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Study of the role of the Sox2 transcription factor in neural and mammary cancer stem cells using Sox2 conditional knock-out in mouse

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GENERAL INTRODUCTION

1. Stem cells and cancer

Stem cells are defined as "cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation" [1]. Stem cells proliferation and differentiation are strictly regulated by the surrounding molecular and cellular environment, the stem cell niche. Stem cells constitute the basis of development and, in adult life, are responsible for tissue homeostasis and repair. Adult tissues contain cells at different stages of differentiation and self-renewal ability. The vast majority of tissues are constituted of differentiated cells, which permit the functioning of the tissue and the organs. This population is sustained by stem cells. Under precise stimuli, stem cells, usually quiescent, re-enter the cell cycle and undergo an asymmetric division: each stem cell gives rise to a stem cell (self-renewal) and a more differentiated cell (differentiation), called progenitor. The progenitor is still an undifferentiated cell; however, it has limited self-renewal ability and it has lost a certain degree of potency. The progenitors, duplicate several times and give rise to terminally differentiated cells. In this way, only the progenitor compartment undergoes massive proliferation, avoiding replication-associated accumulation of mutations in the stem cell compartment. Under different conditions and in response to stress, stem cells can also undergo symmetric divisions; in this case, a stem cell can give rise to two stem cells or two progenitors.

Tumors can be described as aberrant organs originated from a tumor-initiating cell [1]. Unlike normal tissues, tumors are composed of indefinitely proliferating cells. Normal cells, with the partial exception of stem cells, undergo replicative senescence after some divisions (Hayflick limit). Only stem cells have the ability to proliferate indefinitely. This observation leads to the hypothesis that tumor cells may utilize the machinery physiologically used by stem cells to self-renew [1]. Some hints in this direction have already been suggested by a number of studies.

Tumor cells differ significantly from the normal cells that compose the hit tissue, even though they retain a "molecular signature" of the cell that originated the tumor. Moreover, cells within the same tumoral mass differ significantly in their ability to proliferate and to reform a tumor after transplantation [1].

1.1. The stochastic model

The stochastic model describes the tumor as an heterogeneous tissue, composed of highly proliferating, dedifferentiated and tumorigenic cells. Different cells within the tumor can present different proliferation rates and phenotypes, but there is no clear hierarchy; there are no progenitors in tumors, and all cells can give rise to a new tumor.

1.2. The hierarchical model

The hierarchical model focuses the attention on the differences among tumor cells. The tumor is described as an aberrant organ, in which both stem cells and progenitors exist and sustain the growth of the tumor. In this case, the majority of the cells that compose a cancer mass are the ones that proliferate faster; however, they are not able to reform a tumor after transplantation, mainly because they lack self-renewal ability. Therefore, they are exhausted after a limited number of duplications. In order to expand, the tumor needs continuous sustenance from the cancer stem cell-like compartment.

The hierarchical model is sustained by the observation that different populations within the tumor have different efficiency in reforming a tumor after transplantation, and they are characterized by different markers. Cancer stem cells – or tumor initiating cells – have been identified in many classes of tumors, comprising breast cancer, lung cancer and different types of brain cancers [2]. In the classic model of tumorigenesis, any kind of cell could in theory be subject to transformation. The existence of a cancer stem cell has highlighted the question of the cell of origin of cancers. The presence of stem-like cells in tumors has led to the hypothesis that stem cells could be the ideal target for oncogenic mutations. This hypothesis is further suggested by the frequent similarity between markers expressed on normal stem cells and tumor initiating cells.

2. Therapeutic implications of the cancer stem cell hypothesis

The "cancer stem cell" hypothesis could have a significant impact on anti-tumoral drug development. Drugs and other treatments currently used against cancer, such as chemotherapy and radiotherapy, have been developed in accordance with the classical, stochastic model which targets all cancer cells indiscriminately. They clearly exert severe collateral effects on normal proliferating cells. For this reason, these drugs must be used at the lowest efficient dose; moreover, they have to be used in an intermittent way, in order to allow normal tissues to recover. These two limitations often cause tumors to become resistant to chemotherapy and radiotherapy.

Targeting cancer stem cells could represent a major advancement in cancer treatment. The ablation of cancer stem cells would make the tumor incapable of generating new cells. After the complete loss of the stem cell compartment, the tumor would probably go on growing for a short period, sustained by the last divisions of non-stem neoplastic cells; however, these cells would start entering replicative senescence or, in any case, they would exhaust their replicative potential [1]. This would lead to the spontaneous degeneration of the tumor, without using the drugs discussed above.

In the first place, it is fundamental to identify and characterize cancer stem cells in all classes of tumors. Once cancer stem cells have been identified, it is then necessary to find new molecular targets, whose inactivation – or overactivation – would inhibit cancer stem cells functioning. This doesn't necessary mean killing cancer stem cells; for example, it

would be sufficient to induce their differentiation or to eliminate their self-renewal ability. However, it is also necessary to understand the differences between cancer stem cells and normal stem cells or progenitors [3], in order to selectively target cancer stem cells and avoid severe side effects. In addition, it will be necessary to develop new means to delivery drugs inside cancer stem cells without them being expelled by the transport systems specifically expressed by stem cells.

