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Tumor and Stem Cell Biology

Cancer Research

Sox2 Is Required to Maintain Cancer Stem Cells in a Mouse Model of High-Grade Oligodendroglioma

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Abstract

The stem cell–determining transcription factor Sox2 is required for the maintenance of normal neural stem cells. In this study, we investigated the requirement for Sox2 in neural cancer stem-like cells using a conditional genetic deletion mutant in a mouse model of platelet-derived growth factor–induced malignant oligodendroglioma. Transplanting wild-type oligodendroglioma cells into the brain generated lethal tumors, but mice transplanted with Sox2-deleted cells remained free of tumors. Loss of the tumor-initiating ability of Sox2-deleted cells was reversed by lentiviral-mediated expression of Sox2. In cell culture, Sox2-deleted tumor cells were highly sensitive to differentiation stimuli, displaying impaired proliferation, increased cell death, and aberrant differentiation. Gene expression analysis revealed an early transcriptional response to Sox2 loss. The observed requirement of oligodendroglioma stem cells for Sox2 suggested its relevance as a target for therapy. In support of this possibility, an immunotherapeutic approach based on immunization of mice with SOX2 peptides delayed tumor development and prolonged survival. Taken together, our results showed that Sox2 is essential for tumor initiation by mouse oligodendroglioma cells, and they illustrated a Sox2-directed strategy of immunotherapy to eradicate tumor-initiating cells. *Cancer Res; 74(6); 1833–44. ©2014 AACR.*

Introduction

The cancer stem cell (CSC) hypothesis provides a novel point of view on the mechanisms of tumor development and on therapeutic approaches. CSC can reinitiate tumor development following conventional therapeutic approaches (to which they are resistant) and following experimental transplantation into mouse brain. Neural tumors were among the first tumors in which CSCs were identified (1–4). Defining the gene regulatory networks that control the maintenance of the malignant phenotype of CSCs is thus a fundamental objective to understand tumor pathogenesis and to develop novel approaches to targeted therapy.

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Neural CSCs are proposed to originate from normal neural stem cells (NSC) or from the "reprogramming" of more differentiated cells to a stem cell state (5–7).

Sox2 is a transcription factor functionally important for normal stem cells, including pluripotent and tissue-specific stem cell types (8–12). In the nervous system, Sox2 is active in NSCs and progenitors of the embryo and adult (8, 12). By conditional knockout in mouse, we found that Sox2 is required to maintain self-renewal of NSCs *in vitro* and *in vivo* within specific postnatal brain regions (e.g., hippocampus; ref. 13). Sox2 also "reprograms" differentiated fibroblasts to pluripotent iPS cells, acting together with a small number of other molecules (14, 15).

Sox2 is expressed in many neural tumors (including gliomas/glioblastomas, medulloblastomas, and ependymomas), and its expression is consistently detected in the cell fraction displaying properties of CSCs (2, 3, 16, 17).

Oligodendroglioma is a type of glioma consisting primarily of cells resembling oligodendroglia and it is the second most common malignant brain tumor in adults (18); patients affected by high-grade (anaplastic) oligodendroglioma have a median survival of 3 to 4 years (19). Alteration of platelet-derived growth factor (PDGF)-B signaling is a common molecular lesion in oligodendrogliomas, and in gliomas in general, and PDGF-B experimental overexpression in neural stem/progenitor cells of the mouse brain generates neural tumors, in particular oligodendrogliomas (20, 21). We generated a mouse genetic model of oligodendroglioma by overexpression of PDGF-B in mouse embryonic neural precursors, by retroviral transduction of embryonic brains *in utero* (22); these tumors (PDGF-induced high-grade gliomas, pHGG hereafter) display a homogeneous

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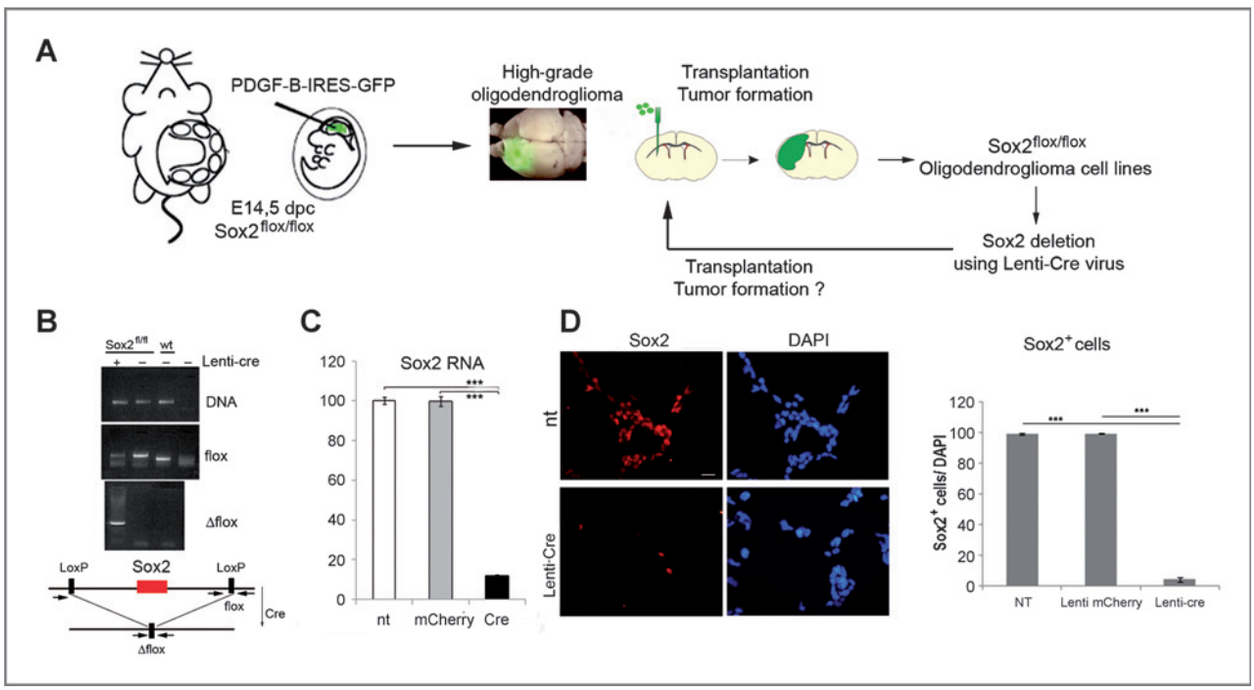

Figure 1. Obtainment of *Sox2*$^{flox/flox}$-induced oligodendrogliomas and Sox2 deletion via lentiviral Cre recombinase. A, schematic representation of the experimental procedure followed to obtain and study *Sox2*$^{flox/flox}$ oligodendrogliomas (pHGGs; modified from ref. 23). B, PCR assay of *Sox2*^{flox} deletion on DNA, with primers detecting a non-mutated DNA sequence for normalization (DNA), the nondeleted (flox), or Cre-deleted (Δflox) *Sox2*^{flox} alleles. Primers (depicted in the bottom diagram) are on genomic sequences just upstream and downstream to the loxP site, absent in the wild-type control DNA (wt), thus giving a band of slightly lower size compared with *Sox2*^{flox} (*Sox2*$^{fl/fl}$). The small amount of nondeleted DNA in Cre-treated cells typically represents less than 10%. C, qRT-PCR assay of Sox2 mRNA in nontransduced (nt; set = 100), control mCherry, or Cre lentivirus-transduced pHGG cells. D, SOX2 immunofluorescence (red) of untransduced (nt) and Cre-transduced (lenti-Cre) pHGG cells. 4′,6-diamidino-2-phenylindole (DAPI; blue) stains nuclei. Scale bar, 20 μm. Sox2 is widely expressed in pHGG cells and efficiently ablated by lenti-Cre. Right, quantification of Sox2-positive cells (***, $P < 0.0001$; Fisher exact two-tailed test).

character of oligodendroglioma, express Sox2 (IA/PM, unpublished data; see Figs. 1 and 2), and reproducibly develop after a latency of several weeks (22, 23). pHGGs contain CSCs that will reform the same tumor type following *in vivo* transplantation of dissociated tumor tissue or *in vitro* cultured pHGG cells (22, 23).

