SOX2 through the activation of the mammalian target of rapamycin complex 1 (mTORC1). The inhibition of mTORC1 by rapamycin in GSCs leads to the inhibition of the SOX2 expression and a decrease in self-renewal activity [16]. SOX2 is also regulated by EGFRvIII, a frequent mutant in GBM that leads to the activation of pro-oncogenic signaling in GBM. Indeed, the expression of EGFRvIII positively correlates with the expression of SOX2 and it is associated with an enhanced self-renewal ability and tumor initiating activity [43]. This correlation has demonstrated that it is carried out by the axis EGFRvIII-STAT3-PEDF-Notch [44].

SOX2 can also be regulated via post-translational modifications such as ubiquitination, phosphorylation and acetylation. Acetylation and phosphorylation enhance the export of SOX2 to

the cytoplasm and inhibit the ability to bind DNA in embryonic stem cells [45,46]. In contrast, the phosphorylation of SOX2 by AKT stabilizes the protein and enhances the transcriptional activity of SOX2 [47]. However, the function of these modifications in GSCs' activity remains elusive.

**Table 1:** Summary of relevant findings of SOX2 in glioblastoma.

Year	Finding	Author [Ref]
2007	SOX2 is overexpressed in human glioma samples	Schmitz [23]
2009	Genetic SOX2 silencing impairs GSC activity	Gangemi [30]
2011	Identification of SOX2 downstream targets in GBM with miRNA145-SOX2 feedback loop	Fang [48]
2011	Genetic and epigenetic regulation of SOX2 in GBM samples	Alonso [25]
2011	Identification of SOX2 as a target for combination treatments with tyrosine kinase inhibitors	Hagerstrand [32]
2011	Identification of SOX2 downstream of TGF- $\beta$ signaling in GSCs	Ikushima [31]
2014	Elevated SOX2 promotes dedifferentiation and acquisition of GSC characteristics in GBM cells	Suva [35]
2007,2014	First results supporting SOX2 mediated immunotherapy in mouse models and human samples	Schmitz[23] Favaro [41]
2016	SOX2 induces chemoresistance, which is inhibited by rapamycin	Garros-Regulez [16]

Additionally, *SOX2* genetic amplification and promoter DNA hypomethylation has been identified in a set of GBM patients, further expanding the mechanism responsible for SOX2 upregulation in glioblastoma samples and GSCs [25]. Moreover, the regulation of SOX2 through different miRNAs, including miRNA21 or miRNA145, has been described in glioma cells, with this axis having relevant functions in GSCs' activity and in the clinic [29,48].

## DOWNSTREAM REGULATION OF SOX2

Several studies have started to characterize downstream targets of SOX2 in glioblastoma. A study of Fang et al. identified SOX2 downstream targets by CHIP-seq and microarray analyses in the LN229 glioma cell line. They found 4,883 SOX2 binding regions in the GBM cancer genome. Moreover, they detected 489 genes whose expression was altered with SOX2 inhibition, including additional SOX family members, cytokines, or BEX members with tumor suppressor activity in glioblastoma. They also identified 105 pre-miRNAs (corresponding to 95 mature miRNAs) that were differentially expressed in SOX2 knockdown glioblastoma cells. Among them, they observed that miRNA145 and SOX2 form a double negative feedback loop in GBM cells [48], demonstrating that the relationship between SOX2 and miRNAs is bidirectional. We have recently observed that several oncogenic SOX2 functions are mediated by SOX9, another member of the SOX family [16], which also carries out important functions in GSC regulation and glioblastoma [26]. This regulation occurs at the post-transcriptional level [16]. Additionally, specific phenotypes associated with SOX2 have been linked to different genes and signaling pathways. Indeed, SOX2-regulated migratory and invasive capacities are mediated by RhoA-dependent pathway and focal adhesion kinase (**FAK**) signaling, whereas proliferation is mediated by CYCLIN D1 expression [34]. WNT signaling pathway, self-renewal and retinoic acid associated genes are within the genes involved in SOX2 mediated glioma cell plasticity and astrocytic differentiation [49].

# THERAPEUTIC APPROACHES TOWARDS SOX2 REDUCTION IN GBM

The current chemotherapeutic agent for newly diagnosed GBM is Temozolomide (**TMZ**), which extends patient survival from 12 to 15 months [50]. A role for SOX2 in TMZ chemoresistance has been deciphered during recent years. Thus, cells with elevated SOX2 expression are more resilient to TMZ, whereas its inhibition sensitizes glioma cells to this agent [16]. This cellular finding is correlated with clinical information. High levels of SOX2 have been included as a marker of the proneural subtype, which has been shown to be the most resistant subgroup to current therapeutic radio- and chemotherapy treatment [10]. The involvement of SOX2 in chemoresistance has been further substantiated through different mechanisms. The inhibition of *SOX2* by miRNA145 decreases the chemoresistance of GSCs increasing the sensitivity to radiation and TMZ [51]. The over expression of ID-4 suppresses the expression of miRNA9\*, which can repress *SOX2*, leading to an increase in the SOX2 expression. SOX2 induction enhances the ATP-Binding Cassette (**ABC**) transporters, ABCC3 and ABCC6, through direct transcriptional regulation. The activation of ABC transporters confers chemoresistance to GSCs [52]. These facts together with the prominent role of SOX2 in the regulation of GSCs suggest that SOX2 might be a key responsible factor for resistance to current chemotherapy and postulate that targeting its activity may offer a novel, attractive therapeutic approach to treat glioblastoma patients.

