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

Original Research

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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 remains 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.

Expert opinion: SOX2 and SOX9 have the potential to become biomarkers for the identification of GSCs and poor patient outcome in the clinic. Their expression might be used for patient stratification. We postulate the combination of rapamycin to conventional therapy in glioblastoma patients whose biopsies express elevated SOX2/SOX9.

Key words: 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 play 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 (HMG) 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 NSCs differentiation [9, 10]. It is also one of the factors necessary for pluripotent and neural stem cell 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.

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 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 GSCs 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 3 healthy donors from the Basque Research Biobank for Research O+EHUN and mRNA was obtained from a mix of 6 adult brains (Ambion). All study participants signed informed consent form approved by the Institutional Ethical Committee.

2.2. Cell lines and cultures

Glioma cell lines U251MG (U251), U87MG (U87), A172 y U373 were purchased from the ATCC. The cell lines were cultured in DMEM (Gibco), supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin for traditional monolayer cultures or in 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) for tumorspheres 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 tumorsphere medium for 10 days. Then, tumorspheres were mechanically and enzymatically disaggregated with accutase (Gibco), seeded for secondary neurospheres and injected in mice at early passage. Moreover, they were maintained in culture for at least 9 passages. Differentiation assays were performed by removing bFGF and EGF and by adding 1%FBS to the DMEM-F12 complete medium.

For neurospheres assays, U87 and U251 were grown in GSCs medium for 10 days. Then, these spheres were disaggregated with accutase, and seeded for secondary neurospheres and

maintained in culture for another 10 days (2ry GSCs). For quantification studies 500cells/well were seeded in non-treated 12-wells flat bottom plates and fresh media was added every 3 days to the plate. After 10 days tumorospheres 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* shRNAs (Origene, *sh1* or *sh75*) using Lipofectamine (Invitrogen) and selected in the presence of puromycin for 3 weeks. A non-specific 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 pWXL-GFP as control. Cells were infected at a MOI of 10 for 6 hour. SOX9 overexpression was achieved by transfection using Fugene with pCAGGS-SOX9. Temozolomide and rapamycine (Sigma) were dissolved in DMSO and cyclopamine in ethanol.

2.3. Flow citometry

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

2.4. Senescence Associated β-galactosidase staining

To measure senescence, senescence-associated β-galactosidase (SA-β-gal) staining was performed using a commercial staining Kit (Cell Signaling), according the manufacturer's guidelines.

2.5. RNA analysis

Total RNA was extracted with Trizol (Life Technologies). 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) in an ABI PRISM 7300 thermocycler (Applied Biosystems). 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) was used, for SOX9 AB5535 antibody (Millipore) and for β-actin AC-15 (Sigma). HRP-linked anti-rabbit or anti-mouse (SantaCruz Biotechnology) secondary antibodies, both at a 1:2000 dilution were used. Detection was performed by chemiluminescence using NOVEX ECL Chemi Substrate (ThermoFisher).

2.7. Immunofluorescence

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

2.8. Immunohistochemistry

Tumors generated in mice were dissected, fixed in 10% formalin for 48h and embedded in paraffin. 4 micrometer-thick sections were stained with hematoxylin and eosin (H&E) using the Varistain Gemini ES machine (ThermoFisher). For immunohistochemistry, sections were rehydrated and heated in citrate buffer for 10 minutes for antigen retrieval. Endogenous peroxidase was blocked with 5% hydrogen peroxide in methanol for 15min. 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 $2.5 \cdot 10^3$ cells per well and treated 24 hours later with the indicated concentrations of temozolomide, rapamycine and cyclopamine (Sigma) for 72 hours 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 hours. 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). Results were presented as the percentage of viable cells relative to controls.

2.10. *In vivo* carcinogenesis assays

For subcutaneous injection, glioma cells were harvested with trypsin/EDTA and resuspended in PBS. $1x10^6$ 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 48h with TMZ 0,1mM, rapamycin 1nM, the combination of both drugs or vehicle (control), previous bilateral implantation in nude mice. One week later, mice were injected intraperitoneally with TMZ (10mg/kg), rapamycin

(5mg/kg) and combination (10mg/kg and 5mg/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 NOD-SCID mice. Briefly, GSCs were disaggregated with accutase and resuspended in PBS. $1x10^5$ cells were injected into the putamen using a stereotaxic apparatus.

2.11. Data evaluation

Data are presented as mean values ± S.E.M. with the number of experiments (n) in parenthesis. Unless otherwise indicated, statistical significance (p-values) was calculated using the Student´s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

3. RESULTS

3.1. SOX2 and SOX9 overexpression correlate 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 up-regulated 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* up-regulation (18 of 27). Moreover, *SOX2* was increased by an average of more than three-fold, while *SOX9* was up-regulated by six-fold in tumors compared to brain tissue (Fig. 1A,B, Fig Suppl. 1). Interestingly, the correlation analysis showed a significant association between *SOX2* and *SOX9* expression (Fig 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 (Fig 1B). Similar results were observed in the publically accessible data from The Cancer Genome Atlas (TCGA) (Fig suppl. 1). 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, 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 (Fig 1C and suppl. 2). 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 (Fig 1D,E and suppl 2). 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 (Fig 1D,E). These results extend the correlation of SOX2 and SOX9 to GSCs.

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 (Fig 1G). Interestingly, the levels of SOX9 correlated with SOX2 (Fig. 1G). Moreover, their levels in U251 cells are within the range of expression observed in GSCs and tumor biopsies (Fig. suppl 1, 2), 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 (Fig 1I,K and suppl 3). 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 (Fig 1H,J and suppl 3). This evidence further demonstrates the correlation between their expression and together reveal that the SOX2-SOX9 axis might define an oncogenic signaling that predict 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 in 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 (Fig. 2A, Fig suppl4), 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* knock-down, 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 (Fig 2B), indicating that the efficiency of silencing was not complete (Fig suppl4). Moreover, we identified that ectopic SOX9 also increased the expression of SOX2 in control cells (Fig. 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* re-activation restored them , *SOX2* knockdown led to a significant decrease of more than 2 fold in cell growth and number of p-Histone3 (P-H3) positive cells (Fig. 2C, suppl 4). Moreover, flow cytometry analysis showed increased number in G0/G1 and decreased in S phase of *shSOX2* compared with control cells (Fig. 2D and suppl 4). 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 (Fig 2E,F, and suppl 4). 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 (Fig. 2G,H). Similar results were obtained in limiting dilution analysis (Fig. suppl 4), 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 (Fig. 2I), senescence associated β-galactosidase activity significantly decreased (Fig. 2J and suppl 5) and the ability to form colonies at low density and spheres increased in SOX9 restored cells (Fig. 2K and suppl 5). 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-Histone3 positive cells (Fig·3A,B, and suppl 6) and generated lower number of foci in soft-agar and formed tumors later than control cells (Fig3C and suppl 6). 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 (Fig. 3D, Fig Suppl7). 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). Phenotipically, cells with increased *SOX2* expression exhibited higher cell growth curves and rates of proliferation compared to control cells, (Fig. 3E,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 and average of over 20 spheres (Fig. 3G). Similarly, transient overexpression of SOX9 was sufficient to increase the number of U87 derived spheres (Fig. 3H,I) and induced the formation of larger tumors (Fig. 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 hypothesized that their high levels could be involved in cellular resistance to TMZ. To test this idea, we first analysed SOX2 and SOX9 expression in U251 and U87 cells cultured with increasing concentrations of TMZ for 24 hours. We found that both SOXs were elevated in response to 100 and 200μM of TMZ, more markedly with the highest concentration (Fig. 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 hours 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%) (Fig. 4B) Together, these findings confirm that high levels of SOX2 and SOX9 correlate with temozolomide resistance.