Here, we ask whether Sox2 is required by oligodendroglioma stem cells, mirroring its requirement for normal NSCs. We used our *Sox2*^{flox} conditional mutation (13), in combination with the pHGG mouse model (22), to address the effects of Sox2 ablation on tumor reinitiation following tumor cell transplantation into brain. Mice transplanted with Sox2-deleted cells remained tumor-free throughout the time window in which controls developed lethal tumors. Loss of tumorigenesis of Sox2-ablated cells is prevented by transduction with a Sox2-expressing virus. Microarray analysis identifies early gene expression changes following Sox2 deletion. Finally, vaccination with Sox2 peptides elicits a response that significantly delays tumor development, pointing to Sox2 itself as a possible therapeutic target.

Materials and Methods

Tumor induction in *Sox2*$^{flox/flox}$ mice

Sox2$^{flox/flox}$ (13) E14.5 embryos from homozygous *Sox2*$^{flox/flox}$ matings were injected in the ventricular space with PDGF-B-IRES-GFP-encoding retrovirus, as in ref. 23. Tumors (pHGGs) arising after 90 days were retransplanted and cultured as described (22).

Lentiviral constructs and infections

Cre-encoding virus was obtained from Cre-IRES-GFP (a gift from S. Brunelli, The University of Milan-Bicocca, Milan, Italy; ref. 24) by GFP deletion (using *Bst*XI/*Sal*I); this avoided GFP toxicity observed while superinfecting oligodendroglioma cells (which synthesize PDGF-B-GFP) with the original GFP-expressing virus. The control mCherry-expression virus (1070.935.hPGK.dNGFR.minhCMV.mCherry.SV40PolyA) was a gift from L. Naldini, San Raffaele Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, Italy. The Sox2-expressing lentivirus was obtained from a Sox2-IRES-GFP lentivirus (25) by *Sal*I/*Bst*xI GFP deletion.

For Cre- or control-lentiviral transduction, cells were plated in 24-well plates at 50,000 cells per well on Matrigel and transduced 4 hours after plating, with a multiplicity of infection (MOI) of 7. Medium was changed 15 hours after transduction. In some experiments (Fig. 2), pHGG cells were initially transduced with Sox2 lentivirus at MOI 7, tested for expression of transduced Sox2 (Supplementary Material), passaged, and further transduced with the Cre virus, as above.

Sox2 PCR, quantitative reverse transcription PCR, and immunofluorescence

PCR primers and procedures are described in Supplementary Materials. Sox2 immunofluorescence was performed as described in ref. 25.

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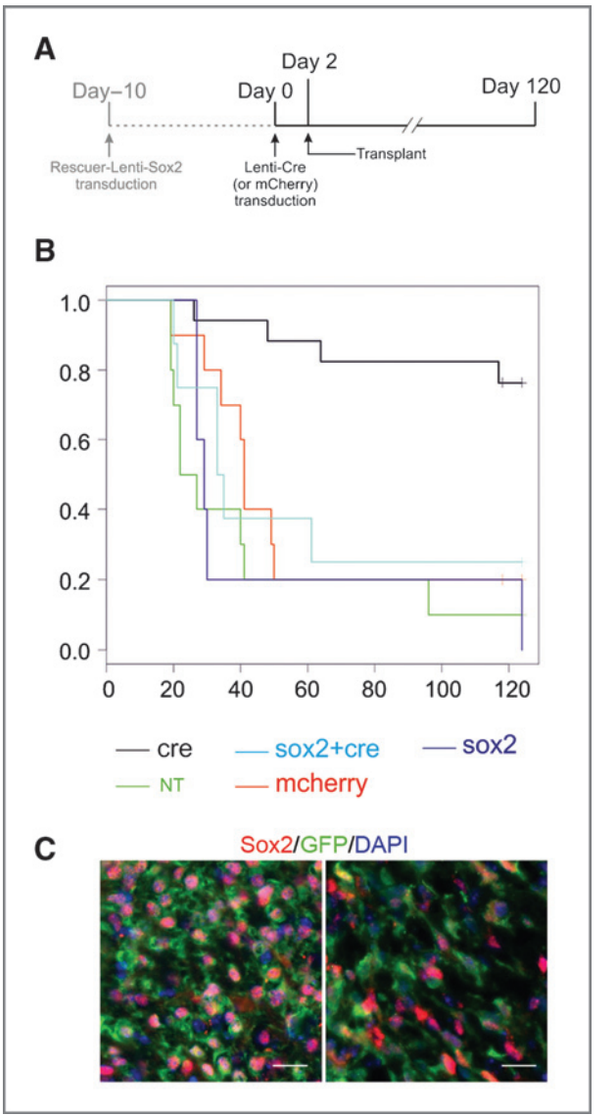

Figure 2. *Sox2*$^{flox/flox}$ deletion before pHGG stem cells transplantation efficiently antagonizes tumor reinitiation and tumor-caused lethality. A, diagram of viral transduction and brain transplantation experiments. B, Kaplan–Meier survival curves for mice transplanted with untransduced pHGG cells (NT, green line) or with pHGG cells transduced with Cre virus (Sox2-deleted, black line), control mCherry virus (red line), Sox2 "rescuing" virus plus Cre virus (clear blue line), or Sox2 "rescuing" virus only (blue line). C, immunofluorescence for SOX2 (red) and PDGF-B-GFP (green) on sections from two pHGGs, showing abundant SOX2-positive cells. Scale bar, 20 μm.

Transplantation of virally transduced cells into mouse brain

Twenty thousand cells were transplanted into the brain of C57Bl/6J mice (23) 36 to 40 hours after viral transduction. The data in Figs. 2 and 6 were obtained in different laboratories (Malatesta, Genova and Finocchiaro, Milano, respectively).

In vitro assays

For *in vitro* assays, pHGG cells were transduced, collected after 96 hours, and plated: (i) at clonal density (60 cells/100 μL/well) in 96-well plates in normal growth medium (22) without Matrigel, to allow the formation of well-individualized clones, with or without EGF and basic fibroblast growth factor (bFGF; Fig. 3A); and (ii) at a density of 5,000 cells per well in Matrigel-coated 4-well chambered slides, in medium devoid of EGF and bFGF, supplemented with 2% fetal calf serum (Fig. 3B). A total of 10 μmol/L EdU (A10044 Molecular Probes, Invitrogen) was administered for 30 minutes before 4% paraformaldehyde fixation and EdU-positive nuclei were detected by a Click-IT EdU Alexa Fluor 549 HCS Assay Kit (Molecular Probes, Invitrogen). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis and GFAP IF were performed as described in ref. 25. O4 and GalC IF used anti-O4 and anti-GalC hybridomas (undiluted supernatant), a gift from C. Taveggia.

