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mTOR inhibition decreases SOX2-SOX9 mediated glioma stem cell activity and temozolomide resistance

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ABSTRACT

Background: SOX2 and SOX9 are commonly overexpressed in glioblastoma, and regulate the activity of glioma stem cells (GSCs). Their specific and overlapping roles in GSCs and glioma treatment remain unclear.

Methods: *SOX2* and *SOX9* levels were examined in human biopsies. Gain and loss of function determined the impact of altering SOX2 and SOX9 on cell proliferation, senescence, stem cell activity, tumorigenesis and chemoresistance.

Results: SOX2 and SOX9 expression correlates positively in glioma cells and glioblastoma biopsies. High levels of SOX2 bypass cellular senescence and promote resistance to temozolomide. Mechanistic investigations revealed that SOX2 acts upstream of SOX9. mTOR genetic and pharmacologic (rapamycin) inhibition decreased SOX2 and SOX9 expression, and reversed chemoresistance.

Conclusions: Our findings reveal SOX2-SOX9 as an oncogenic axis that regulates stem cell properties and chemoresistance. We identify that rapamycin abrogate SOX protein expression and provide evidence that a combination of rapamycin and temozolomide inhibits tumor growth in cells with high SOX2/SOX9.

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Glioma stem cell; SOX2; SOX9; senescence; rapamycin and temozolomide

1. Introduction

Glioblastoma multiforme is the most common and malignant adult primary brain tumor with an incidence ranging from 2 to 10 cases per 100,000 people per year. The incorporation of temozolomide (TMZ) to clinical practice resulted in improved quality of life, delayed tumor progression, and extended patient survival.[1] Current standard treatment includes multimodal therapy of surgery followed by concomitant radiotherapy and TMZ. However, most patients develop refractory disease and tumor recurrence because of the intrinsic or acquired chemoresistance of glioma cells. There are several characteristics of glioblastoma that are responsible for difficulties of current therapies, including genetic, molecular and morphological heterogeneity,[2,3] the presence of a subpopulation of cancer cells [called glioma stem cells (GSCs)] that drives tumor formation and maintenance [4], and the resistance of GSCs to therapeutic treatments.[5,6] Therefore, it is critical to elucidate the molecular mechanisms underlying the chemoresistance of glioma cells to discover more efficient therapeutic treatments.

GSCs share phenotypic and functional characteristics with neural stem cells (NSCs), such as self-renewal and multipotency. Accumulating evidence indicates that dysregulation of genes and pathways controlling normal NSCs plays a role in the regulation of GSCs. SOX [sex-determining region Y (SRY)-box] are a family of transcription factors characterized by a conserved high-mobility group DNA-binding domain. They control several developmental processes and are involved in the maintenance of stem cell activity in a wide range of tissues during embryogenesis and adult stages.[7] Their functions are particularly relevant in the central nervous system (CNS). Moreover, mutation and dysfunction of SOX factors are implicated in a broad variety of cancers, including glioblastoma.[8]

SOX2 is necessary at early stages of neurodevelopment, it is highly expressed in the areas where NSCs are present during embryogenesis and in the adult stages, and its genetic inactivation leads to NSC differentiation.[9,10] It is also one of the factors necessary for pluripotent and NSC reprogramming.[11–13] In regards to glioblastoma, SOX2 is highly expressed in clinical samples [2, 14–16], and these high levels identify a subset of patients with poor clinical outcome.[17] SOX2 activity is required to sustain stem cell identity with its knockdown, significantly impairing GSCs self-renewal and ability to form tumors *in vivo*.[18,19] SOX2 is also one of the master transcription factors responsible for the reprograming of differentiated glioblastoma cells into induced GSCs,[20] together establishing a major functional relevance of SOX2 in the maintenance of GSCs and glioblastoma progression. However, its function in response to therapy remains poorly understood.

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Supplemental data for this article can be accessed here.

Article highlights

- SOX2 has the potential to become a useful biomarker in the clinic for patient outcome and tumor recurrence.
- The expression of SOX2 might be used for patient stratification and for new therapeutic opportunities targeting it directly or through signals upstream or downstream.
- We postulate the combination of rapamycin with temozolomide in glioblastoma as a therapy strategy, particularly in the subset of patients whose biopsies express elevated SOX2 and SOX9.

This box summarizes key points contained in the article.

SOX9 belongs to the related SOXE subgroup, whose expression is also associated with NSCs.[21] It is essential for directing cells to late NSC stages when gliogenesis is prominent.[22] The activity of SOX9 has also been associated to brain primary tumors. Thus, SOX9 levels are more elevated in glioma than in healthy brain tissue and increasing expression correlates with higher WHO grade gliomas.[23] In glioblastoma, strong SOX9 staining is associated with lower Karnofsky score, lower disease-free and overall patient survival rates.[24,25] Functionally, ectopic expression of SOX9 cooperates to transform NSCs and form tumors.[26]

Different studies have shown an association between SOX2 and SOX9 expression within the developing CNS neurogenic areas in the retina, spinal cord, and dorsal telencephalon.[21,27] Similar effects have been shown in adult stem cells in the subventricular zone and cerebellum.[21,28,29] However, it is unknown whether these two SOX factors cooperate in GSC's self-renewal and/or in glioblastoma chemoresistance.

2. Patients and methods

2.1. Patients and tumor samples

The Basque Biobank for Research O+EHUN provided the human glioblastoma samples. The study included biopsies from 27 patients seen at Donostia University Hospital (San Sebastian, Spain) and diagnosed as primary glioblastoma grade IV according to the WHO criteria. The control group consisted of three healthy donors from the Basque Research Biobank for Research O+EHUN and mRNA was obtained from a mix of six adult brains (Ambion). All study participants signed the informed consent form approved by the Institutional Ethical Committee.

2.2. Cell lines and cultures

Glioma cell lines U251MG (U251), U87MG (U87), A172, and U373 were purchased from the ATCC (American Type Culture Collection). The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin for traditional monolayer cultures or DMEM/F-12 supplemented with N2, B27 supplements (Fisher), and growth factors [20 ng/ml basic fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF)] (Sigma, St. Louis, MO, USA) for spheres cultures. Cells were maintained at standard conditions of 37°C, 5% CO_2 in humidified atmosphere. Glioblastoma primary tumors were dissociated and cells grown in sphere medium for 10 days. Then, spheres were mechanically and enzymatically disaggregated with accutase (Gibco), seeded for secondary spheres, and injected in mice at early passage. Moreover, they were maintained in culture for at least nine passages. Differentiation assays were performed by removing bFGF and EGF and by adding 1% FBS to the DMEM-F12 complete medium.

For spheres assays, U87 and U251 were grown in GSCs medium for 10 days. Then, these spheres were disaggregated with accutase and seeded for secondary and maintained in culture for another 10 days (2^{ry} GSCs). For quantification studies, 500 cells/well were seeded in nontreated 12-well flat-bottom plates and fresh media was added every three days to the plate. After 10 days, spheres were counted. For 2^{ry} GSC assay, the same procedure was repeated.