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 (Fig. 4C), whilst SOX2 knockdown increased the chemosensitivity of U251 glioma cells to TMZ (Fig. 4D). To 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* (Fig. 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 10nM of rapamycin noting that the expression of SOX2 was markedly reduced at protein and mRNA levels specifically in U251 and U373 cells with endogenous high levels of SOX2 (Fig. 5A,B, Fig suppl 7). Similar effect was detected in SOX9 expression, extending the action of this agent on SOX proteins from healthy to cancer cells. The inhibitory effect of rapamycin was concentration dependent (from 1 to 100nM) and in time dependent manner (24-48h) (Fig. 5A,B Fig suppl 7). The reduction in *SOX9* levels was more intense (between 60 and 80%) than in *SOX2* (30 to 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 in SOX expression even at concentration 10 times below (1nM) the ones usually employed in cell culture. 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. 72 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 (Fig. 5C,D), demonstrating the efficient silencing of *mTOR* machinery in our U251 glioma model (Fig. 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 (Fig. 5E,F) further confirming the impact of mTOR in self-renewal and GSC maintenance [36]. Moreover, these studies reveal that genetic inhibion of mTOR and SOX proteins display 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 SonicHedhog molecular pathway (Fig. suppl 8), 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 sensitizes cells with elevated levels of SOX2. Accordingly, we performed MTT assays in which U87 and U251 cells were treated with a constant dose of 100μM TMZ together with 1 and 10nM of rapamycin or 5 and 10μM of cyclopamine (concentrations that significantly inhibited *SOX2*

expression). First, we detected that the citotoxic effect of 5 and 10μM of cyclopamine in U251 cells was higher (18 and 24%) than U87 (14 and 19%), athough we did not observe an additive effect of the combination of TMZ and cyclopamine treatment compared to single treatment (Fig. suppl 8). On the other hand, combined treatment of rapamycin and TMZ achieved a stronger citotoxic effect than with single agents alone (Fig. 5G). Moreover the concomitant treatment of rapamycin and TMZ exerted a greater tumor suppressive effect in SOX2-SOX9 high expressing than in low expressing cells (Fig. 5G). Indeed, the percentage of toxicity in U251 cells was 55 and 57% in TMZ plus rapamycin 1 and 10nM, respectively, compared to 43 and 46% in U87 cells. Of note, the synergestic action of rapamycin and TMZ was achieved even at the low concentration of 1nM and was of similar degree than 10nM. To determine whether this effect was mediated by SOX2 and SOX9, we measured their expression in cells cultured with TMZ (100μM), rapamicin (1nM) or the combination of them for 48hours. Remarkably, SOX2 and SOX9 were much lower in rapamycin or in combination than in non treated or TMZ alone cells (Fig 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 in cells with elevated SOX2 and SOX9 expression, we studied their efficacy in tumor formation *in vivo*. Thus, we injected U251 cells in athymic inmunodeficient mice subcutaneously and since one week later, mice received intraperitoneally TMZ (10mg/kg), rapamycin (5mg/kg) and combination (10mg/kg and 5mg/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 (Fig 5I).

Together, these data demonstrate that combining rapamycin with TMZ enhances the efficacy of TMZ against glioma cells, particularly in the subset with high levels of SOX2 and SOX9.

4. DISCUSSION

Different studies by us and others have shown that expression of SOX2 is often increased in glioblastoma and that this up-regulation is due to genetic amplification and epigenetic mechanisms [2], [14-16]. Notably, beyond high expression of SOX2 in 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 differentitated 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. Morever, we show that overexpression of SOX2, in addition to promote other relevant phenotypic properties such as invasiveness and migration [16], it 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 a dependence on this factor to survive.

In this work, we have identified that SOX2 and SOX9 expression correlate 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 that this

regulation occurs at post-transcriptional levels and that there is a feed back 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.

Temozolomide (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 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 which has been demonstrated to be resistant to the conventional therapeutic regimen of radiotherapy and temozolomide, SOX2 might be postulated as one of the key 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 pathway is aberrantly active in a high percentage of GBMs [14]. Our results indicate that their action might be modulated through SOX2 and SOX9. Consistent with the strategy to silence SOX2 activity in glioma, down-regulation of *SOX2* conferred sensitivity to treatment with PDGF 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 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.

5. FINANCIAL AND COMPETING INTERESTS DISCLOSURE

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6. ACKNOWLEDGEMENTS

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7. BIBLIOGRAPHY

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*** Of considerable interest*

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8. FIGURE LEGENDS

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 GB2 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 ($P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$***).

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 *IL1α* 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). HNumber 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).

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.

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 72h (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 72h (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 72h later (n=5). **(E)** Cell viability in *shSOX2* and *shSOX2*+*SOX9* U251 cells (n=3). Statistical significance was obtained with Student's T test ($P \leq 0.05$*; $P \leq 0.01$**; $P \leq 0.001$***).

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 (24,48h) 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-S6K 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 72h (n=3). **(H)** Kaplan meier curve showing generation of tumors from subcutaneously injected U251 cells after 12 weeks of treatment with TMZ (10mg/Kg) (n=8), rapamycin (5mg/Kg) (n=8) and combination of both (10mg/Kg and 5mg/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