Gene expression analysis

Total RNA was prepared as described (22) from triplicate independent cultures of *Sox2*$^{flox/flox}$ pHGG cells transduced with Cre virus or control (nontransduced, or transduced, with mCherry virus). Cre-transduced cells were harvested 40 or 96 hours after transduction and mCherry-transduced and non-transduced control cells were harvested 40 hours after transduction.

RNA extraction, microarray hybridization, and analysis were performed as described previously (for details, data analysis and Gene Ontology annotation, see Supplementary Files; ref. 22).

Sox2 peptide design and vaccination

For SOX2 immunotherapy, we used four SOX2 peptides: TLMKKDKYTL (26), SGPVPGTAI (Score 21); VSALQYNS (Score 14); GGGGNATA (Score 16), and four OVA control peptides: $OVA_{257-264}$ SIINFEKL (Sigma Aldrich), OVA_{55-62} KVVRFDKL (Score 22); $OVA_{107-114}$ AEERYPIL (Score 22); $OVA_{176-183}$ NAIVFKGL (Score 22) that were expected to bind the murine MHC class I (H-2Db). The new peptides were designed using SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) binding-motif algorithms and were synthesized by Primm srl (Milano).

C57BL/6N (5-week-old females) mice were injected (day 0) with 20,000 tumor cells (stereotactic coordinates with respect to the bregma: 1 mm anterior, 1.5 mm left lateral, and 2.5 mm deep).

Injected mice were treated with temozolomide (Sigma Aldrich) and/or peptide vaccinations. Temozolomide was administered by intraperitoneal injections (5 mg/kg). Peptides were emulsified with Montanide ISA 51 VG (1:1; SEPPIC) and administered by subcutaneous injections of the four peptides separately (15 μg/peptide) into different areas of the flank.

We tested four different conditions: group I: vehicle only (montanide); group II: three peptide vaccinations spaced 1 week apart (days 14, 21, 28); group III: temozolomide alone, five daily injections (day 10–14); group IV: peptide vaccination combined with temozolomide. Groups I, II, and IV also

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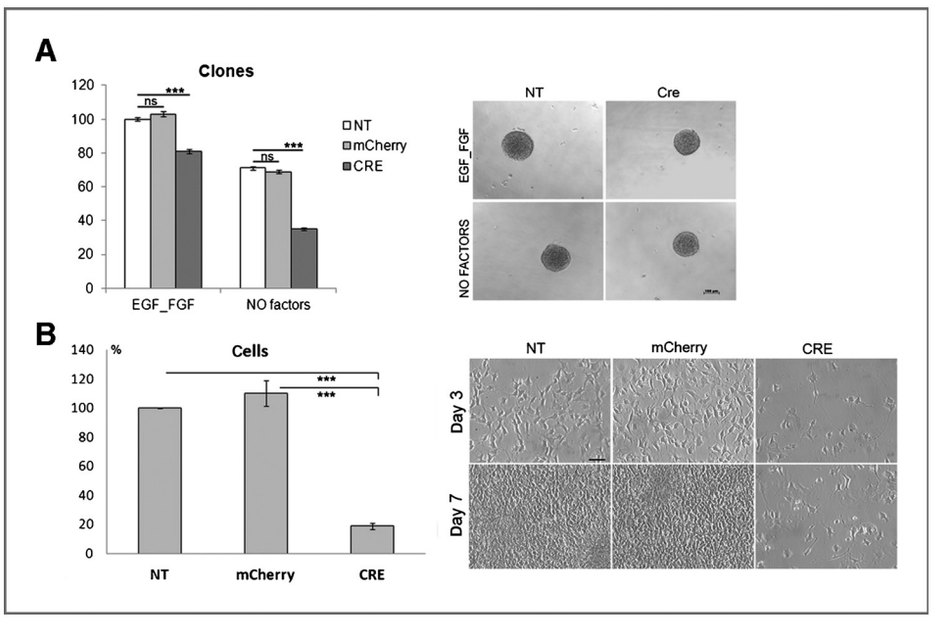

Figure 3. *Sox2*$^{flox/flox}$ deletion reduces *in vitro* growth of pHGG stem cells. A, pHGG oligodendroglioma clone numbers obtained in EGF+bFGF-containing medium (EGF_FGF), or factor-free medium (NO Factors), with nontransduced cells (NT), or cells transduced with control (mCherry) or Cre-expressing (CRE) virus. The number of clones obtained with NT cells (representing >1,400 clones counted for each experiment) was set at 100%. More than 1,400 clones were counted for each experiment replicate. The results shown are the average of $n = 2$ independent experiments performed in duplicate (***, $P < 0.0001$; ns, nonsignificant; $P > 0.05$; Wilcoxon test). The images show examples of clones. Scale bar, 100 μm. B, pHGG cell numbers, obtained with nontransduced cells (NT) or following transduction with control (mCherry) or Cre virus (CRE), after 7 days in serum-containing medium. The cell number obtained with untransduced (NT) cells (>1,000 cells counted for each experiment) is set at 100%. The results shown are the average of $n = 2$ independent experiments performed in duplicate (***, $P < 0.0001$; Fisher exact test). Representative images showing cell density at day 3 and 7 are shown.

received a total of 3 μg of recombinant murine granulocyte macrophage colony-stimulating factor as described (27). Cumulative survival curves were obtained using the Kaplan–Meier method (MedCalc 12.7).

For cytotoxicity assay, isolation of tumor-infiltrating lymphocytes (TIL) and flow cytometry, see ref. 27 and Supplementary Materials.

Results

We generated oligodendrogliomas in mouse by transduction of a PDGF-B-IRES-GFP-encoding retrovirus within the brain at embryonic day (E) 14.5 (22, 23). Embryos were homozygous carriers of a *Sox2*^{flox} mutation, allowing subsequent Sox2 excision via Cre recombinase (Fig. 1A; ref. 13). Tumors developed, at different times after birth: early-onset, showing low-grade tumor features and late-onset, displaying high-grade glioma characteristics, as expected from our previous data (23). We focused on tumors arising at least 90 days after birth, as our previous analyses showed that low-grade tumors arising before day 90 can be hardly grown in culture and are not tumorigenic (23). Indeed, 4 of 7 tumors appearing after 90 days reinitiated tumorigenesis following transplantation into adult mouse brain. We cultured *in vitro* three of these secondary tumors (pHGGs), in conditions allowing the long-term maintenance of TICs, i.e., presence of EGF and bFGF and absence of serum (16, 22), and we used for subsequent analyses one cell population derived from such tumor.