Lentiviral infections were performed as previously described.[30] For *SOX2* or *mTOR* knockdown, cells were infected with *pLKO.1 shSOX2* (a gift from Matthew Meyerson, Addgene plasmid 26353), *shmTOR1* (a gift from David Sabatini, Addgene plasmid 1855), or empty vector. Infected cells were selected in the presence of 2 µg/ml puromycin and then maintained with 0.2 µg/ml puromycin (Sigma). For *SOX9* knockdown, cells were transfected with a *SOX9* shRNA (Origene, *sh1* or *sh75*) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) and selected in the presence of puromycin for 3 weeks. A nonspecific shRNA (*pRS*) was used as a control. For stable overexpression of *SOX2*, lentiviral transductions were performed with a *pLM-mCitrine-SOX2* construct (a gift from Ander Izeta, Biodonostia Institute) with *pWPXL-GFP* as control. Cells were infected at a muiltiplicity of infection of 10 for 6 h. SOX9 overexpression was achieved by transfection using Fugene with *pCAGGS-SOX9*. TMZ and rapamycin (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and cyclopamine in ethanol.

2.3. Flow cytometry

For cell cycle assay, cells were fixed with ethanol and incubated with RNaseA and propidium iodide. Data were acquired in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and processed using FACSDiva software.

2.4. Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase staining was performed using a commercial staining kit (Cell Signaling, Danvers, MA, USA), according to the manufacturer's guidelines.

2.5. RNA analysis

Total RNA was extracted with Trizol (Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed using random priming and Superscript Reverse Transcriptase (Life Technologies), according to the manufacturer's guidelines. Quantitative real-time PCR was performed using Absolute SYBR Green mix (Thermo Scientific, Waltham, MA, USA) in an ABI PRISM 7300 thermocycler (Applied Biosystems, Foster City, CA, USA). Variations in input RNA were corrected by subtracting the number of PCR cycles obtained for *GADPH*.

2.6. Western blot analysis

Immunoblots were performed following standard procedures. For SOX2 detection, AB5603 antibody (Millipore, Billerica, MA, USA) was used, for SOX9 AB5535 antibody (Millipore), and for β-actin AC-15 (Sigma). HRP-linked (Horseradish Peroxidase) anti-rabbit or anti-mouse (SantaCruz Biotechnology, Dallas, TX, USA) secondary antibodies, both at a 1:2000 dilution, were used. Detection was performed by chemiluminescence using NOVEX ECL Chemi Substrate (ThermoFisher, Waltham, MA, USA).

2.7. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline (PBS) supplemented with 0.3% Triton X-100 and 1% FBS for 5 min at 4°C. Subsequent to blocking for 1 h with PBS and 1% FCS, cells were incubated with p-Histone3 (P-H3) (Abcam, Cambridge, UK) or SOX9 (Millipore) antibodies for 2 h. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (Sigma).

2.8. Immunohistochemistry

Tumors generated in mice were dissected, fixed in 10% formalin for 48 h and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin and eosin using the Varistain Gemini ES machine (ThermoFisher). For immunohistochemistry, sections were rehydrated and heated in citrate buffer for 10 min for antigen retrieval. Endogenous peroxidase was blocked with 5% hydrogen peroxide in methanol for 15 min. Anti-SOX2 (Abcam), SOX9 (Millipore), and Ki67 (Abcam) primary antibodies were used.

2.9. Cell viability MTT assay

Cells were seeded in 96-well plates at a density of 2.5×10^3 cells per well and treated 24 h later with the indicated concentrations of TMZ, rapamycin, and cyclopamine (Sigma) for 72 h in sextuplicates. Then, cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 0.25 mg/ml (Sigma) for 3 h. Formazan produced by viable cells was dissolved in 150 µl of DMSO and absorbance determined at 570 nm in a microplate reader (Multiskan Ascent Thermo Electron Corporation, Waltham, MA, USA).

2.10. In vivo *carcinogenesis assays*

For subcutaneous injection, glioma cells were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA) and resuspended in PBS. 1×10^6 cells were injected subcutaneously into both flanks of $Foxn1^{nu}/Foxn1^{nu}$ nude mice (8 weeks old). Mice were observed on a daily basis and external calipers were used to measure tumor size at the indicated time points from which tumor volume was estimated. For therapy experiment, U251 were cultured for 48 h with TMZ 0.1 mM, rapamycin 1 nM, the combination of both drugs and vehicle (control), previous bilateral implantation in nude mice. One week later, mice were injected intraperitoneally with TMZ (10 mg/kg), rapamycin (5 mg/kg), and combination (10 and 5 mg/kg, respectively) twice per week for 12 weeks. Tumors were considered positive when palpable and the diameter was bigger than 3 mm.

For xenotransplantation, GSCs were injected stereotactically into the frontal cortex of 6–8-week-old non-obese diabetic severe combined immunodeficiency (NOD-SCID) immunodeficient mice. Briefly, GSCs were disaggregated with accutase and resuspended in PBS. 1×10^5 cells were injected into the putamen using a stereotaxic apparatus.

2.11. Data evaluation

Data are presented as mean values ±SEM with the number of experiments (*n*) in parenthesis. Unless otherwise indicated, statistical significance (*p*-values) was calculated using the Student's *t*-test. *, **, and *** indicate statistical significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3. Results

3.1. SOX2 and SOX9 overexpression correlates in glioblastoma samples and GSCs

We analyzed the expression of SOX2 and SOX9 in a cohort of human glioblastoma samples and compared them with healthy brain tissue. The expression of *SOX2* and *SOX9* was significantly upregulated in glioblastoma. Indeed, 70% of the tumor biopsies showed overexpression (fold change higher than 1.5) of *SOX2* (19 biopsies of 27), while 65% of them presented *SOX9* upregulation (18 of 27). Moreover, *SOX2* was increased by an average of more than threefold, while *SOX9* was upregulated by sixfold in tumors compared to brain tissue (Figure 1A and B, and Supplementary Figure S1). Interestingly, the correlation analysis showed a significant association between *SOX2* and *SOX9* expression (Figure 1B). In fact, 85% of the biopsies with *SOX2* overexpression also presented increased levels of *SOX9* (16 out of 19), whilst 75% of cases with moderate or low *SOX2* (6 out of 8) presented low *SOX9* as well (Figure 1B). Similar results were observed in the publically accessible data from The Cancer Genome Atlas (Supplementary Figure S1). Together, these results demonstrate that high levels of *SOX2* and *SOX9* are associated in glioma biopsies.

Next, we determined the association between SOX2 and SOX9 in freshly derived GSCs cultures from human patients. For this, cells dissociated from glioblastoma biopsies were plated in serum-free medium in the presence of EGF and bFGF growth factors. Two independent cultures (GB1 and GB2) gave rise to long-term expanding cultures. These cultures were able to grow as tumorspheres, displayed multipotency, and generated tumors when injected orthotopically in the brain of immunodeficient mice (Figure 1C and Supplementary Figure S2). Importantly, both SOX2 and SOX9, in addition to *CD133* and *OCT4*, were highly expressed in these nondifferentiating conditions, and their levels were higher in GB1 cells, which generated tumors earlier (Figure 1D, and E, and Supplementary Figure S2). When we checked their expression in differentiation conditions, in the absence of growth factors and addition of 1% serum, both SOX2 and SOX9 decreased significantly (Figure 1D and E). These results extend the correlation of SOX2 and SOX9 to GSCs.