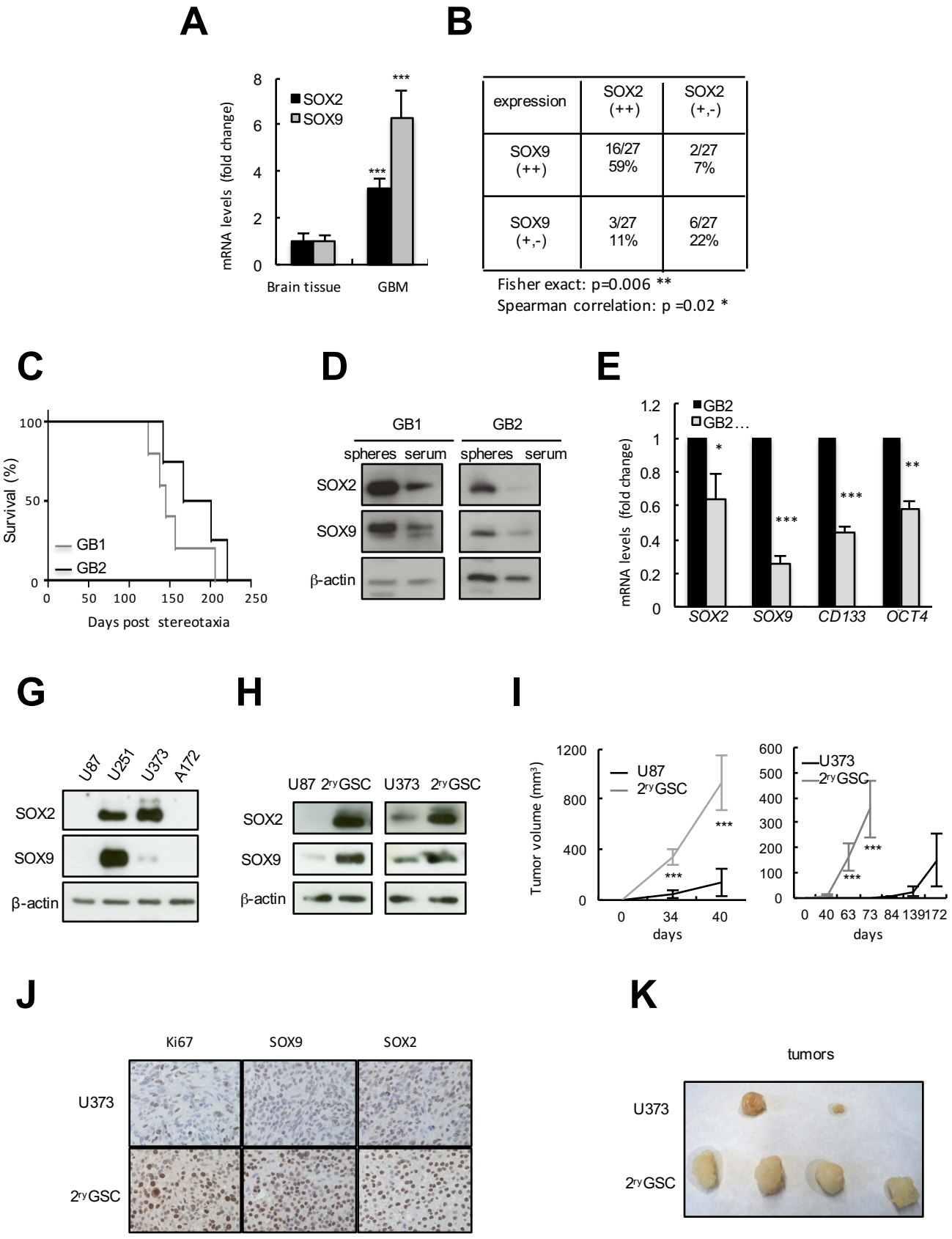
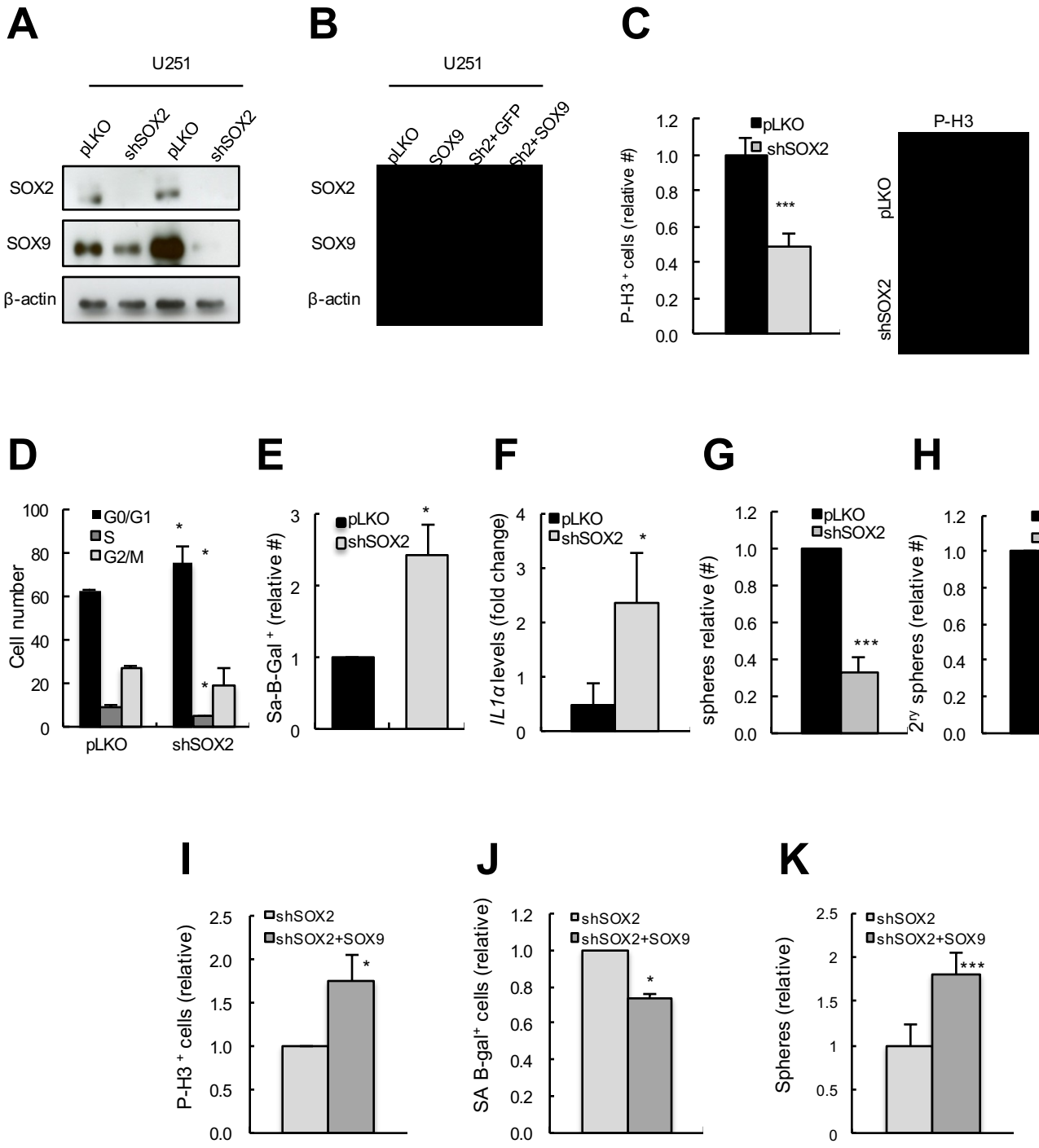


Fig. 1 Garros L et al.,



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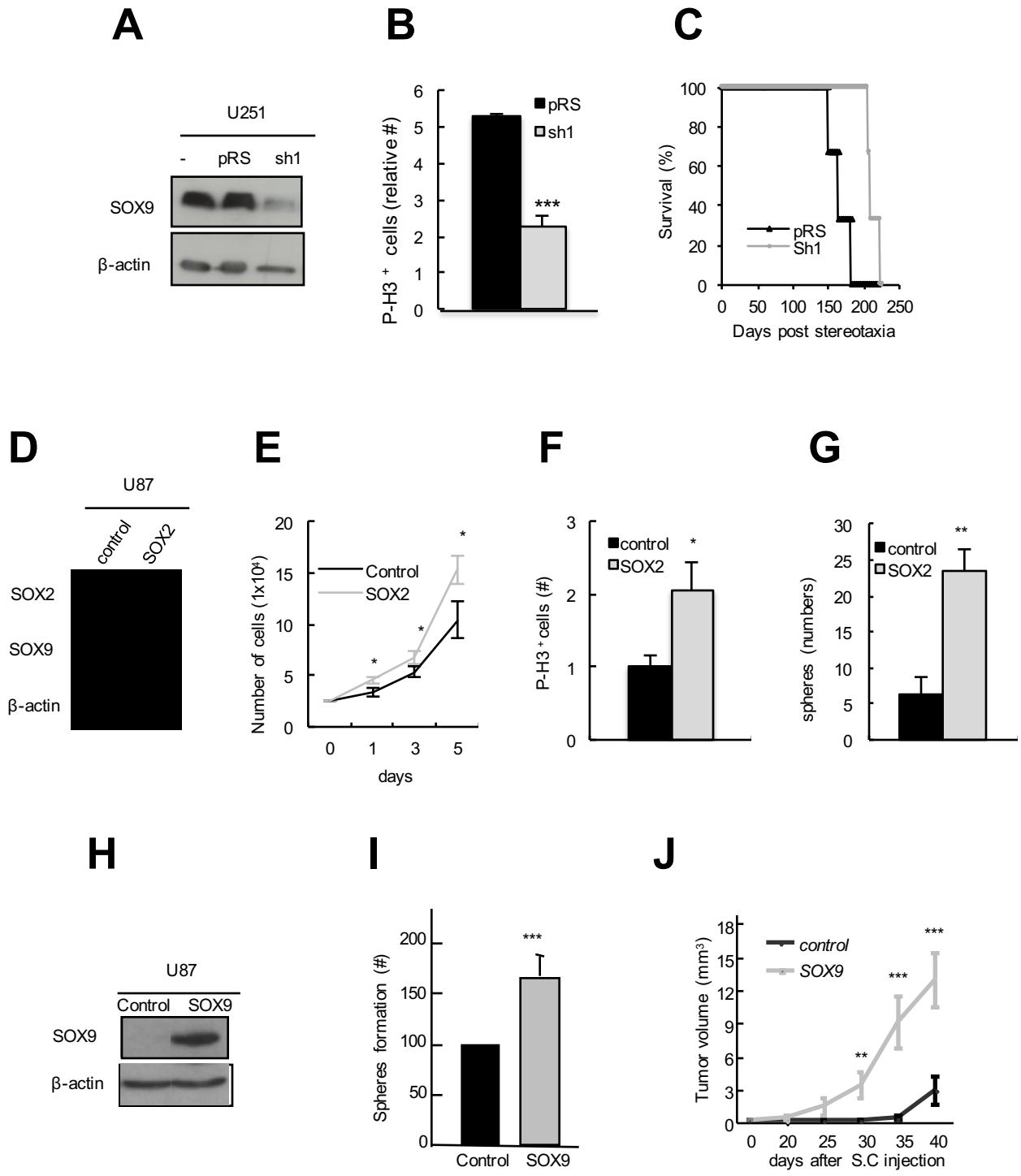