Sox2 deletion impairs tumor reinitiation by pHGG cells following *in vivo* transplantation in the brain

To evaluate the role of Sox2 in tumor initiation, we deleted the "floxed" *Sox2* gene from tumor-derived cells by transduction with lentiviruses expressing Cre recombinase or mCherry as a control (Fig. 1A). Transduction of Cre recombinase (but not of control virus) induced efficient deletion of *Sox2* (>95% by DNA analysis; Fig. 1B), leading to loss of Sox2 mRNA (>90% by real-time RT-PCR; Fig. 1C) and protein (>95% by immunofluorescence) by 36 hours after transduction (Fig. 1D). We then transplanted Cre-transduced, or control mCherry-transduced, or nontransduced cells into the brain of adult C57/Bl6 mice, 36 hours after viral transduction (Fig. 2). Control mCherry-transduced and nontransduced cells caused the development of tumors (17/20 mice, 85%; of which 8/10 mCherry, 9/10 nontransduced), resulting in an overall median survival of the control mice of 40 days, consistent with previous reports with similar tumor-derived cells (Fig. 2B; Table 1; ref. 23). However, mice injected with Cre-transduced cells were almost all alive (13 of 17; 76.5%) at day 118 after transduction (Fig. 2B; log-rank test, $P < 10^{-4}$). When sacrificed and analyzed at day 121 ± 3, these mice were found tumor-free. Analysis of the 4 mice injected with Cre-transduced cells that had died (2 by day 50, 1 on day 64, 1 on day 117; Table 1) showed that they had developed tumors that demonstrated a nondeleted status of the *Sox2*^{flox} gene upon genotyping (not shown), quantitative reverse transcription (qRT)-PCR (Supplementary

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Table 1. Transplanted animals and observed tumors

Transplanted cells	Lethal tumors/ transplanted animals
NT	9/10 (90%)
Lenti-mCherry	8/10 (80%)
Lenti-Sox2	5/5 (100%)
Lenti-Sox2; Lenti-CRE	6/8 (75%)
Lenti-CRE	4[a]/17[b] (23.5%)

[a]The four tumors were tested by PCR for the presence of the undeleted and deleted *Sox2* locus; all four were Sox2-positive (undeleted Sox2); one also presented a band for the deleted *Sox2* locus, indicating that some Sox2 deleted cells are part of the tumor mass.
[b]The 13 surviving mice were tumor-free at day 120.

Fig. S1), and immunofluorescence (Fig. 2C), indicating their likely origin from the few non–Sox2-deleted cells.

To address whether the loss of TIC properties was specifically due to loss of Sox2, rather than to nonspecific effects (Cre-toxicity, etc.) we performed a control experiment. Before Cre transduction, we transduced the tumor-derived cells with a Sox2-encoding lentivirus (Fig. 2A and B; ref. 13). After subsequent ablation of endogenous Sox2 by Cre (leading to loss of endogenous Sox2 mRNA, as verified with specific primers, see Supplementary Materials), we transplanted the cells into host mouse brains and compared their survival with that of controls, i.e., cells transduced with Sox2 virus but not with Cre, or with the control mCherry virus, or untransduced (Fig. 2B). Sox2-transduced cells demonstrated tumorigenic ability similar to that of controls, with tumors developing in 6 of 8 mice (75%) within 120 days and a median survival of 34 days (Fig. 2B).

We conclude that the tumor-initiating ability of PDGF-B–induced oligodendroglioma cells requires Sox2 function.

Consequences of Sox2 deletion on *in vitro* growth of pHGG cells

To obtain information on the mechanisms of loss of tumor-initiating ability of the Sox2-deleted pHGG cells, we studied the *in vitro* growth of intact or Sox2-deleted cells (Figs. 3 and 4). We tested cells in three growth conditions: the first one optimized for maintenance of stem cell properties, and corresponding to the initial condition in which the cultures had been derived (with EGF and bFGF and without serum: +EGF/bFGF); a second one in the same medium with no added growth factors (no factors); and a third one in medium without added factors, but with 2% serum (no factors + serum). The latter represents "differentiating" conditions normally used to obtain terminal differentiation of normal NSC (neurospheres; refs. 1, 25, 28, 29). Cells were plated at clonal density (96 hours after viral transduction) and scored for clone numbers after 7 days. In EGF/bFGF, the number of clones obtained with Sox2-deleted cells was only slightly, although significantly, decreased compared with undeleted controls; however, when plated without factors, Sox2-deleted cells were reduced to less than 50% of controls (Fig. 3A). Cells plated in serum adhered to the substrate and did not form individualized clones. We thus plated the cells, 96 hours after transduction, in 2% serum-containing medium without added factors, at nonclonal density, on Matrigel, allowing more efficient growth. Under these conditions, untreated pHGG cells continue to grow, although to a rate somewhat lower (20%–30% increase in duplication time) than in medium with added growth factors. Following Sox2 deletion, the total cell number at day 7 was strongly reduced relative to controls (<20% that obtained with untransduced or mCherry transduced cells; Fig. 3B). We also assessed proliferative ability (at day 2 and 7) by administering EdU for 30 minutes and measuring the percentage of cells that incorporated EdU (Fig. 4A). While controls (mCherry transduced or nontransduced) cells had similar high levels of EdU incorporation (30%–35%), Sox2-deleted cells showed significant reduction of EdU incorporation (to about 10%; Fig. 4A). We then evaluated apoptosis by the TUNEL assay (Fig. 4B). TUNEL-positive cells were more than 4-fold increased following Sox2 deletion relative to controls (Fig. 4B). Immunofluorescence for differentiation markers of oligodendroglia, O4 and GalC, and astroglia, GFAP, revealed widespread positivity, together with an altered morphology of Sox2-deleted cells, with features suggestive of aberrant differentiation (branching, flattening), as compared with the relatively undifferentiated morphology of undeleted pHGG cells (Fig. 4C). We conclude that in "differentiating" growth conditions, Sox2 ablation leads to progressive exhaustion of *in vitro* cell proliferation, increased apoptotic cell death, and morphologic changes, suggesting aberrant differentiation.

Sox2 deletion causes alterations in the gene expression program of pHGG cells

The dependence on Sox2 of tumor-initiating properties of oligodendroglioma cells raises the hypothesis that Sox2 may act by regulating the transcription of critical downstream genes. We analyzed gene expression in Sox2-deleted and control cells (nontransduced, m-Cherry virus-transduced) by microarray analysis, at 40 and 96 hours following Cre transduction (Fig. 5A; Supplementary Table S1). The gene expression profile of $Sox2^{flox/flox}$ pHGG cells closely matched the "oligodendroblast" program that we previously reported for several independent PDGF-B–induced oligodendrogliomas (Fig. 5B; refs. 22, 23). At 96 hours, the expression of 146 genes was substantially deregulated (more that 2-fold); at 40 hours, few, if any, gene changed its expression level significantly (Fig. 5A). This suggests that our analysis at 96 hours likely includes the earliest changes in gene expression that follow Sox2 loss, presumably including those genes that directly rely on Sox2. Following Sox2 ablation, 12 genes are downregulated, compatibly with an activator function of Sox2; 134 genes are upregulated (Fig. 5A and D), possibly reflecting a repressor function of Sox2 (25), or indirect effects. Gene ontology analysis of the deregulated genes indicated a significant enrichment in the functional categories of