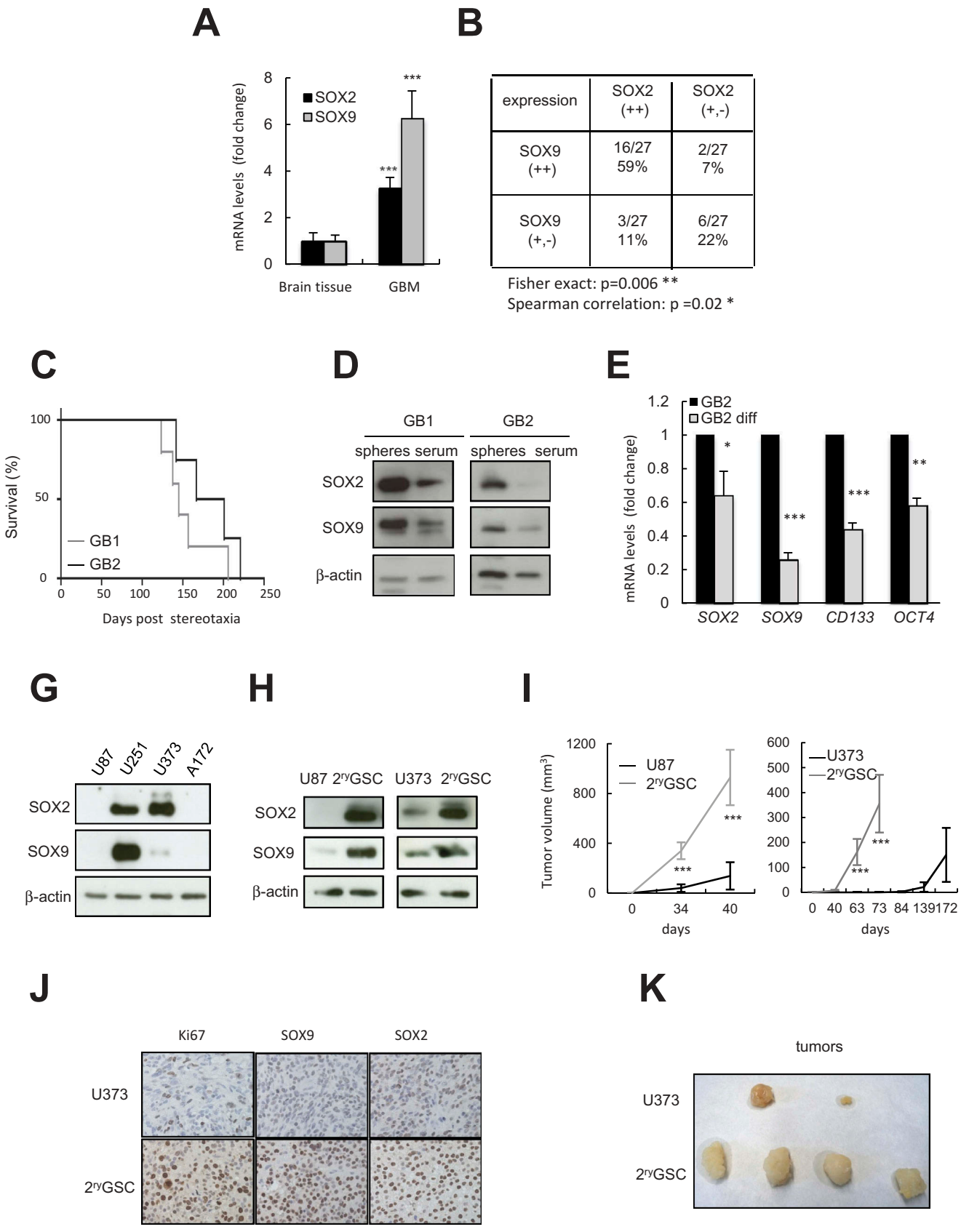

Figure 1. SOX2 and SOX9 are co-expressed in human glioblastoma samples, GSC and glioma cell lines.
(A) *SOX2* and *SOX9* mRNA levels were assayed in a set of healthy brain tissue as control ($n = 9$) and GBM ($n = 27$) samples. q-PCR data are normalized to *GAPDH* expression and expression in tumors is relative to healthy brain tissue **(B)** Analysis of the correlation of SOX2 and SOX9 expression in human glioblastoma samples (Fisher exact Test = 0.006; Spearman correlation = 0.02). **(C)** Kaplan-Meier curve representing the survival of NOD-SCID mice that were xenotransplantated with GB cell lines (n = 5) **(D)** Representative image of higher levels of SOX2 and SOX9 in GB1 and GB2 cells grown in stem cell medium compared to differentiation conditions (n = 3). **(E)** mRNA expression of the indicated GSC markers were analyzed in GB1 and GB2 cells (n = 3). **(F)** Representative immunoblots of SOX2 and SOX9 expression in different glioma cell lines (n = 5). **(G)** SOX2 and SOX9 expression levels in U87 and U373 grown in serum (parental cells) or in stem cells medium (2^{ry} GSC) (n = 5). **(H)** U87 and U373 parental cells and those grown as tumorspheres were injected subcutaneously in nude mice (n = 8 for condition) and growth of the tumors was scored at the indicated time points. **(I)** Representative images of Ki67, SOX2 and SOX9 immunohistochemical staining in U373 derived tumors (n = 4). **(J)** Comparative of the size of the tumors generated by U373 parental and 2^{ry} GSCs. Statistical significance was obtained with Student's T test ($P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$***).

Then, we studied their expression in a set of glioma cell lines. Western blotting and quantitative PCR revealed that the expression of SOX2 was very high in U251 and U373, while U87 and A172 expressed low levels (Figure 1G). Interestingly, the levels of SOX9 correlated with SOX2 (Figure 1G). Moreover, their levels in U251 cells are within the range of expression observed in GSCs and tumor biopsies (Supplementary FiguresS1 and S2), suggesting that these high levels are of biological relevance. Cells with stem cells characteristics have been isolated in several glioma cells lines.[31] Therefore, we cultured U87 and U373 cell lines under NSC growth conditions. These cells grew as tumorspheres and produced tumors faster and larger than parental cells when injected in immunodeficient mice (Figure 1I and K, and Supplementary Figure S3). In this context, the levels of SOX2 and SOX9 were strikingly elevated in the tumors and in the cultures of tumorspheres compared to U87 and U373 parental cells (Figure 1H and J, and Supplementary Figure S3). This evidence further demonstrates the correlation between their expression and together reveal that the SOX2-SOX9 axis might define an oncogenic signaling that predicts the presence of malignant GSCs.

3.2. SOX2-regulated proliferation, senescence, and self-renewal is mediated by SOX9

To directly address the impact of SOX2 on the regulation of glioma cells and SOX9 expression, we knocked down *SOX2* by using RNA interference in U251, cell line with the highest levels of SOX2 and SOX9. Western immunoblotting confirmed effective inhibition of SOX2 and revealed a marked reduction of SOX9 protein levels in *shSOX2* cells (Figure 2A and Supplementary Figure S4), suggesting that SOX9 might act downstream of SOX2 in glioma cells. To further characterize the regulation of SOX9 by SOX2, we measured *SOX9* mRNA levels in cells with *SOX2* knockdown, not detecting significant differences in relation to control cells (data not shown). Thus, the effect of SOX2 seems to be at translational instead of transcriptional level.

To extend this finding, SOX9 was ectopically re-expressed in *shSOX2* cells. Western blot assay showed that SOX9 restoration in U251 cells re-established the expression of SOX9 and increased the levels of SOX2 (Figure 2B), indicating that the efficiency of silencing was not complete (Supplementary Figure S4). Moreover, we identified that ectopic SOX9 also increased the expression of SOX2 in control cells (Figure 2B), together suggesting that a feedback loop might exist between SOX2 and SOX9.