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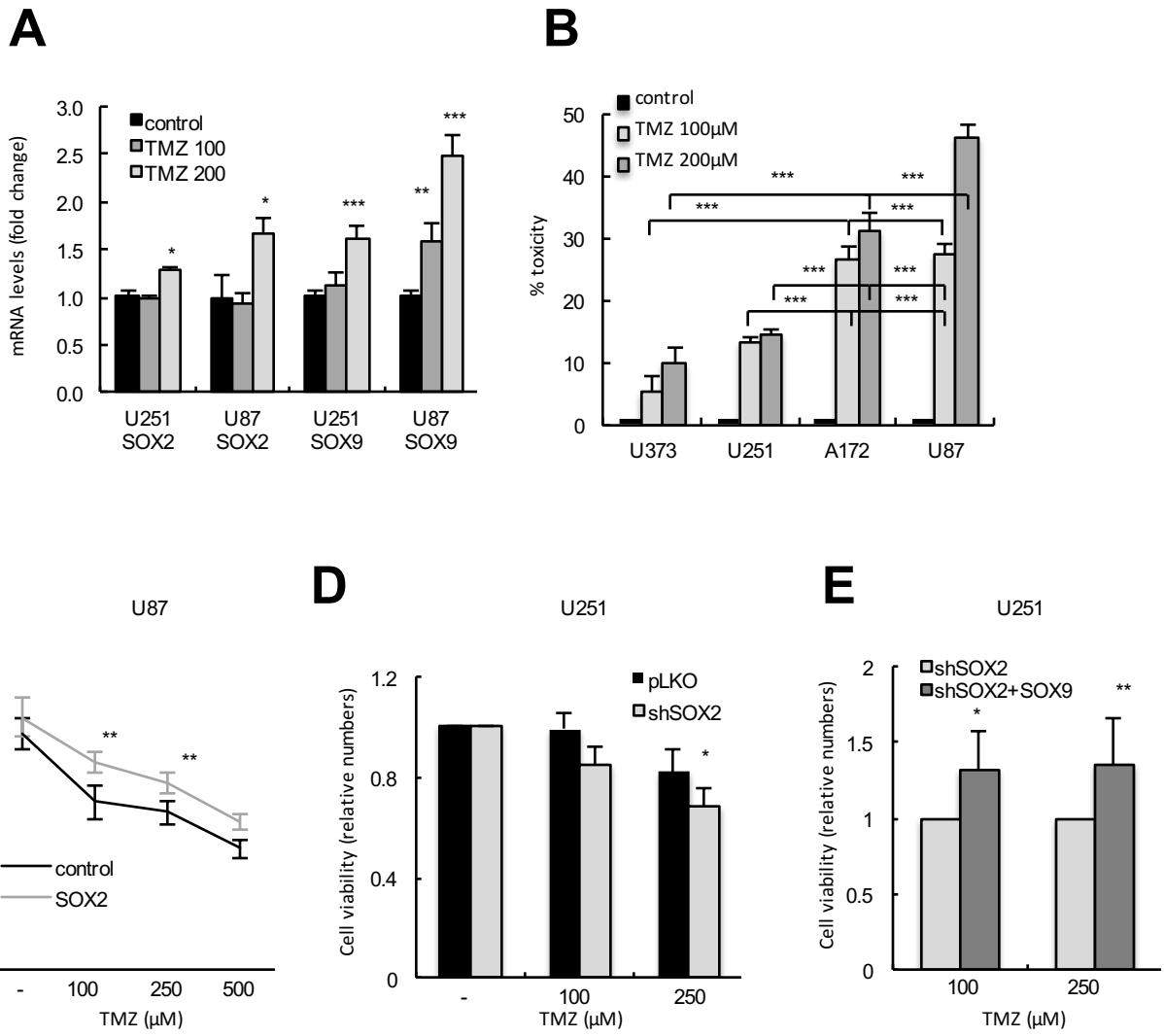

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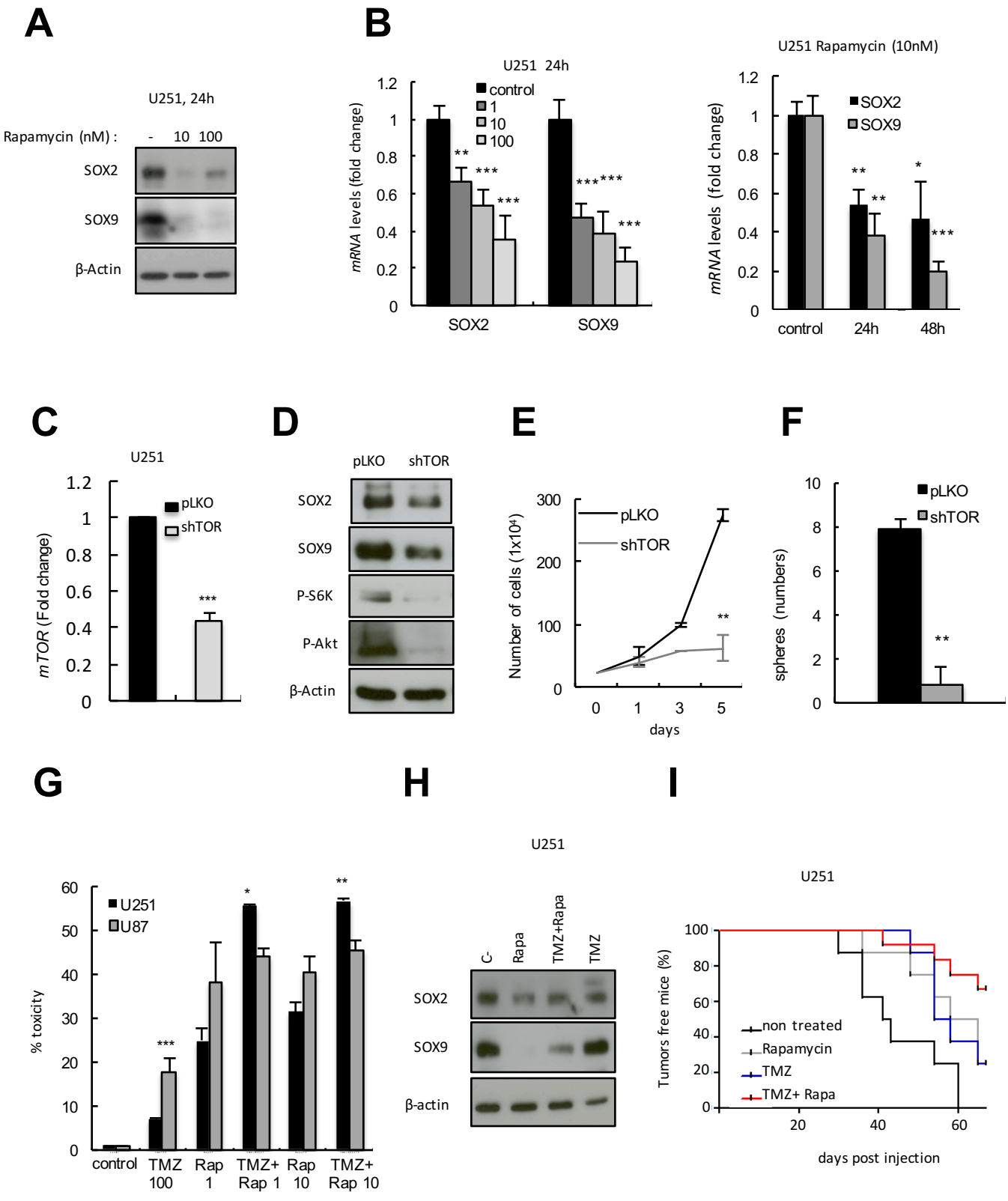


Fig. 5 Garros L et al.,

Supplementary Figures

Figure 1. Characterization of *SOX2* and *SOX9* levels in GBM

(A) Expression of *SOX2* and *SOX9* mRNA in each of the 27 human glioblastoma samples. Healthy brain tissue as control is included in a circle. **(B)** Determination of *SOX2* and *SOX9* in 11 human glioblastoma samples (GBM-GBM11), two independent healthy tissues (HT) and U251 and U87 cell lines. **(C)** Expression of SOX2 and SOX9 in human glioblastoma samples included in the TCGA (The Cancer Genome Atlas) studies.