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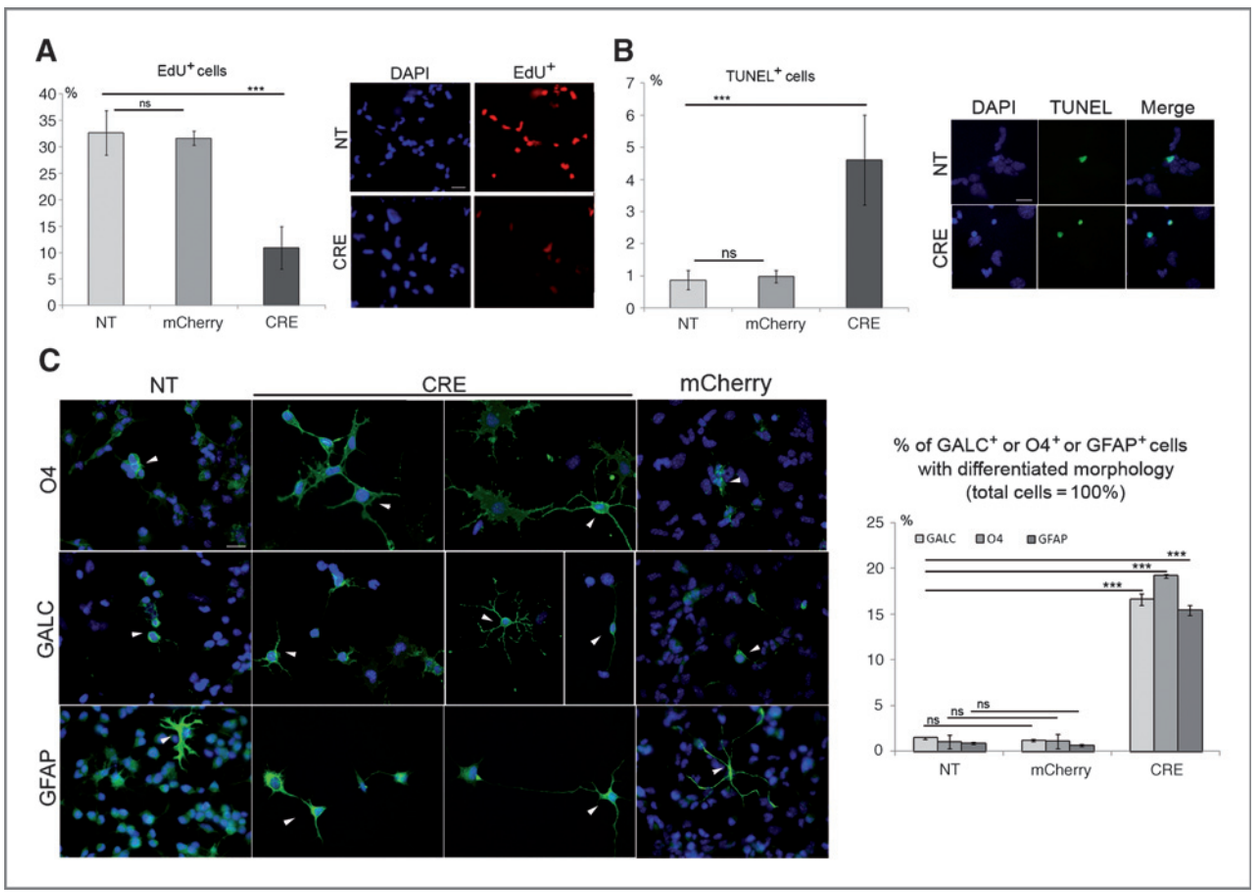

Figure 4. *In vitro* effects of *Sox2*$^{flox/flox}$ deletion on pHGG cells. A, EdU incorporation obtained following transduction with control (mCherry) or Cre virus after 2 days in serum-containing medium. Histograms report the percentage of EdU-positive nuclei over the total number of (DAPI-positive) nuclei. Representative images showing EdU-positive cells are shown besides the histograms (scale bar, 20 μm). B, TUNEL analysis of cells 2 days after transduction with Cre or control mCherry virus. Representative images are shown, with TUNEL-positive nuclei in green (scale bar, 20 μm). C, immunofluorescence with antibodies against O4, GalC, or GFAP (green) of cells transduced with control mCherry or Cre virus, or untransduced (NT), after 7 days in culture in serum-containing medium (scale bar, 20 μm). The results in A–C are the average of $n = 2$ experiments performed in duplicate, with more than 700 cells per sample counted (A and B, ***, $P < 0.0001$; Fisher test; C, ***, $P < 0.001$; ns, nonsignificant; $P > 0.05$, two-way ANOVA). Arrowheads, some examples of cells with "differentiated" morphology.

Developmental Processes, Response to Stimulus, Cell Proliferation, Communication and Signaling, and Cell Differentiation (Fig. 5C).

"Vaccination" with SOX2 peptides significantly prolongs survival and induces specific antitumor effector response

The requirement for Sox2 by oligodendroglioma CSC raises the possibility that Sox2 itself may qualify as a target for CSC-directed therapeutic strategies. While Sox2 is highly expressed in oligodendroglioma-initiating cells, its expression in the normal brain is very limited. This led us to try an immunotherapy approach, to "vaccinate" immunocompetent mice after the transplantation of TICs (Fig. 6). After cell transplantation into the brain, we administered the mice 4 SOX2 peptides, including one previously shown to elicit T-cell activation against Sox2-expressing glioblastoma cells *in vitro* (26). Vaccination with peptides was performed on days 14, 21, and 28 (Fig. 6A). Mice also received temozolomide (five daily injections on days 10–14, alone or in combination with peptide vaccination) as an immunologic adjuvant for enhancing immunogenicity of tumor cells (Fig. 6A; ref. 30). Peptide vaccination alone significantly increased survival time, and combined temozolomide and peptide treatment doubled mice survival, as compared with vehicle-treated and non-specific OVA peptide-treated control mice (Fig. 6B). The tumors that eventually developed in vaccinated mice were widely SOX2-positive by immunohistochemistry (Fig. 6C). These observations indicate that Sox2 requirement by oligodendroglioma CSC may be potentially relevant from a therapeutic perspective.

To examine the direct effects of peptide vaccination on T-cell activation, splenocytes, and infiltrating lymphocytes from freshly harvested tissues of immunized and control mice were characterized by flow cytometry. The frequency of $CD8^+$ (Fig. 6D) and $CD4^+$ T cells (Supplementary Fig. S2) in spleens and tumor-infiltrated brains increased significantly in immunized mice compared with vehicle-treated controls. We also investigated whether pHGG-specific effector cells

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were generated in response to SOX2 peptide vaccination. Prestimulated splenocytes were assayed for *in vitro* cytotoxic activity against pHGG cells or NIH 3T3 cells (negative control) using a cytotoxicity MTT assay. The splenocytes from immunized mice, but not from vehicle-treated mice, displayed cytotoxic activity against tumor cells (Fig. 6E). The specificity of the effector immune response was confirmed by the absence of cytotoxicity against NIH 3T3 cells (Fig. 6E).

Discussion

We report that Sox2 is required by oligodendroglioma stem cells to reinitiate tumor development within the transplanted mouse brain, and, in some conditions, for *in vitro* growth. Cells cultured from PDGF-B–induced mouse oligodendroglioma will reform a lethal tumor following transplantation in mouse brain; however, the majority of animals transplanted with Sox2-deleted cells remain tumor-free. Transduction of Sox2-deleted tumor cells with a Sox2-expressing lentivirus maintains tumor-initiating capacity, confirming that this is dependent on Sox2 activity. Finally, vaccination against Sox2 significantly delays tumor development, pointing to Sox2 (and its downstream targets) as a potential therapeutic target.

In adult mouse, Sox2 is expressed only in a minority of cells, mainly stem/progenitor cells within various tissues (8). In contrast, Sox2 is expressed in many tumor types, both in the brain and in other organs (mammary gland, lung, esophagus, bone; refs. 31–33).

In the majority of these tumors, Sox2 is not primarily altered/mutated, with the exception of its amplification in lung and esophageal squamous cell carcinoma (31). Sox2 deregulation is, in rare cases, the immediate downstream consequence of the primary lesion (34); more frequently, it is part of the altered transcriptional program of the tumor. As Sox2 is important for pluripotency and for reprogramming, these observations suggests an analogy between the role of Sox2 in CSC and in the normal development of stem cells (6).