To determine whether *SOX9* is necessary for *SOX2* oncogenic activity, we next investigated the phenotypes associated to SOX2 silencing, and whether *SOX9* reactivation restored them, *SOX2* knockdown led to a significant decrease of more than twofold in cell growth and number of p-Histone3 (P-H3)-positive cells (Figure 2C and Supplementary Figure S4). Moreover, flow cytometry analysis showed increased number of cells in G0/G1 and decreased in S phase of *shSOX2* compared with control cells (Figure 2D and Supplementary Figure S4). This impairment in *shSOX2* cell proliferation was accompanied by a significant increase in senescence measured by cytoplasmic β-galactosidase activity and *IL1α*, interleukin associated to senescence-associated secretory phenotype [32] both elevated by more than 2.5-fold in cells with *SOX2* silencing (Figure 2E, F and Supplementary Figure S4). Thus, impaired proliferation and increased senescence account for the reduction in cellular growth of *SOX2*-silenced cells. Moreover, SOX2 knockdown diminished sphere-formation and self-renewal activities (Figure 2G and H). Similar results were obtained in limiting dilution analysis (Supplementary Figure S4), further providing evidence for a decrease in self-renewal activity in the absence of SOX2.[19] When SOX9 was ectopically re-expressed in *shSOX2* cells, cell proliferation was significantly increased (Figure 2I), senescence-associated β-galactosidase activity significantly decreased (Figure 2J and Supplementary Figure S5), and the ability to form colonies at low density and spheres increased in SOX9 restored cells (Figure 2K and Supplementary Figure S5). However, SOX9 reactivation did not restore completely the numbers observed in control cells (data not shown), indicating that the oncogenic activity of SOX2 is, at least in part, mediated by SOX9.

In order to further characterize the significance of this axis in glioma cells, we knocked down *SOX9* activity in U251 cells. *shSOX9* (*sh1*) transduced cells presented significantly lower number of P-H3-positive cells (Figure·3A and, B, and Supplementary Figure S6) and generated lower number of foci in soft-agar and formed tumors later than control cells (Figure 3C and Supplementary Figure S6). Together, our results demonstrate that genetic silencing of SOX2 and SOX9 suppresses proliferation and tumorigenicity of glioma cells and indicate that their inhibition might be a novel therapeutic strategy for glioblastoma.

3.3. Overexpression of SOX2 and SOX9 promotes proliferation and stem cell activity

Next, we introduced ectopic *SOX2* in U87 cells with the lowest levels of endogenous SOX2 and SOX9. We confirmed the overexpression of SOX2, and interestingly, SOX9 levels were also elevated (Figure 3D and Supplementary Figure S7). Together with the above data, these results strongly indicate that SOX2 modulates the activity of SOX9 expression. We also measured *SOX9* mRNA levels in cells with *SOX2* overexpression without detecting significant differences compared to control cells (data not shown). Phenotypically, cells with increased *SOX2* expression exhibited higher cell growth curves and rates of proliferation compared to control cells (Figure 3E and F). Moreover, we assessed the effect of SOX2 on self-renewal and found that *SOX2* overexpression led to an increase in the generation of tumorspheres. While control cells formed an average of 5 spheres, *SOX2* overexpressing cells generated an average of over 20 spheres (Figure 3G). Similarly, transient overexpression of SOX9 was sufficient to increase the number of U87-derived spheres (Figure 3H and I) and induced the formation of larger tumors (Figure 3J). Collectively, our data revealed that SOX2 and SOX9, acting in the same axis, are not only necessary for the maintenance but their elevated activity also facilitates self-renewal activity and tumor growth in glioma cells.

3.4. SOX2 expression modulates TMZ sensitivity

The evidence of GSCs as responsible for resistance to therapeutic treatments,[33] together with our data of SOX2/SOX9 expression associated to malignant GSCs, prompted us to hypothesize that their high levels could be involved in cellular resistance to TMZ.