Figure 2. Characterization of SOX2 and SOX9 levels in GB1 and GB2 cells

(A) Representative image of Hematoxilin/Eosin and SOX9 staining in GB1 and GB2 derived tumors. **(B)** Comparison of *SOX2* and *SOX9* levels in conventional glioma cell lines and GSCs. Indicated values are the average of 2 independent experiments and are normalized to their expression in U87. **(C)** *SOX2*, *SOX9*, *CD133* and *OCT4* expression were assayed in GB1 and GB2 grown as spheres or in differentiation media.

Figure 3. SOX expression in U87 and U373 GSCs.

(A) *SOX2*, *SOX9*, *CD133* and *OCT4* mRNA levels were assayed in U87 and U373 cells and compared to their respective 2^{ry} GSC population. (B) Quantification for SOX2 and SOX9 in U87MG and U373MG cells and their respective 2ryCSC (N=5). **(C)** Representative image of subcutaneously generated tumors from U87 2^{ry} and U87 parental cells. **(D)** Immunohistochemistry of SOX2 and SOX9 in these tumors.

Figure 4. **Downregulation of SOX2 in U251 cells.**

(A) Quantification of SOX2 and SOX9 protein levels in *shSOX2* cells. Value is relative to pLKO transduced cells (n=5). **(B)** Cell growth of U251 cells at the indicated time points (n=4). **(C, D)** Representative images of P-H3 positive senescence associated β-galactosidase cells in *shSOX2* and pLKO U251cells (n=4). **(E)** Data from cell cycle assay showing an arrest in G0/G1 phase and a reduced S phase in *shSOX2*. **(F)** Representative image of 1^{ry} GSC generated from control and *shSOX2* cells. (**G)** Absolute number of spheres (1^{ry}) formed from the indicated numbers of cells

Figure 5. Effect of SOX9 restoration in *shSOX2* cells

(A,B) Image of P-H3 and senescence associated β-galactosidase positive cells in *shSOX2* and shSOX2 restored with SOX9 in U251 cells. **(C)** *shSOX2* and *shSOX2+SOX9* transduced U251 cells ($2x10^3$ cells) (n=4) were plated, and the number of colonies scored after two weeks.

Figure 6. Downregulation of SOX9 in U251 cells.

(A) *SOX9* reduced A levels in *shSOX9 (sh1*) cells (n=3) compared to pRS control cells. (B) Representative inmunofluorescence of SOX9 in *shSOX9 (sh1)* cells. (C) Quantification of foci generated by *sh1* and *pRS* cells in soft agar (n=4).

Figure 7. Effect of SOX2 overexpression in glioma cell proliferation and self-renewal

(A) Quantification of SOX2 and SOX9 protein in control and SOX2 overexpressed cells, corresponding to western blot of Figure 3E (n=4) **(B,C)** Representative image of P-H3 positive cells and spheres (1^{ry}) in the indicated conditions of U87 cells

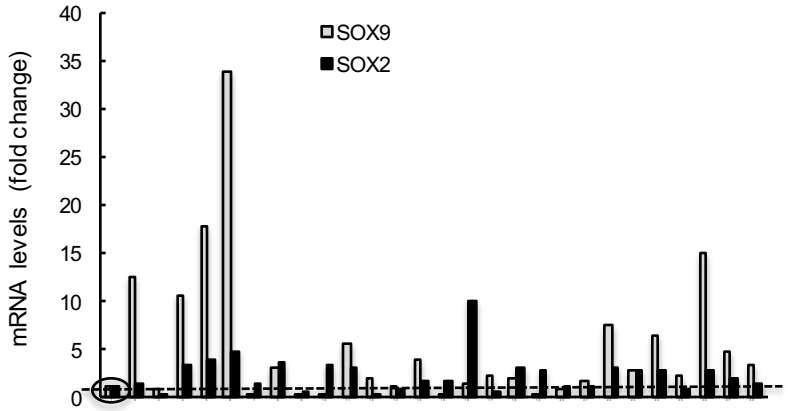
Figure 8. SOX2 and SOX9 expression in glioma cells cultured with rapamycin,

(A) Reduced *SOX2* and *SOX9* levels at increasing dosages of rapamycin (10nM and 100nM) in U373 cells (n=2). **(B)** Representative image of the action of rapamycin (1nM), TMZ (100μM) and both (1nM+100μM respectively) for 48 hours over SOX2 and SOX9 expression in U87MG and U251MG cells. Treatment with corresponding DMSO concentration was used as control treatment (n=3).

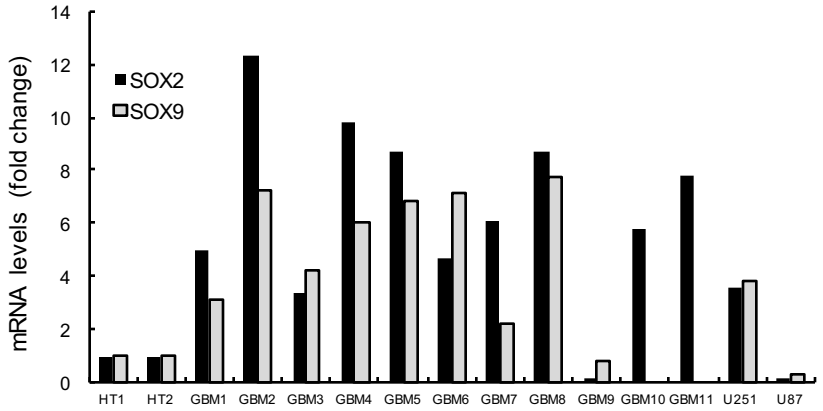
Figure 9. Effect of cyclopamine in cells with variable SOX expression

(A) Western blot of SOX2 and SOX9 in U251 glioma cells cultured for 48 h in the absence (*-)* or presence of 5μM of cyclopamine. **(B)** qRT-PCR of SOX2 and SOX9 mRNA levels in U251 cells cultured with increasing concentrations of cyclopamine for 24 and 48 hours (n=3). **(C)** MTT assay of U87 and U251 glioma cell lines cultured with TMZ (100μM), increasing dosages of cyclopamine (5-10μM) and combination of both for 72h (n=5).