Several strategies are starting to target SOX2 directly or indirectly to target GSCs (Figure 1B). PDGFR signaling has been involved in glioblastoma biology through studies based on analyses of human tumor tissue, cultured glioblastoma cells, and mouse glioblastoma models [22]. Similarly, IGF1-R signaling has been described in glioblastoma, and findings from preclinical studies suggest favorable combination effects when IGF1-R inhibitors have been combined with other receptor tyrosine kinase (**RTK**)-targeting agents. Interestingly, a combination therapy with PDGF and IGF-1 receptor inhibitors (imatinib and NVP-AEW541) produces a significant tumor growth reduction through SOX2 downregulation and GSC sensitization [32].

Rapamycin is an allosteric inhibitor of mTOR, which has been shown to dramatically reduce the self-renewal and tumorigenic activity of glioma cells and GSCs [53]. In agreement with these results, several phase I and II clinical trials with some rapalogs, first generation mTOR inhibitors such as everolimus or temsirolimus alone or in combination, showed radiographic and symptomatic evidence of improvement in delaying tumor progression without provoking high toxicity in patients with newly diagnosed or recurrent glioblastoma [54]. It has recently been demonstrated that mTOR regulates the expression of SOX2. Genetic mTOR silencing or pharmacological treatment with rapamycin markedly reduced SOX2 levels in glioma cells [16]. Interestingly, the same work showed that the combination of rapamycin and TMZ was more efficient and displayed increased cytotoxicity in cells with high endogenous SOX2 levels [16]. Cyclopamine, an inhibitor of the SHH pathway, was also proved effective in reducing SOX2

expression and inducing cytotoxicity in *in vitro* studies, but unlike rapamycin, its combination with TMZ did not increase the sensitivity of glioma cells to chemotherapy [16]. Furthermore, several studies have demonstrated the capability of the GSCs to transdifferentiate into tumor-derived vascular endothelial and mural-like cells in a VEGF independent manner, making the tumor resistant to anti-vascular therapy [55,56]. This transdifferentiation relies on high levels of NESTIN and CD133 stem cell markers; however, SOX2 has not been evaluated. It would be of interest to determine the role of SOX2 in transdifferentiation and if knocking down its expression levels is sufficient to prevent the process.

Alternative treatments are arising using SOX2 protein as the principal target of new therapies. Immunotherapy represents a promising treatment option to improve the clinical outcome of patients suffering from GBM. In 2014, Dr. Nicolis' group did transplants of GSCs in mice brains using peptide vaccination against SOX2. Peptide vaccination alone increased the mice's survival and the vaccination in combination with the current treatment with TMZ doubled the mice's survival time [41]. Earlier, Schmitz and coworkers had identified SOX2 as a glioma-associated antigen abundantly and specifically overexpressed in glioma cells. In addition, they had identified an immunogenic HLA-A\*0201-restricted T-cell epitope derived from SOX2 that effectively activated tumor directed cytotoxic T lymphocytes [23]. These results highlight the suitability of SOX2 for a novel strategy based in immunotherapy alone or in combination with current therapies for the treatment of patients with glioblastoma.

The use of miRNA delivery could be another therapy linked to SOX2 for cancer cells in the brain. As described above, miRNA145 is associated with SOX2. Interestingly, miRNA145 delivery in GSC and in xenograft studies *in vivo* demonstrated the ability to suppress tumorigenicity by direct downregulation of SOX2 protein with cells becoming more sensitive to chemotherapeutic agents such as TMZ or cisplatin [51]. The promising therapeutic prospect of miR-145 might improve current cancer treatments, especially for those tumors that have developed a resistance to conventional therapeutic methods. However, a note of caution needs to be included since the use of viral vectors for gene delivery may be accompanied by several problems, including an immune response.

## CONCLUSION

Glioblastoma is the most common and malignant adult primary brain tumor with a dismal patient prognosis. It is characterized by presenting significant heterogeneity at the genetic, molecular, cellular and morphological level, which severely affects clinical practice. Tumor bulk is formed by differentiated cells targetable with chemo- and radiotherapy and GSCs, which need to be eradicated in order to achieve effective therapeutic response.

SOX2 transcription factor is important during embryonic development and for the maintenance of stem cell properties of the CNS in adult and aged stages [3,57,58]. It is also a key regulator of



stemness in CSCs and its biological function has been widely described in GBM, associated with stemness activity and a poor clinical outcome (Table 1). Therefore, SOX2 is a strong molecular candidate to be targeted in glioblastoma samples and the potential benefit of SOX2 targeting in the scenario of such a lethal tumor is not negligible. However, a point to take into account with regards to SOX2 targeting for GBM treatment is that other SOX2 expressing cell populations as astrocytes, which develop relevant roles in neurogenesis, could be susceptible to the treatments. The consequences of this must be evaluated and other molecules acting downstream of SOX2, more specific of GSCs, should be identified and considered as targets.

Transcriptional regulation and/or post-transcriptional suppression of SOX2 through miRNA regulation are promising approaches. There are also encouraging results with SOX2 immunotherapy or combining tyrosine-kinase and IGF1 inhibitors. These results show the preclinical proof of concept that silencing SOX2 activity is an effective strategy in glioblastoma. More solid and extensive preclinical results and clinical trials with the postulated combination of therapies in glioblastoma patients whose biopsies express elevated SOX2 are needed to establish their clinical impact.

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