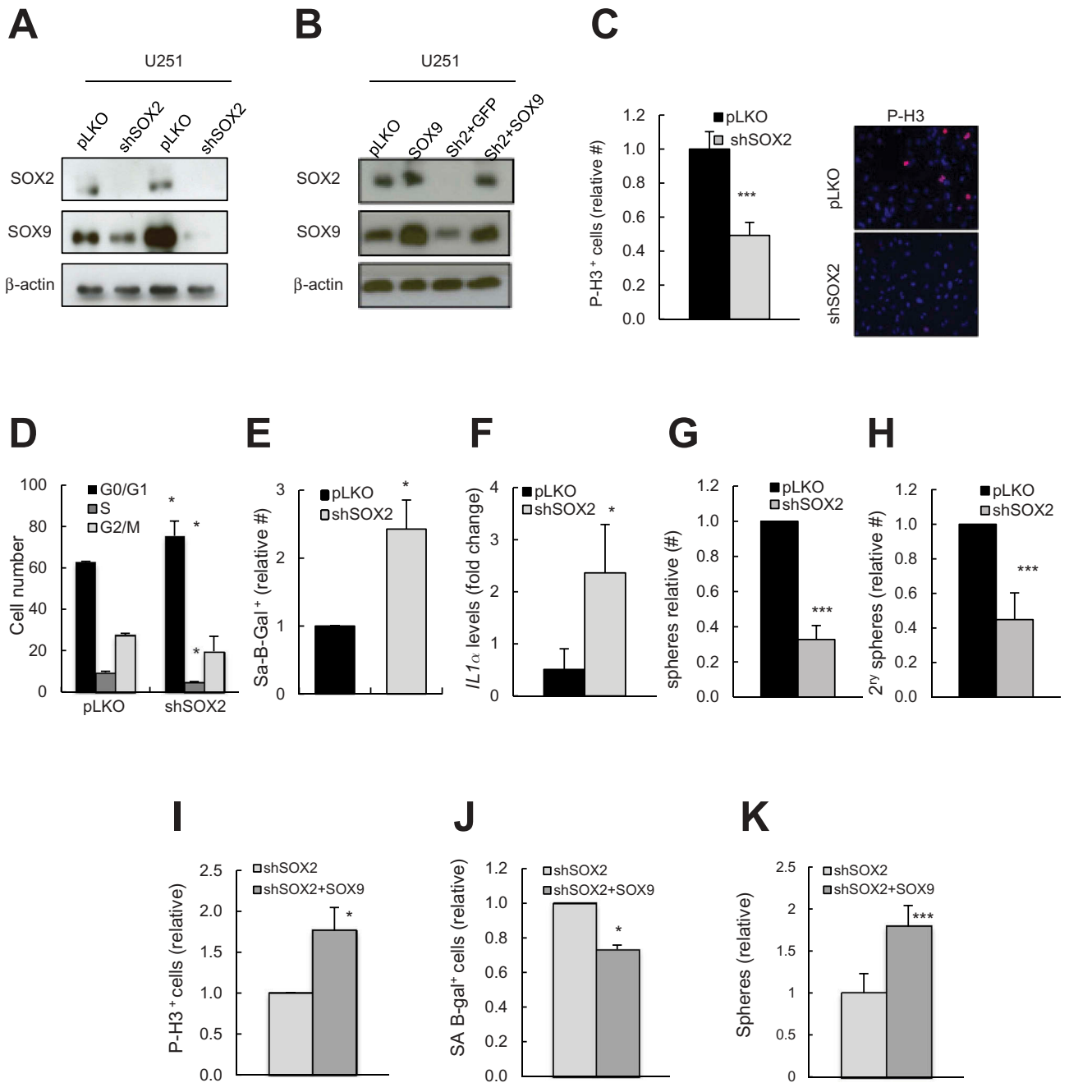

Figure 2. Downregulation of SOX2 leads to decreased proliferation and self-renewal in U251 cells via SOX9.
U251 cells were infected with a *shSOX2* or *shSOX9* and cells examined for protein expression and functional assays (at least n = 4). **(A)** Representative Immunoblots of SOX2 and SOX9 derived from two different and independent lentiviral infections with a shSOX2 construct (n = 4). **(B)** Representative western blot of SOX2 and SOX9 in U251 cells transduced with the indicated conditions (n = 3). **(C)** *shSOX2* impairs proliferation as shown by the quantification and representative image of P-H3 positive cells (n = 4). **(D)** Cell number in each cell cycle phase in empty vector and *shSOX2* condition (n = 2). **(E)**Quantification of senescence associated β-galactosidase positive cells in *shSOX2* and control cells (n = 4). **(F)** Expression of *IL1a* mRNA levels in *shSOX2* cells. qRT-PCR data are normalized to GAPDH expression and are expressed relative to the pLKO control condition (n = 3). **(G)** Quantification of spheres (1^{ry}) forming capacity in *shSOX2* cells after 10 days in culture. The numbers are relative to empty vector transduced cells (n = 4). **H**Number of 2^{ry} spheres generated in both control and *shSOX2* conditions after 8 days in culture, and relativized to the control (n = 3). **(I)** Numbers of P-H3 positive cells were quantified in *shSOX2* and *shSOX2+SOX9* transduced U251 cells (n = 3). **(J)** SOX9 restoration decreases senescence associated β-galactosidase activity in U251 cells (n = 4). **(K)** Quantification of tumorspheres forming capacity in *shSOX2+SOX9* cells after 7 days in culture. The numbers are relative to U251 *shSOX2* cells (n = 4).

To test this idea, we first analyzed SOX2 and SOX9 expression in U251 and U87 cells cultured with increasing concentrations of TMZ for 24 h. We found that both SOXs were elevated in response to 100 and 200 μM of TMZ, more markedly with the highest concentration (Figure 4A), suggesting that this axis may be involved in the underlying resistance to current chemotherapy. To further determine this hypothesis, cell lines with high and low SOX2/SOX9 were exposed to different concentrations of TMZ for 72 h and cell chemosensitivity was measured by MTT assay. U251 and U373 cells, with high levels of both SOX factors, were more resistant (% of toxicity lower than 15% in both lines) than A172 and U87 cells (% of toxicity between 30 and 50%) (Figure 4B) Together, these findings confirm that high levels of SOX2 and SOX9 correlate with TMZ resistance.

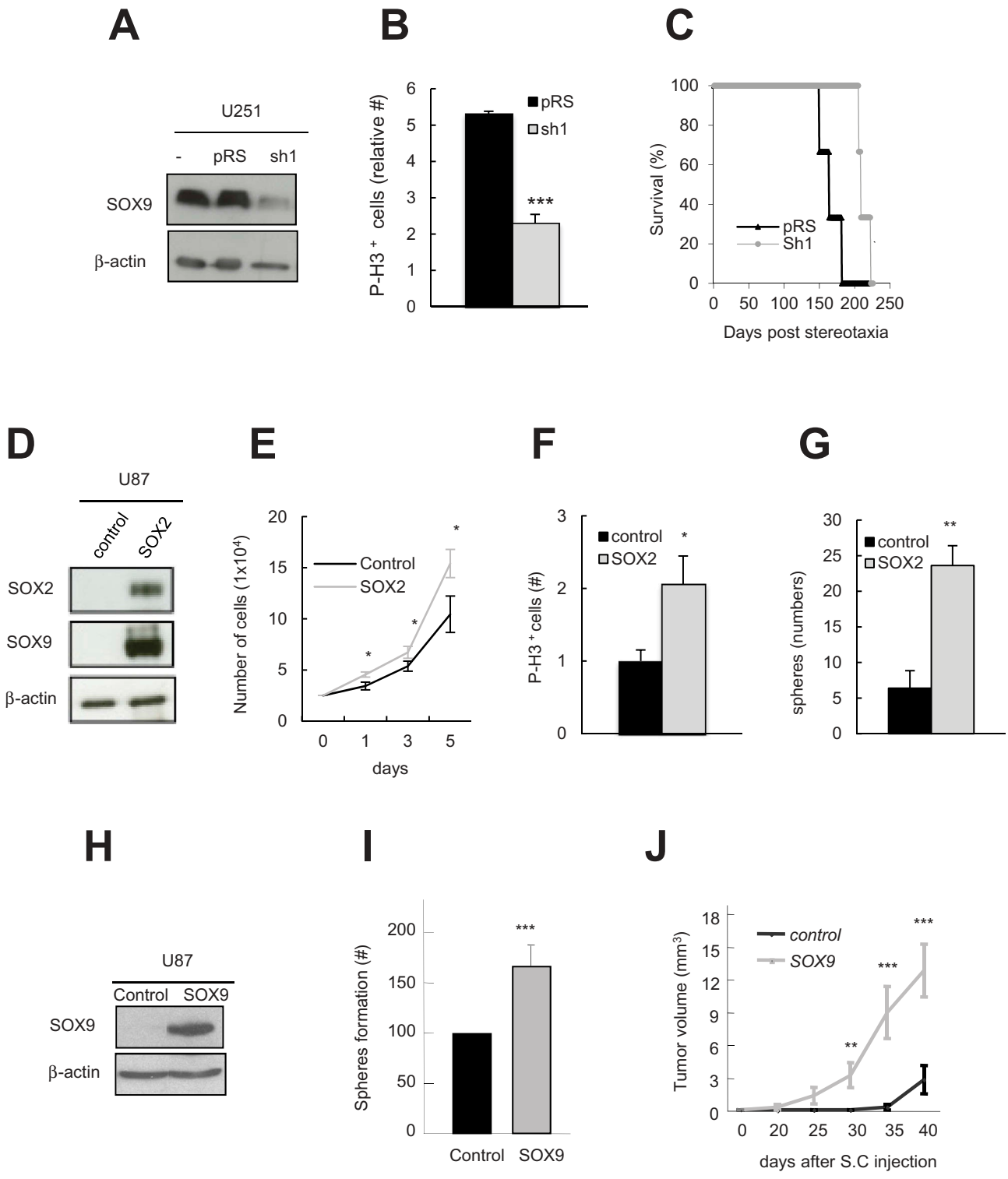

Figure 3. Effect of SOX2 and SOX9 gain of function in glioma cells.
(A) Representative image of SOX9 levels in pRS or *shSOX9* (sh1) cells **(B)** Quantification of P-H3 positive cells in pRS or sh1 cells. **(C)** Kaplan-Meier curve representing the survival of NOD-SCID mice that were xenotransplantated with pRS control or sh1 cells (n = 4). **(D)** Representative western blot of SOX2 and SOX9 in U87 cells lentivirally transduced with *pLM-mCitrine-SOX2* or control construct (n = 3). **(E)** Cell growth assay comparing control and SOX2 overexpressing U87 cells. (n = 5). **(F)** Number of P-H3 positive cells detected in the indicated U87 cells. (n = 5). **(G)** Quantification of tumorsphere formation capacity of cells with ectopic SOX2 compared to control cells (n = 4). **(H)** Representative western blot of SOX9 levels in U87 cells tranfected with *pCAGGS SOX9* or empty vector (control). **(I)** Quantification of spheres generated in SOX9 and control U87 cells (n = 4). **(J)** Control and *SOX9* U87 cells were injected subcutaneously (s.c) in nude mice (n = 6) and growth of the tumors was scored at the indicated time points.