A



B



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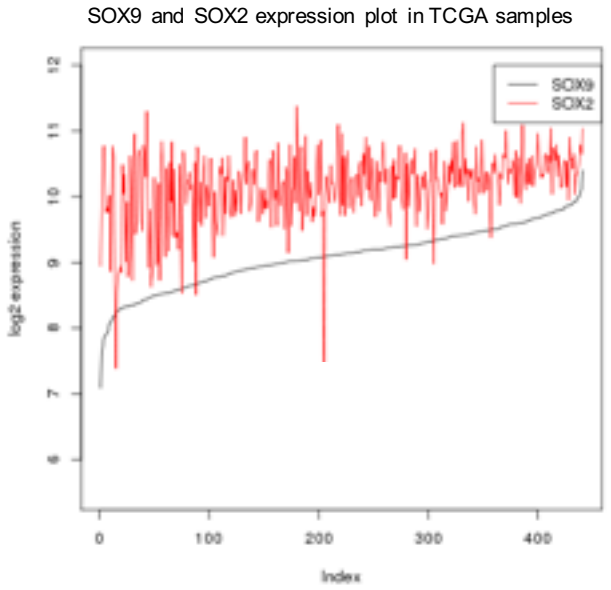
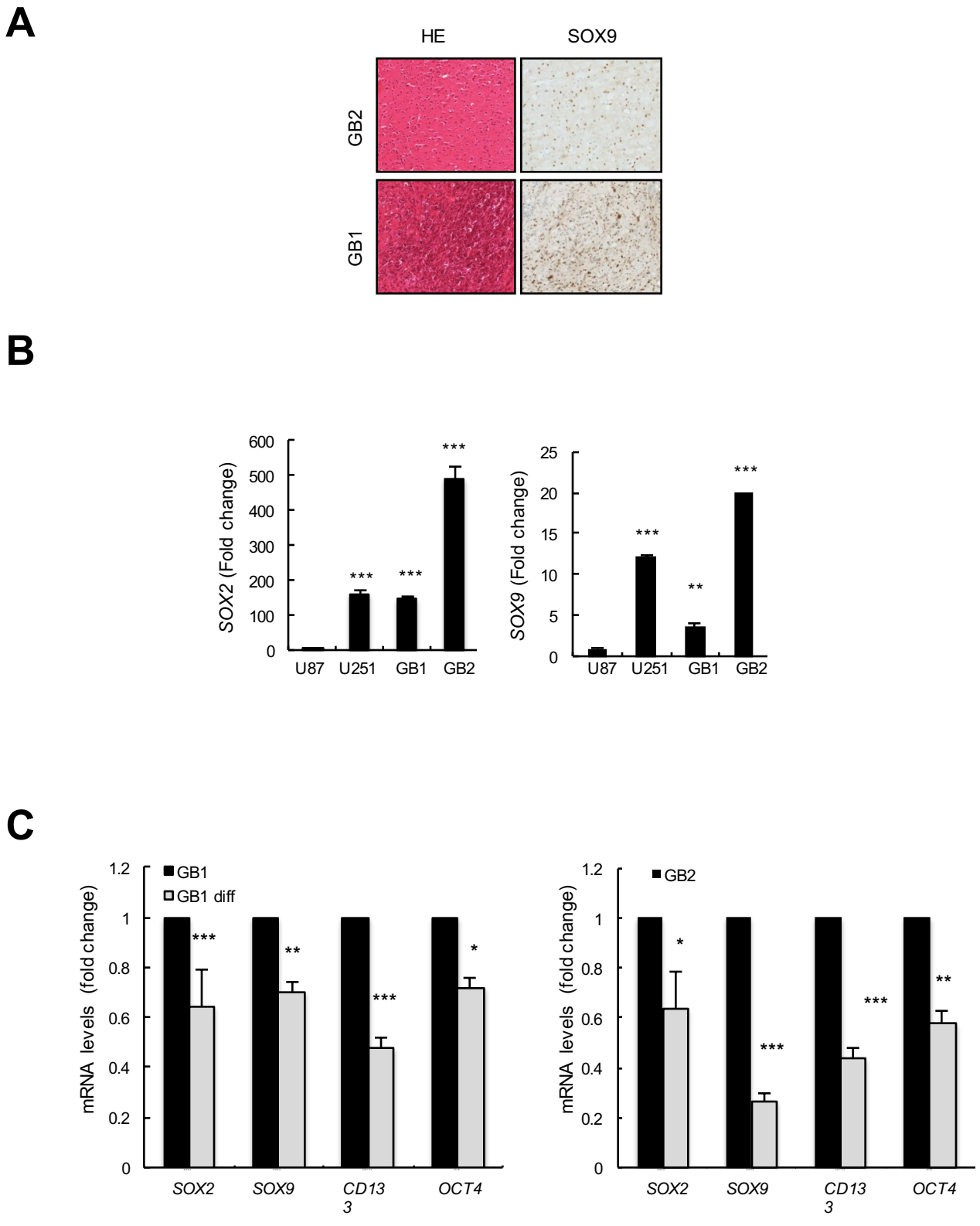


Fig. 1 Supplementary Garros L et al.,



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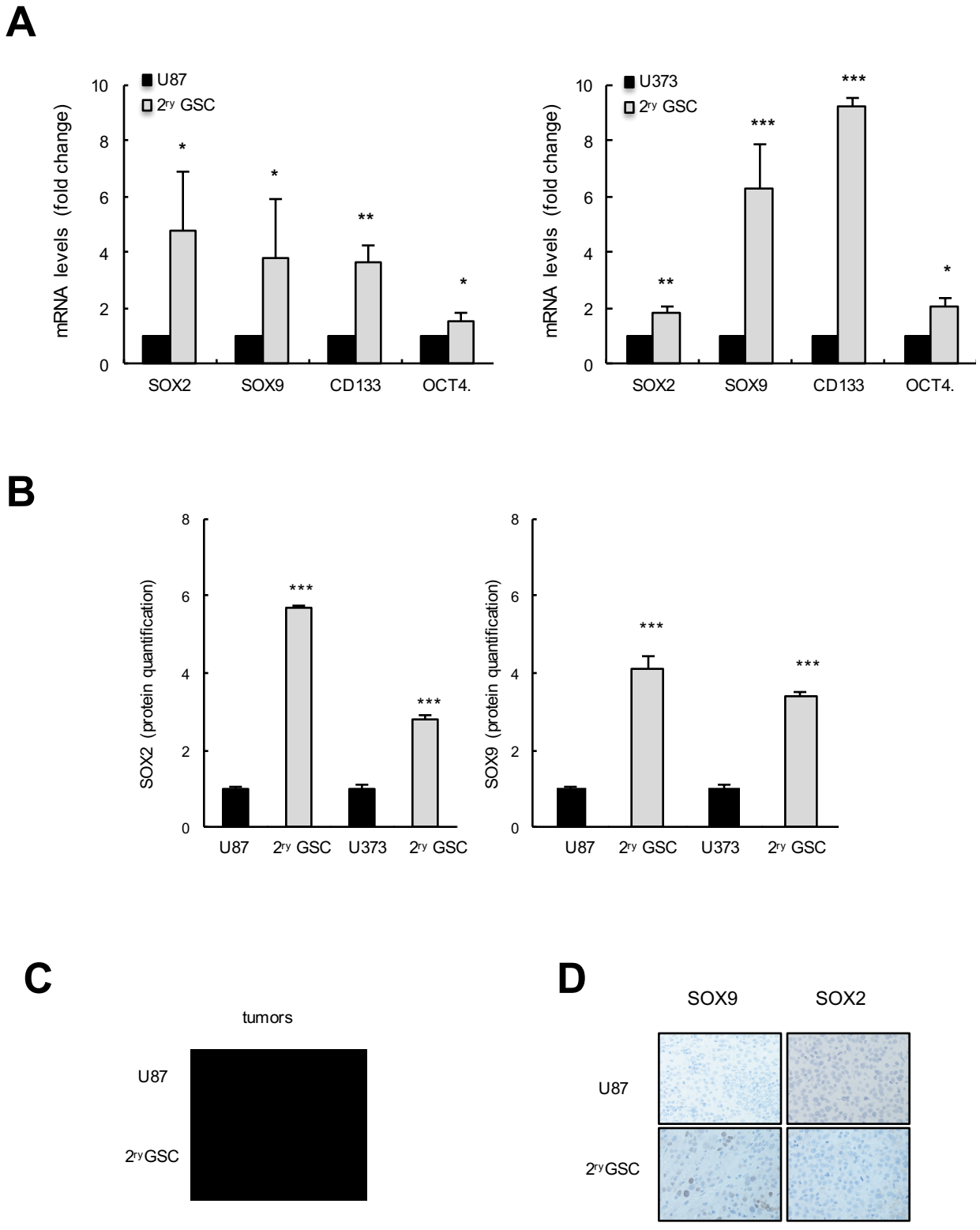