Sox2 is required for the propagation of CSC in oligodendroglioma

In our oligodendroglioma model, Sox2 is necessary for the maintenance of CSC, in agreement with its requirement in normal NSC (13). Previous work showed that in human glioblastoma-derived cell lines, Sox2 downregulation by shRNAs impaired tumorigenesis following transplantation (35). In other patient-derived glioblastoma cells, Sox2 was described to be downstream to Sox4 in a TGF-β signaling-dependent tumorigenicity pathway and was required for *in vitro* maintenance of tumorigenic cells although its *in vivo* requirement was not tested (36). Interestingly, TGF-β promotes proliferation of tumors, including gliomas and osteosarcomas, through induction of PDGF-B (37).

While our results agree with those of Gangemi and colleagues (35), in that both glioblastoma and oligodendroglioma require Sox2 for *in vivo* tumorigenicity, some important differences should be noted. Sox2 ablation in glioblastoma (by shRNA) causes significant loss of cell proliferation *in vitro*, in media with added growth factors; instead, in oligodendroglioma, we noted only a small decrease in the presence of added growth factors in the proliferation of Sox2-ablated cells, both in clonal tests (Fig. 3) and in mass culture (not shown). However, omission of growth factors, and particularly combined addition of serum, a condition favoring NSC differentiation *in vitro* (25, 28, 29), strongly decreased Sox2-ablated oligodendroglioma cell proliferation, increased cell death, and caused important morphologic changes. These culture conditions might mimic conditions more similar to those encountered by tumor cells in the brain, with absence of abundant amounts of EGF/bFGF and presence of various cytokines and factors. Sox2-deleted cells showed morphologic changes (branching and flattening), together with marked positivity for differentiation markers (Fig. 3). Immunopositivity for oligodendrocyte differentiation markers was also observed *in vivo* within the very small number of Sox2-deleted tumor cells found 10 days after transplantation (Supplementary Fig. S3). "Priming" by Sox2 of "differentiation" genes in NSCs was reported (38), and NSCs expressing reduced levels of Sox2 (from mouse hypomorphic mutants) showed morphologic and gene expression abnormalities when induced to differentiation (25). Thus, as in normal NSCs (13, 25), Sox2 may be required in pHGGs in the presence of differentiation stimuli to prevent abnormal differentiation and apoptosis. Prodifferentiative stimuli (bone morphogenetic proteins, BMP, especially BMP4) efficiently antagonize glioblastoma development in mice (39) and targeting of molecules maintaining an undifferentiated state, such as EphA2 receptor, induced differentiation and loss of tumor-initiating capacity in mouse glioblastoma (40). Also in pHGGs, we previously documented a correlation between loss of tumor-initiating ability (following Pax6 overexpression) and the acquisition of differentiated features (41).

Overall, these results suggest that mechanisms causing tumor cell loss after Sox2 ablation may differ between different tumors (glioblastoma and oligodendroglioma), pointing to multiple molecular mechanisms of action of Sox2 in these cells.

On the other hand, Sox2 expression in tumor cells does not always correlate with a strict functional requirement for tumorigenesis. Sox2 is expressed in medulloblastoma, a cerebellar tumor most frequent in childhood; medulloblastoma CSCs express Sox2 in humans, and in mouse models (3, 42, 43). A class of medulloblastomas is associated with mutations activating the SHH pathway; these include SmoM2, a mutation in the SHH-receptor Smo leading to its constitutive activation; in mice, Cre-mediated activation of a SmoM2 transgene leads to medulloblastoma development (44). In these mice, we concomitantly deleted Sox2 ($Sox2^{flox}$) by Cre; yet, Sox2-negative medulloblastoma still developed (42). The discrepancy with our present work might be explained in several ways. First, Sox2 might act upstream to Smo signaling; indeed, Sox2 was found to activate SHH expression in NSCs and neural cells (13, 45). Second, the close homolog Sox3 is expressed in medulloblastoma and might act redundantly with Sox2 in CSC maintenance (42). Third, in this system, SmoM2-induced medulloblastoma development *in vivo* may likely arise from multiple SmoM2-expressing cells

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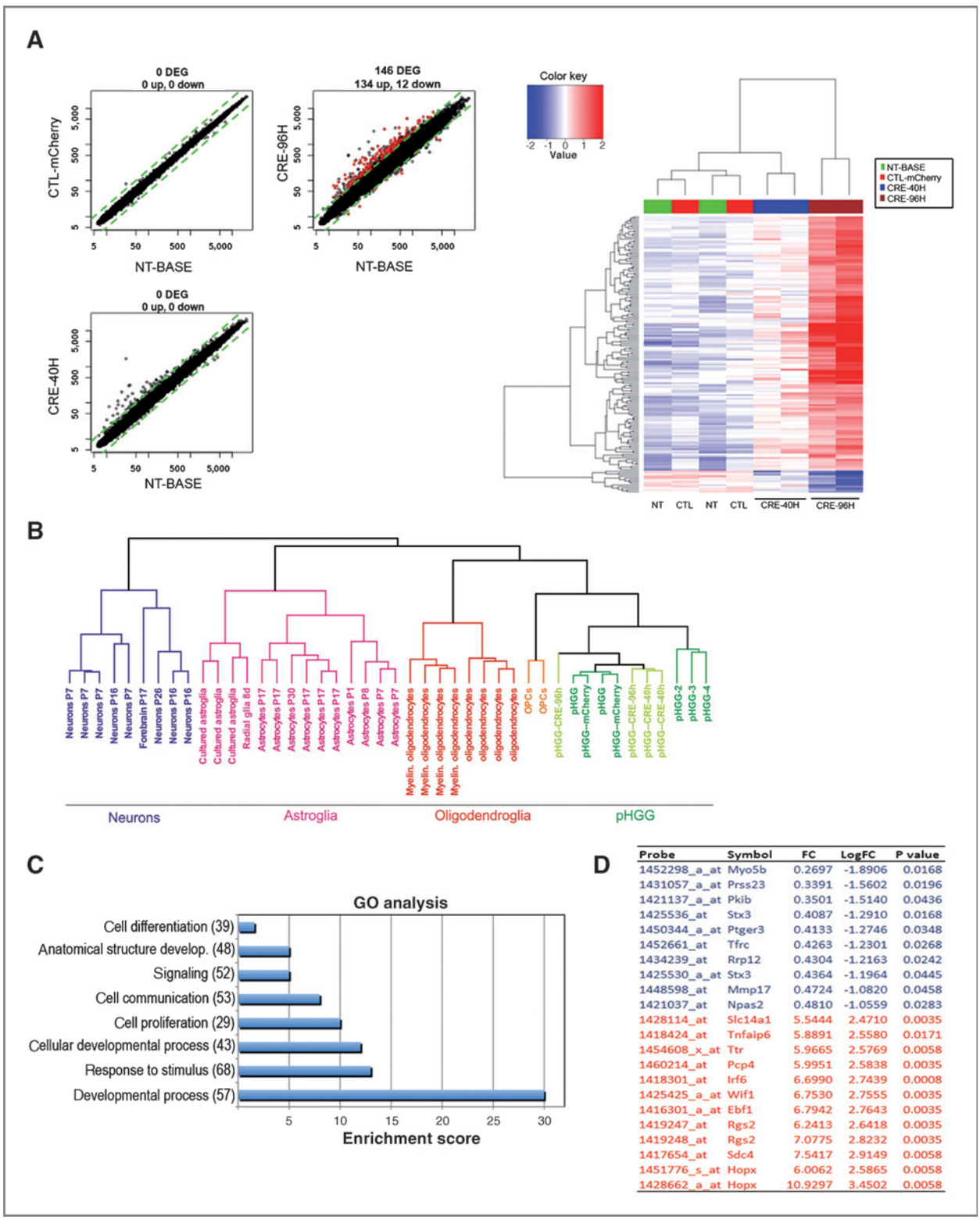