Next, we characterized the role of SOX2 in response to TMZ performing additional MTTs assays. *SOX2* overexpression significantly increased the resistance of U87 cells, as observed by the enhancement of cell growth to increasing concentrations of TMZ (Figure 4C), whilst SOX2 knockdown increased the chemosensitivity of U251 glioma cells to TMZ (Figure 4D). To

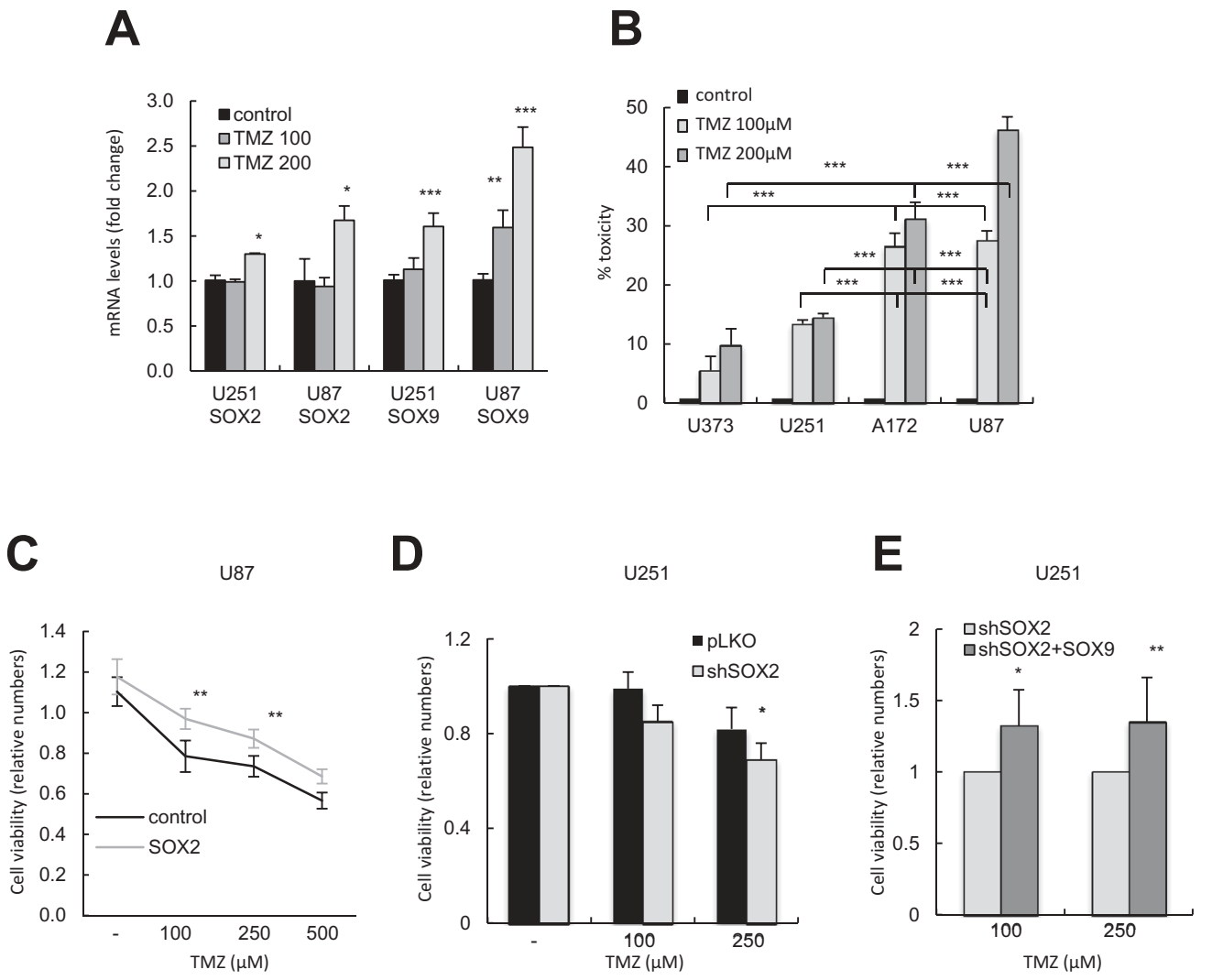

Figure 4. Effect of TMZ treatment in glioma cells with different activity of SOX2 and SOX9.
(A) SOX2 and SOX9 expression levels in U87 and U251 cells cultured with increasing doses (100 and 200uM) of TMZ (n = 3). Data are relative to DMSO treated condition. **(B)** MTT assay of different glioma cell lines in the presence of increasing doses of TMZ for 72 h (n = 6). Values are relative to control cells treated with DMSO. **(C)** *pLM-mCitrine-SOX2* U87 infected cells were cultured with the indicated doses of TMZ for 72 h (n = 3). Cell viability was expressed as the percentage of MTT reduction, assigning the 100% value to the absorbance of the control cells. **(D)** *shSOX2* transduced U251 cells were treated with the indicated doses of TMZ and cell viability measured 72 h later (n = 5). **(E)** Cell viability in *shSOX2* and *shSOX2+SOX9* U251 cells (n = 3). Statistical significance was obtained with Student's T test (P ≤ 0.05*; P ≤ 0.01**; P ≤ 0.001***).

identify whether SOX9 regulated SOX2 response to TMZ, we repeated the MTT experiment with U251 *shSOX2* cells with or without SOX9 restoration. Interestingly, *shSOX2* with SOX9 exhibited a growth advantage in the presence of different doses of TMZ compared to *shSOX2* (Figure 4E). The above-mentioned data indicate that SOX2 activity modulates the sensitivity of glioma cells to TMZ by regulating SOX9 expression and suggest that pharmacological inhibition of SOX2 might be a novel strategy to overcome TMZ resistance in a subset of glioblastoma with high levels of SOX2-SOX9.

3.5. Rapamycin treatment decreases SOX2 expression and TMZ resistance

In an effort to identify agents that could silence the expression of SOX2 in glioma cells, we tested the effect of rapamycin, an inhibitor of the mTOR complex 1, which is known to affect viability and proliferation of glioma cells, and has been shown to inhibit the expression of SOX2 for cell reprogramming. [34,35] First, we cultured several cell lines with 10 nM of rapamycin, noting that the expression of SOX2 was markedly reduced at protein and mRNA levels mainly in U251 and U373, cells with endogenous high levels of SOX2 (Fig 5A;B), extending the action of this agent on SOX2 protein from healthy to cancer cells. Similar effect was detected on SOX9 expression. The inhibitory effect of rapamycin was concentration-dependent (from 1 to 100 nM) and time-dependent (24–48 h) (Figure 5A and B, and Supplementary Figure S7). The reduction in *SOX9* levels was more intense (between 60% and 80%) than in *SOX2* (30–60%), suggesting that rapamycin-induced SOX9 inhibition is not exclusively directed through SOX2. The above concentration–response curves further reveal that rapamycin exerted a negative effect on SOX expression even at very low concentration (1nM). We, therefore, evaluated

whether the effect on SOX2 and SOX9 expression was directly mediated by mTOR signaling inhibition, and knocked down *mTOR* expression in U251 cells. Seventy-two hours after antibiotic selection, we observed a severe decrease in *mTOR* mRNA levels and a striking decline in phosphorylation of AKT and S6, well-established mTOR effectors (Figure 5C and, D), demonstrating the efficient silencing of *mTOR* machinery in our U251 glioma model (Figure 5D). In this context, SOX2 and SOX9 protein levels were also reduced, identifying that SOX2 and SOX9 are downstream targets of mTOR pathway as shown by genetic and pharmacological studies.