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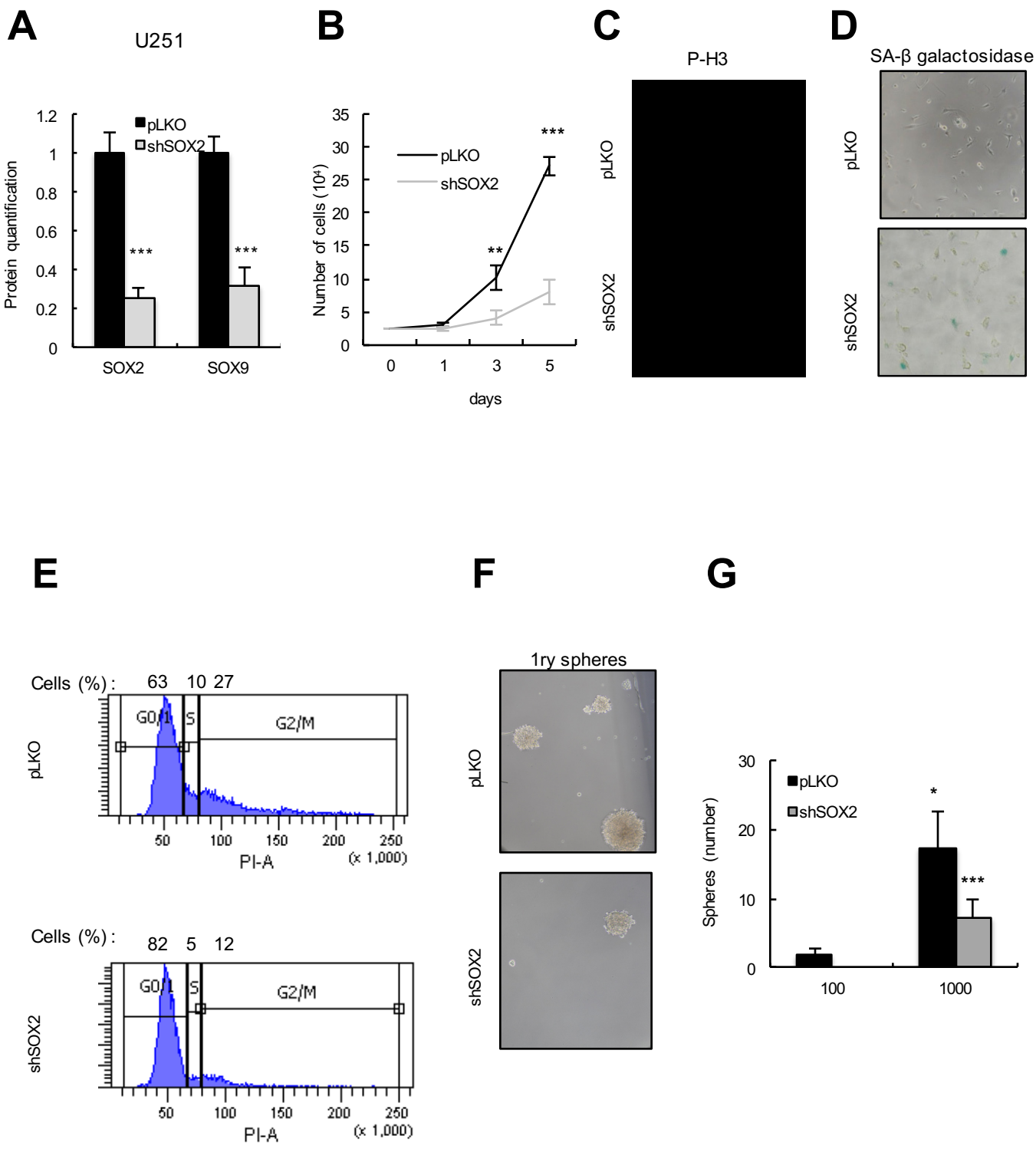

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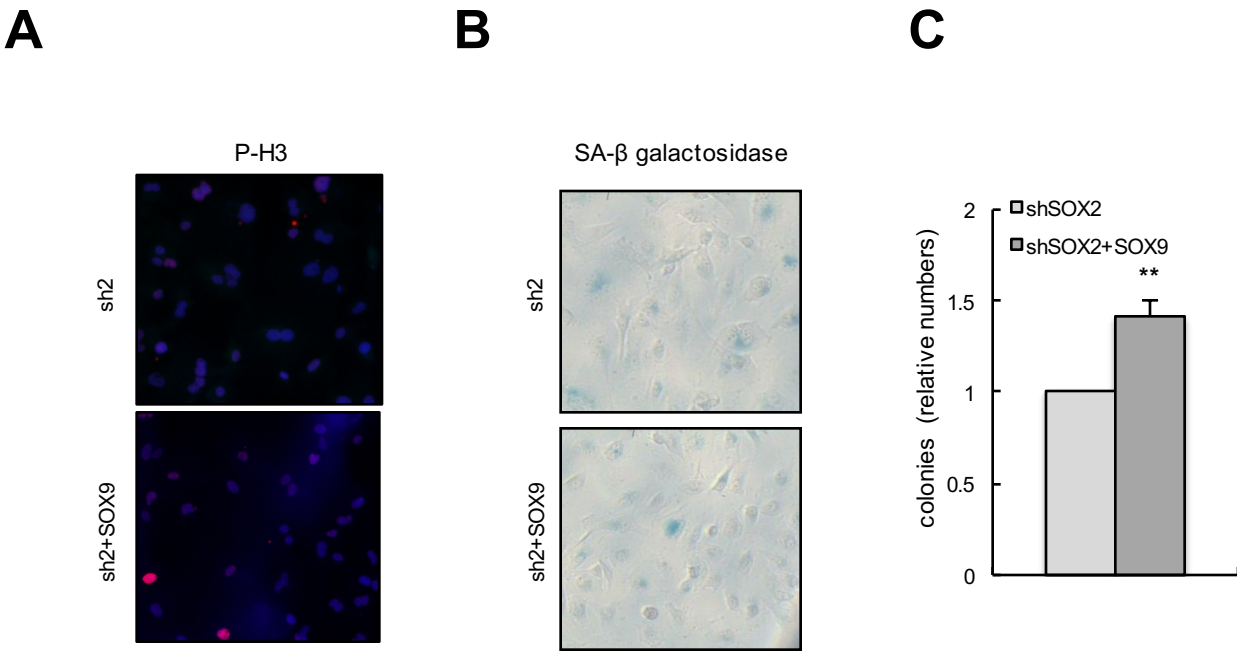

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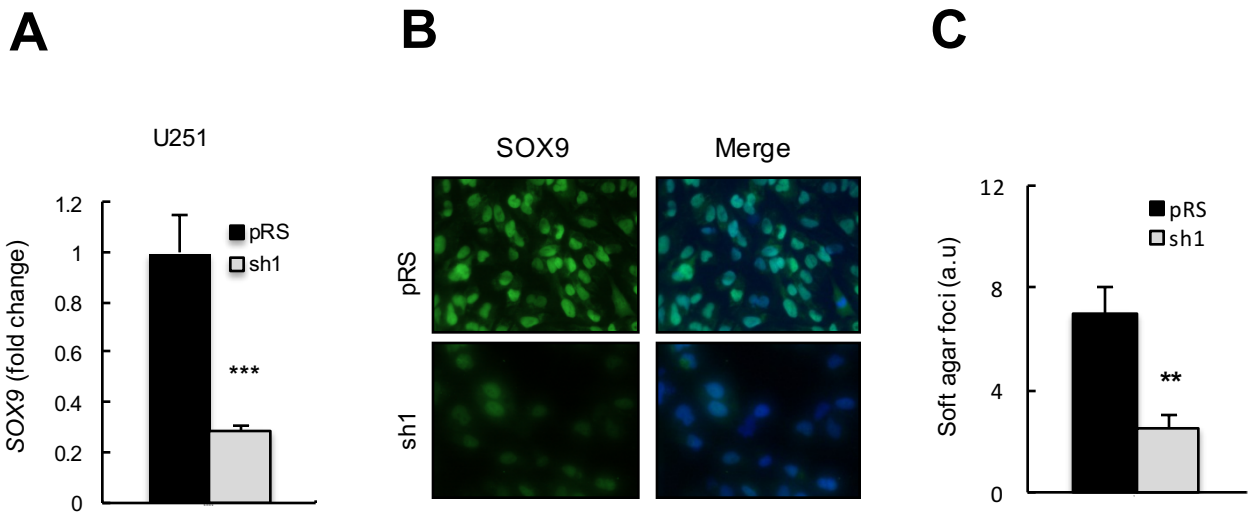