Probe	Symbol	FC	LogFC	P value
1452298_a_at	Myo5b	0.2697	-1.8906	0.0168
1431057_a_at	Prss23	0.3391	-1.5602	0.0196
1421137_a_at	Pkib	0.3501	-1.5140	0.0436
1425536_at	Stx3	0.4087	-1.2910	0.0168
1450344_a_at	Ptger3	0.4133	-1.2746	0.0348
1452661_at	Tfrc	0.4263	-1.2301	0.0268
1434239_at	Rrp12	0.4304	-1.2163	0.0242
1425530_a_at	Stx3	0.4364	-1.1964	0.0445
1448598_at	Mmp17	0.4724	-1.0820	0.0458
1421037_at	Npas2	0.4810	-1.0559	0.0283
1428114_at	Slc14a1	5.5444	2.4710	0.0035
1418424_at	Tnfaip6	5.8891	2.5580	0.0171
1454608_x_at	Ttr	5.9665	2.5769	0.0058
1460214_at	Pcp4	5.9951	2.5838	0.0035
1418301_at	Irf6	6.6990	2.7439	0.0008
1425425_a_at	Wif1	6.7530	2.7555	0.0035
1416301_a_at	Ebf1	6.7942	2.7643	0.0035
1419247_at	Rgs2	6.2413	2.6418	0.0035
1419248_at	Rgs2	7.0775	2.8232	0.0035
1417654_at	Sdc4	7.5417	2.9149	0.0058
1451776_s_at	Hopx	6.0062	2.5865	0.0058
1428662_a_at	Hopx	10.9297	3.4502	0.0058

Figure 5. Gene expression analysis identifies an early transcriptional response to Sox2 deletion in oligodendroglioma stem cells. A, left, scatter plots for differentially expressed genes (DEG) identified by pairwise comparisons between the normalized probe expression values for untransduced cells (NT-BASE), control mCherry virus-transduced cells (CTL-mCherry), and for Cre-transduced cells at 40 (CRE-40H) and 96 hours (CRE-96H) after transduction, with the fold change threshold of 2 (red dots). (*Continued on the following page.*)

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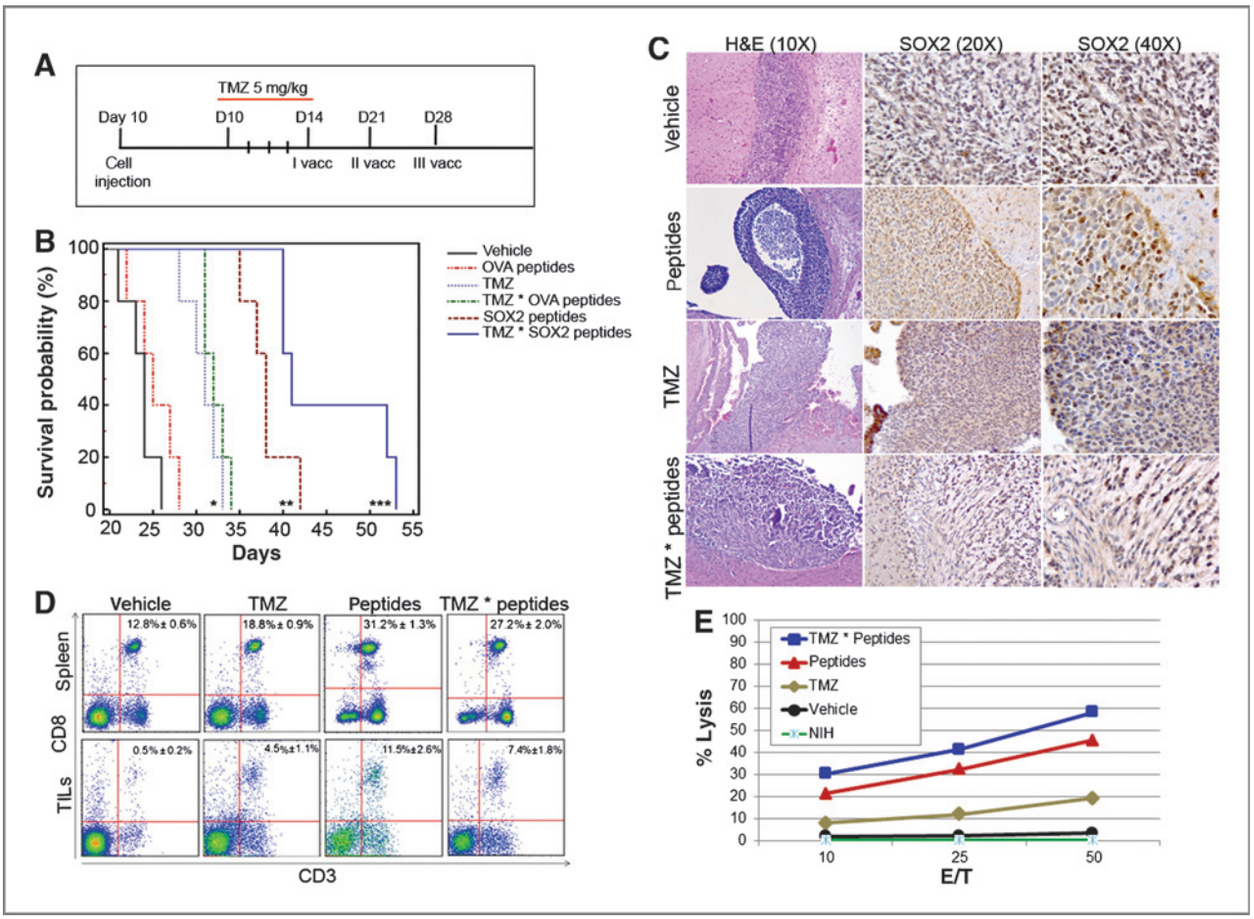

Figure 6. Vaccination with Sox2 peptides causes a significant delay in tumor development and lethality following transplantation. A, schedule of Sox2 peptide and temozolomide (TMZ) administration and cell transplantation. Peptide vaccinations (vacc) were on day 14, 21, 28; temozolomide: five daily injections on days 10 to 14. B, Kaplan–Meier survival curves for mice treated with: vehicle ($n = 5$, mean ± SD, 23.6 ± 1.8; median, 24); OVA peptides ($n = 5$; mean ± SD, 25.2 ± 0.9; median, 25); temozolomide ($n = 5$; mean ± SD, 30.8 ± 1.9; median, 31); temozolomide + OVA peptides ($n = 5$; mean ± SD, 32.2 ± 0.5; median, 32); Sox2 peptides ($n = 5$; mean ± SD, 38.0 ± 2.5; median, 38); temozolomide + SOX2 peptides ($n = 5$; mean ± SD, 45.2 ± 6.7; median, 41; *, $P < 0.001$; **, $P < 0.005$; ***, $P < 0.001$ temozolomide + SOX2 peptides vs. vehicle or OVA peptides). C, hematoxylin and eosin (H&E) staining and Sox2 immunohistochemistry (brown) of sections from tumors obtained after the indicated treatments. D, flow cytometry on splenocytes (top) and TILs (bottom; $n = 4$ mice per group; data reported in dot plots as the mean% ± SD; $P = 0.0003$ and $P = 0.003$ for temozolomide +SOX2 peptides versus vehicle in splenocytes and TIL, respectively). E, *in vitro* MTT cytotoxicity assay performed using splenocytes from mice treated with SOX2 peptide with or without temozolomide, temozolomide, and vehicle as effector cells and pHGG or NIH 3T3 cells as target using different effector:target (E:T) ratios (10:1, 25:1, and 50:1).