To confirm the role of mTOR signaling in glioma cell activity, we further characterized the effect of mTOR silencing in functional studies. Interestingly, cell growth and the number of spheres were dramatically diminished (Figure 5E and F), further confirming the impact of mTOR in self-renewal and GSC maintenance.[36] Moreover, these studies revealed that genetic inhibition of mTOR and SOX leave as mTOR and SOX2 displays the same cellular phenotype, further extending the association between them. In summary, our results show that SOX2/SOX9 expression can be silenced with the pharmacological inhibition of mTOR machinery. Similar results were obtained with cyclopamine, inhibitor of the Sonic Hedgehog (SHH) molecular pathway (Supplementary Figure S8), together demonstrating that pharmacological silencing of SOX2 and SOX9 activity is plausible with current agents.

Combined therapeutic approaches acting synergistically have been proven more effective than individual treatments. We, therefore, tested whether rapamycin (or cyclopamine) could represent a potential enhancer of the cytotoxic effects of TMZ and sensitize cells with elevated levels of SOX2. Accordingly, we performed MTT assays in which U87 and U251 cells were treated with a constant dose of 100 μM TMZ together with 1 and 10 nM of rapamycin or 5 and 10 μM of cyclopamine (concentrations that significantly inhibited *SOX2* expression). First, we detected that the cytotoxic effect of 5 and 10 μM of cyclopamine in U251 cells was higher (18% and 24%) than in U87 (14% and 19%), although we did not observe an additive effect of the combination of TMZ and cyclopamine treatment (Supplementary Figure S8). On the other hand, combined treatment of rapamycin and TMZ achieved a stronger cytotoxic effect than with single agents alone (Figure 5G). Moreover, the concomitant treatment of rapamycin and TMZ exerted a greater anti tumorogenic effect in SOX2-SOX9 high-expressing than in low-expressing cells (Figure 5G). Indeed, the percentage of toxicity in U251 cells was 55% and 57% in TMZ plus rapamycin 1 and 10 nM, respectively, compared to 43% and 46% in U87 cells. Of note, the synergistic action of rapamycin and TMZ was achieved even at the low concentration of 1 nM and was of similar degree than 10 nM. To determine whether this effect was mediated by SOX2 and SOX9, we measured their expression in cells cultured with TMZ (100 μM), rapamycin (1 nM), or their combination for 48 h. Remarkably, SOX2 and SOX9 levels were much lower in rapamycin or in combination than in nontreated or TMZ alone cells (Figure 5H). These results indicate a sensitization of TMZ-resistant cells by rapamycin likely through SOX2 and SOX9 downregulation.

To corroborate the synergistic effect of TMZ and rapamycin on cells with elevated SOX2 and SOX9 expression, we studied their efficacy in tumor formation *in vivo*. Thus, we injected U251 cells in athymic immunodeficient mice subcutaneously since 1 week later the mice received intraperitoneally TMZ (10 mg/kg), rapamycin (5 mg/kg), and combination (10 and 5 mg/kg, respectively) twice per week. In the case of untreated animals, tumors started to be detected 30–40 days after injection and 100% mice developed them after 2 months. In contrast, treatment with rapamycin or TMZ delayed the formation of the tumors, with around 50% of them presenting tumors 2 months after injection. Remarkably, these numbers were lower in the combined treatment group with only 25% of mice with tumors (Figure 5I). Together, these data demonstrate that combining rapamycin with TMZ enhances the efficacy of each one against glioma cells, particularly in the subset with high levels of SOX2 and SOX9.

4. Discussion

Different studies have shown that expression of SOX2 is often increased in glioblastoma and that this upregulation is due to genetic amplification and epigenetic mechanisms.[2, 14–16] Notably, beyond high expression of SOX2 in glioblastoma multiforme (GBM) biopsies, the genetic inhibition of *SOX2* expression decreases tumor cell proliferation, causes depletion of self-renewal, and subsequently tumor regression.[18,19] In this study, we have identified that SOX2 inhibition induces cellular senescence in differentiated U251 cells. Moreover, the increased levels of IL1α observed in *shSOX2* U251 cells suggest that SOX2 might be involved in paracrine senescence.[32] Gangemi and collaborators did not observe an increase in senescence-associated β-galactosidase activity when SOX2 was silenced in human-derived GSCs,[19] indicating that SOX2 might exert different actions within the cellular heterogeneity of the tumor bulk. These results suggest that inactivation of SOX2 in GSCs induces differentiation whilst in differentiated ones facilitates senescence or apoptosis. Moreover, we show that overexpression of SOX2, in addition to promote other relevant phenotypic properties such as invasiveness and migration,[16] is a necessary condition for maintaining GSCs and therefore essential for GBM propagation. Further supporting this notion, SOX2 belongs to the core set of transcription factors (with POU3F2, SALL2, and OLIG2), which are sufficient to reprogram differentiated cells into GSCs.[20] Altogether, these data confirm that tumor cells harboring high levels of SOX2 protein are addicted to it and have dependence on this factor to survive.

In this work, we have identified that SOX2 and SOX9 expression correlates in glioma cells and that the oncogenic activity of SOX2 is at least partially mediated by the latter. In support of these actions, it has been previously shown that SOX9 plays a key role in the regulation of cellular proliferation, senescence, and self-renewal.[26,37,38] Moreover, we show