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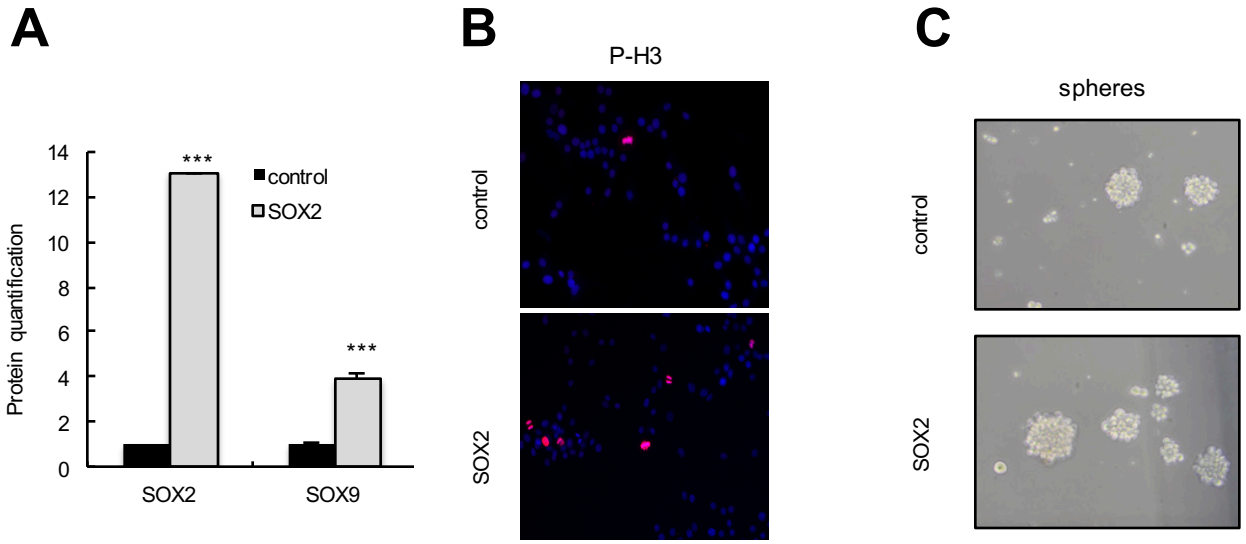

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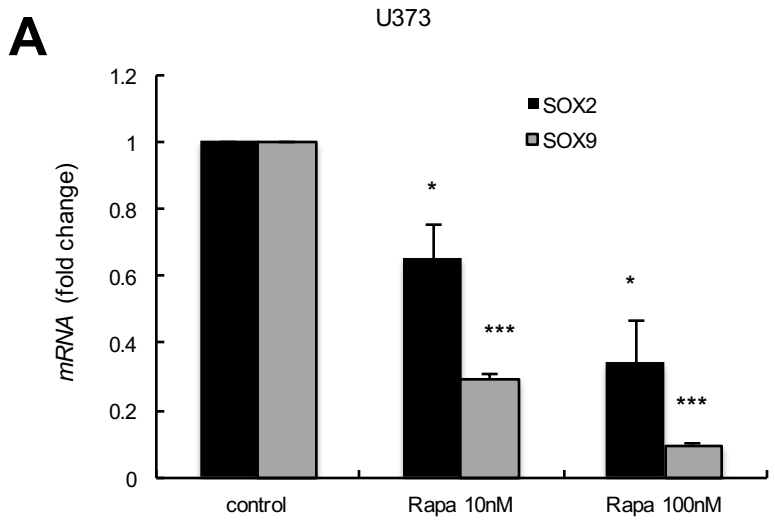

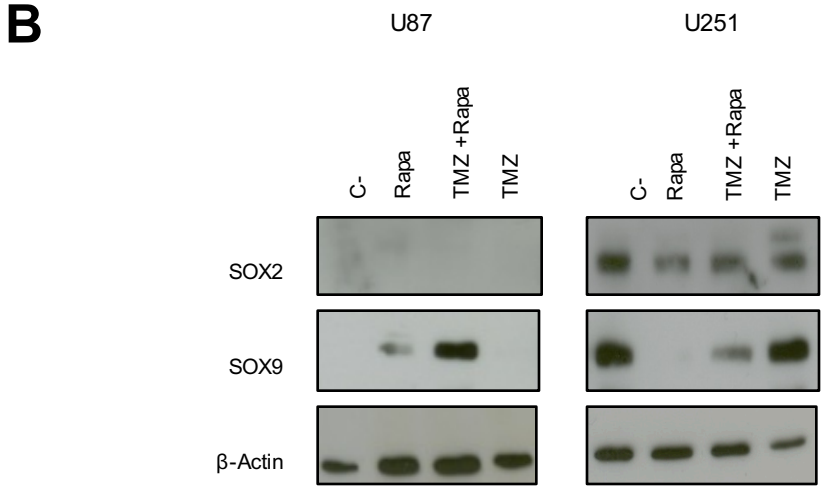

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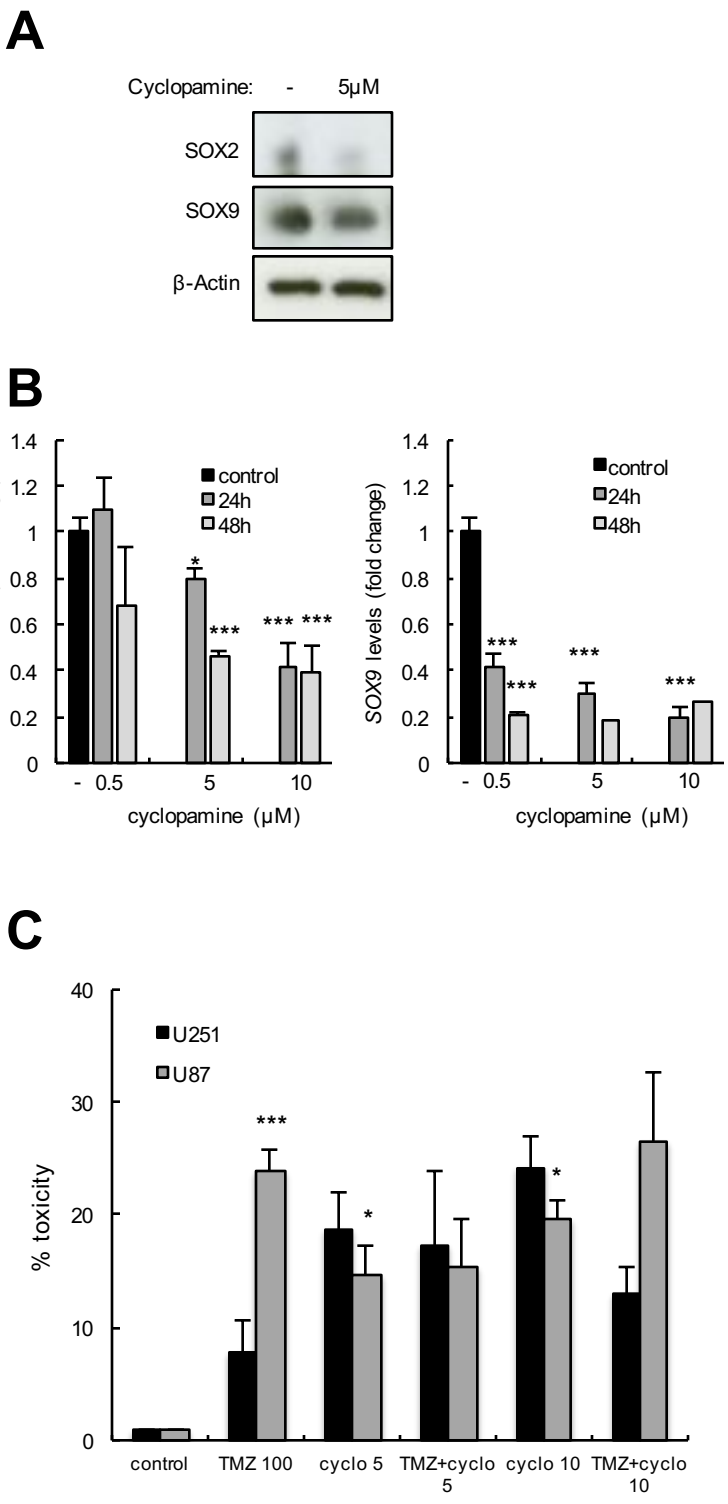

Fig. 9 Supplementary Garros L et al.,