and it is possible that additional mutations in a subset of these cells allow to bypass Sox2 requirement. Finally, although Sox2 activity was not strictly required for medulloblastoma development, experimental increase of Sox2 levels was found to correspondingly affect medulloblastoma cell proliferation (42).

The differences between neural tumors with respect to the degree of their Sox2 requirement are reminiscent of the

(*Continued.*) Data represent the mean of probe expression values of the replicates samples in the considered condition. Right, heatmap diagram of gene expression changes (red, increased expression; blue, reduced expression) in Cre-treated cells, as compared with the indicated controls. Probesets (rows) and samples (columns) are clustered on the basis of their similarity by hierarchical clustering using complete linkage (Euclidean distance). The top dendrogram (*x*-axis) indicates the pairwise comparisons between the cell types identified by the different colors. NT, nontransduced cells; CTL, control mCherry-transduced cells; CRE40 and CRE96, Cre-transduced cells at 40 and 96 hours after transduction. B, dendrogram representation of the results of the hierarchical clustering analysis between the gene expression profiles of our pHGG cells (pHGG = untransduced, pHGG-mCherry or pHGG-Cre-transduced), and previously analyzed pHGGs (pHGG-2, 3, 4; ref. 22), as well as neurons, astroglia, oligodendroglia, and OPC gene expression profiles as described in ref. 22. C, analysis of Gene Ontology (GO) biologic processes enriched in DEGs. The most representative GO functional annotations for DEGs from each experimental condition are identified by determining the probability of random occurrence of functional terms (hyper geometric distribution). On the basis of this probability ranking, only the top eight statistically most significant annotation terms are reported. The enrichment scores identify the functional categories that are overrepresented. Enrichment scores <6 indicates enrichment *P* values of 10^{-6}, scores between 5 and 13 *P* values of 10^{-7}, scores >15 *P* values of 10^{-8}. D, list of the 10 top-down (blue) and top-upregulated (red) genes following Sox2 deletion. FC, fold change as compared with undeleted cells.

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differences in Sox2 requirement between different regions of the normal, developing nervous system. Sox2 is expressed ubiquitously in neural stem/progenitor cells, yet its deletion *in vivo* has region- and stage-specific effects in the brain (hippocampus, ventral telencephalon; refs. 13, 46). These observations point to specificities in the downstream gene expression networks controlled by Sox2 in tumorigenic as well as in normal neural (stem) cells.

Oligodendrogliomas may arise within the committed oligodendrocyte lineage, by "reprogramming" to a CSC state. Oligodendrocyte precursor cells (OPC) can be "reprogrammed" to a neural stem-like state, by sequential treatment with PDGF and EGF, and this process requires Sox2 reactivation (47). A future in-depth molecular investigation of Sox2 function in our model system may uncover if Sox2 regulates genes critical for reprogramming committed cells to a stem cell status, acting as a pioneer factor in ways related to its action in iPS cell generation (6).

Sox2 as a potential therapeutic target

The requirement for Sox2 by CSC raises the possibility that Sox2 itself may qualify as a target for therapeutic intervention. Targeting CSC may be a strategy to increase the potential efficacy of immunotherapy (27). Sox2 vaccination significantly prolongs survival enhancing systemic and local immune response (Fig. 6). Sox2 is localized in the nucleus, and is thus not, *a priori*, the most accessible molecule to target. However, recent data suggest that intracellular oncoproteins can be targeted by vaccination, as some intracellular antigens may be released and expressed on the surface of cancer cells (48). Antibodies and T-cell immune responses against SOX2 have been detected in patients with monoclonal gammopathy (MGUS), a premalignant condition to myeloma, where Sox2 expression marks the clonogenic compartment (49), and, recently, in about 50% of patients with non–small cell lung carcinoma (NSCLC; ref. 50). Cellular anti-SOX2 immunity inhibited the growth of MGUS cells *in vitro* and the presence of anti-SOX2 T cells predicted favorable clinical outcome (49); in NSCLC, T-cell response against SOX2 was associated with NSCLC regression upon immunotherapy with anti-PD-1 antibodies (50). These observations suggest that the immune system may be able to "discover" tumor-associated SOX2. Furthermore, an immune reaction by T cells elicited by SOX2-derived peptides (one of which was used here) was reported to lyse human glioblastoma-derived cells in culture (26). Finally, we previously found that vaccination against GLAST, a protein retaining significant expression in the adult brain, elicited an immune reaction specifically targeted to the tumor, not damaging the surrounding tissue (27).

The fact that late-arising tumors that eventually developed in vaccinated animals were widely Sox2-positive (Fig. 6) is consistent with the hypothesis of a failure of the immune system to completely eradicate Sox2-positive tumor cells, rather than with escape mechanisms developed by the tumor, allowing it to develop without Sox2. Collectively, these observations suggest that targeting Sox2-expressing cells may provide a basis for therapeutic approaches. Complementing Sox2 immunotherapy with action directed against some downstream Sox2 targets in oligodendroglioma might further increase the efficacy of this approach.

The observations about Sox2 requirement in neural tumors are extended by the reported requirement for Sox2 in a wider sample of tumor types. These include tumors of the osteoblast lineage, as shown in osteosarcoma cell lines (32); here, Sox2 is required also in the normal tissue stem cell counterpart, osteoblast stem/progenitor cells (10), as seen with neural cells. CSC from mammary tumors cultured as tumorigenic "mammospheres" express Sox2 and Sox2 knockdown impairs mammosphere formation and delays tumor formation following transplantation (33).

We conclude that targeting Sox2, likely in combination with selected downstream targets, may provide an effective strategy to antagonize the development of oligodendroglioma, and, perhaps, other tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Favaro, S. Ottolenghi, P. Malatesta, S.K. Nicolis
Development of methodology: R. Favaro, G. Finocchiaro, S.K. Nicolis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Favaro, I. Appolloni, S. Pellegatta, A.B. Sanga, P. Pagella, E. Gambini, F. Pisati, M. Foti, G. Finocchiaro, P. Malatesta, S.K. Nicolis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Favaro, I. Appolloni, A.B. Sanga, M. Foti, G. Finocchiaro, P. Malatesta, S.K. Nicolis
Writing, review, and/or revision of the manuscript: R. Favaro, I. Appolloni, S. Pellegatta, S. Ottolenghi, M. Foti, G. Finocchiaro, P. Malatesta, S.K. Nicolis
Study supervision: S.K. Nicolis

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Sox2 Is Required to Maintain Cancer Stem Cells in a Mouse Model of High-Grade Oligodendroglioma

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