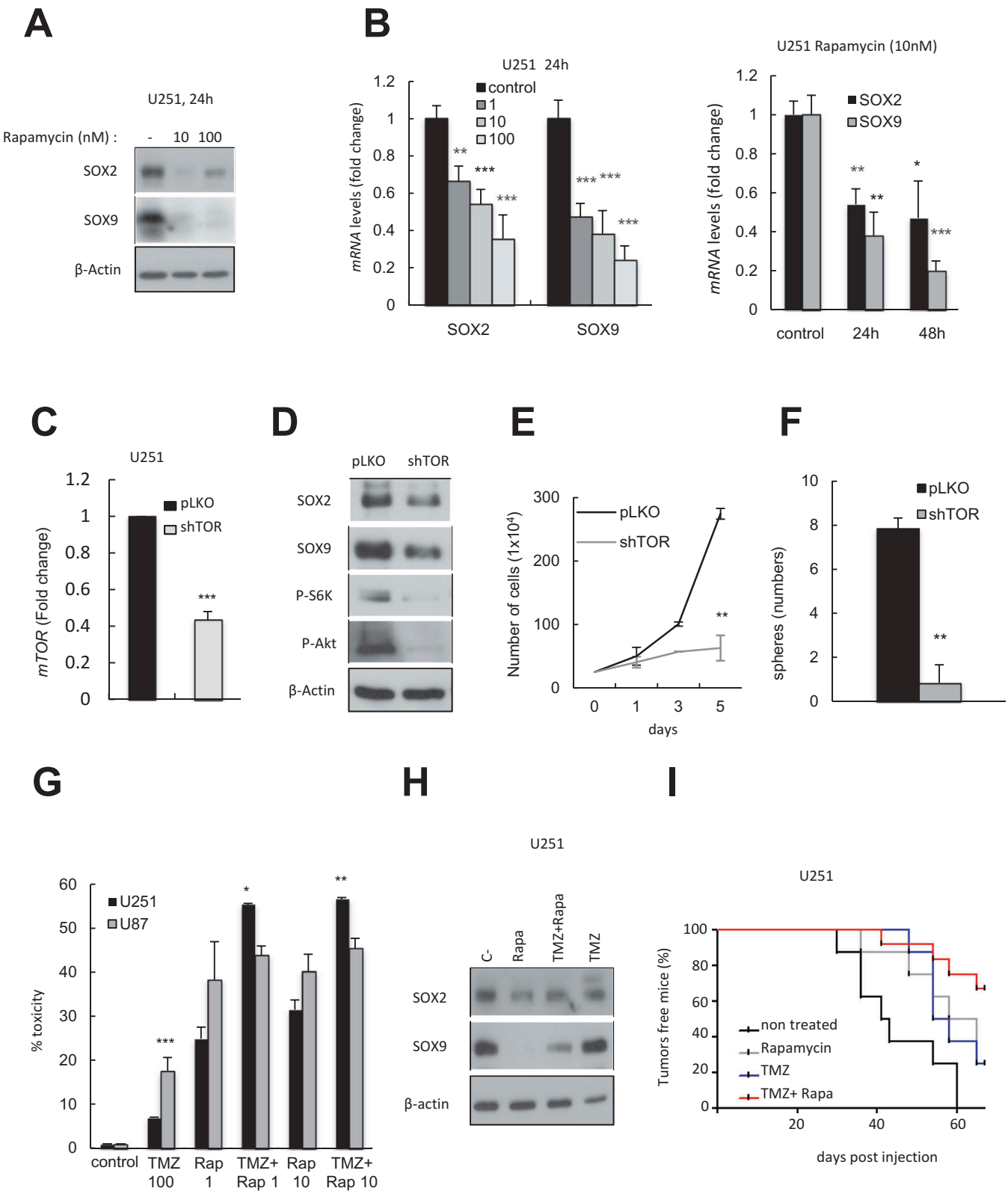

Figure 5. mTOR signaling inhibition reduces SOX2 and SOX9 and cooperates with TMZ.
(A) Representative western blot of the effect of 10 and 100nM doses of rapamycin in SOX2 and SOX9 in U251 cells. **(B)** Dose (1, 10 and 100nM) and time (24,48 h) dependent effect of rapamycin in *SOX2* and *SOX9* mRNA levels in U251 cells. **(C)** *mTOR* mRNA in U251 cells lentivirally transduced with pLKO or *mTOR shRNA (shTOR)* (n = 2). **(D)** Representative image of SOX2, SOX9, P-S6 K and P-Akt in the indicated U251 genotypes. **(E)** Cell growth assay comparing control and *shTOR* (n = 2). **(F)** Sphere formation capacity in *shTOR* and control cells (n = 2) **(G)** MTT assay of U87 and U251 cells cultured with TMZ (100μM), rapamycin (1-10nM) and combination of both for 72 h (n = 3). **(H)** Kaplan meier curve showing generation of tumors from subcutaneously injected U251 cells after 12 weeks of treatment with TMZ (10 mg/Kg) (n = 8), rapamycin (5 mg/Kg) (n = 8) and combination of both (10 mg/Kg and 5 mg/Kg respectively) (n = 12). Non-treated (n = 8) mice were used as control. LogRank Test is 0.0323 for TMZ, 0.040 for rapamycin and 0.0003 for the combination of both compared to non treated.

that this regulation occurs at post-transcriptional levels and that there is a feedback loop between them. A recent study observed that Sox2 regulates Sox9 protein at the level of mRNA translation in oligodendrocytes, identifying miR-145 as a candidate mediator in this process.[39] It is possible to surmise that the same pathway is acting in glioma cells. Indeed, it has been shown that SOX2 inactivation induces the expression of miR-145,[40] while this miRNA regulates and inhibits SOX9 to function as a tumor suppressor.[25] Our results also highlight that SOX transcription factors act sequentially in the regulation of GSCs, mimicking the action of those in neural lineage development,[18,41] and indicate that SOX2 is a master regulator of GSCs, which together with SOX9 might form a relevant molecular node that sustains tumor maintenance and progression.

TMZ is currently the most efficient chemotherapy for GBM. Indeed, its addition extended patient median survival from approximately 12 to 15 months.[42] Damage generated by TMZ can be repaired by O6-Methylguanine-DNA methyltransferase (MGMT), thus inducing treatment resistance, while methylation of the MGMT promoter leads to an increase in TMZ sensitivity. Our results show that cells with high levels of SOX2 are more resistant to TMZ and silencing it sensitizes against this chemotherapeutic agent *in vitro* and *in vivo*. Of note, the cell lines used in our experiments exhibit MGMT promoter hypermethylation status. Given that SOX2 is included in the proneural subset in different glioblastoma classifications [43,44], group that has been demonstrated to be resistant to the conventional therapeutic regimen of radiotherapy and TMZ, SOX2 might be postulated as one of the key terms responsible for resistance to current chemotherapy in glioblastoma. Therefore, targeting the activity of SOX2 may offer a new promising therapeutic treatment modality.

In an effort to identify drugs or molecules that might inhibit efficiently the expression of SOX2 (direct or indirectly), we found that inhibitors of the SHH signaling cascade (cyclopamine) and mTOR (rapamycin) reduced significantly, between 40% and 80%, the activity of SOX2 and SOX9, demonstrating that the pharmacological silencing of SOX2 is feasible using inhibitors of these signaling pathways. It is important to note that SHH and particularly PI3 kinase/mTOR pathways are aberrantly active is aberrantly active in a high percentage of glioblastomas.[14] Our results indicate that their action might be modulated through SOX2 and SOX9. Consistent with the strategy to silencing SOX2 activity in glioma, downregulation of *SOX2* conferred sensitivity to treatment with platelet-derived growth factor and IGF1 receptor inhibitors [44] and vaccination with Sox2 peptides elicited a response that significantly delayed tumor development in mice,[45] underscoring the feasibility of using SOX2 as a target in different therapeutic approaches. Furthermore, it has been shown that elevated expression of SOX2 protein desensitizes tumor cells to current therapies present in the clinic such as hormone therapy in breast cancer [46] and chemotherapy in medulloblastoma.[47]

A growing number of evidence indicates that combining drugs with chemotherapeutic agents is becoming a more effective therapeutic option in cancer. Our results identified that the concomitant treatment of rapamycin and TMZ exerted a higher cytotoxic effect *in vitro* and *in vivo* in cells expressing endogenous high levels of SOX2-SOX9, suggesting that the addition of rapamycin to TMZ treatment could potentially enhance the efficacy of this therapy against human glioblastoma, particularly in the subset of patients whose biopsies express elevated levels of SOX2 and SOX9.

Clinically, we have observed that there is a strong correlation between *SOX2* and *SOX9* expression in patient biopsies. Independent studies demonstrated that elevated levels of SOX2 and SOX9 are associated with a subgroup of patients with lower median survival and also that they are part of a signature of stem cell markers related with worse prognosis in glioblastoma.[17,24] Our results, together with this evidence, demonstrate that the assessment of the activity of SOX2-SOX9 might be a useful prognostic and predictive marker in glioblastoma. Moreover, our results postulate the incorporation of the expression of SOX factors to patient stratification and the concept of personalized medicine, providing a rationale for the combination of rapamycin with TMZ in glioblastoma, particularly in the subset of patients with high levels of SOX2 and SOX9.

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•• of considerable interest

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