3. Sox2

Sox2 is a member of the Sox (SRY-related HMG box) gene family that encodes transcription factors characterized by a single HMG DNA binding domain. Sox2 belongs to the B1 subgroup, comprising also Sox1 and Sox3, based on homology inside and outside the HMG domain [4]. Sox2 is required for stem cell maintenance, functionality and differentiation during both development and adult life in the nervous system; moreover, it has been recently demonstrated that Sox2 is expressed in a wide variety of tumors and can correlates with high malignancy and poor prognosis.

3.1. Roles of Sox2 in normal development and differentiation

Sox2, in mouse, is expressed both during development and during adult life, in different types of highly undifferentiated cells [5]. In earlier development, Sox2 is expressed by pluripotent embryonic stem cells of the inner cell mass [5]. Later in development, Sox2 expression is restricted to the developing nervous system, mainly in the most undifferentiated precursors [5]. In adult life, Sox2 is mainly expressed in neural stem cells. Sox2 positive cells, isolated from both the developing nervous system and the adult neurogenic regions and cultured under appropriate conditions, are able to grow as neurospheres [5]. These cells are able to self-renew indefinitely in culture, and they are able to differentiate to all neural lineages - neurons, astrocytes and oligodendrocytes [5].

Sox2 requirement in different types of stem cells has been addressed mainly by knock-out experiments. The generation of Sox2-null mutants showed that Sox2 is strictly required for the maintenance of the pluripotent stem cells of the epiblast [6]; Sox2 knock-out impairs the formation of the blastocyst, inducing cells from the inner cell mass to differentiate into trophoblastic cells. Sox2 knock-out is therefore embryonic lethal [6]. On these basis it was then demonstrated that Sox2 acts together with Oct4, Nanog and N-myc to maintain pluripotency in embryonic stem cells; this four genes are able to induce pluripotency in terminally differentiated cells [7], reprogramming them to induced pluripotent stem cells (iPSCs). Given the embryonic lethality of the Sox2 complete knock-out, Sox2 roles in later development and in stem cell physiology have been investigated using a conditional knock-out mouse model.

Sox2 conditional knock-out experiments have shown that Sox2 is necessary for neural stem cell maintenance *in vitro* (in neurosphere cultures) and *in vivo* (in the dentate gyrus of the hippocampus), and its action is partially mediated by the direct regulation of the

Shh-pathway [8]. Sox2 knock-out *in vivo* in mouse brain at embryonic day 12.5 (E12.5) – by a Nestin-Cre transgene – results in loss of hippocampal neural stem cells and neurogenesis, and this phenotype is partially rescued by a Shh pharmacological agonist [8]. Sox2 knock-out on neurosphere cultures induces complete exhaustion of the culture after a few passages [8]. These data indicate Sox2 as a fundamental gene for neural stem cell maintenance and physiology; its key role in reprogramming and dedifferentiation has attracted attention on its potential role in tumorigenesis, notably in cancer stem cells.

3.2. Sox2 and cancer

It is now known that Sox2 is expressed in the majority of tumors, with some exceptions. It has been demonstrated that Sox2 is an amplified oncogene in lung and esophageal squamous cell carcinomas [9]; stimulates proliferation and has an oncogenic role also in breast cancer [10,11]; and is aberrantly expressed in neural tumors, such as gliomas and medulloblastoma [12-14].

The combination of Sox2 role in stem cell maintenance and reprogramming, and its diffuse overexpression in cancer make Sox2 an extremely interesting target for cancer stem cell-directed therapies. Stem cell-like populations represent an extremely variable fraction of the overall tumor mass in different classes of tumors. Independently of their abundance, the presence of Sox2 positive cells in tumors arising from Sox2 negative tissues could indicate a certain degree of dedifferentiation that could be dependent on Sox2 reactivation. After birth, Sox2 is expressed in limited stem cell populations, notably neural stem cells. Targeting Sox2 positive cells would probably exert much less severe side effects that targeting indiscriminately all proliferating cells, the targets of most therapies. Moreover, it could selectively eliminate cancer stem cells, which are thought to be responsible for the sustenance of tumor growth, the formation of metastasis and tumor relapse; as already said, these cells are often resistant to current anti-tumoral treatments. These findings and observations indicate Sox2 as a promising stem-cell specific target for therapy.

4. Neural tumors and stem cells

Normal neural stem cells are found in different areas of the brain, mainly the subventricular zone and the dentate gyrus of the hippocampus [14]. Neural stem cells are defined functionally: they are able to self-renew and to give rise, by differentiation, to neurons, astroglia and oligodendroglia within the clonal progeny of a single cell [15]. When grown in non-differentiating conditions, they proliferate extensively.

Cancer stem cells are able to self-renew, to proliferate indefinitely and to differentiate. Multipotency is not necessary, given that some tumors show just a single differentiation lineage [14]. Although cancer stem cells show many similarities with neural stem cells, there are also significant differences between them, and, notably, between cancer stem cells isolated from different tumors. Neural stem cells are extremely rare in the nervous

system (< 1% of total cell population), while cancer stem cells can represent 1-25% of the overall tumoral mass [16], and they are extremely dependent on the context. Moreover, cancer stem cells can resemble neural stem cells in different proportions, depending on the tumor and, notably, on the cell of origin of that class of cancer [14].

4.1. PDGF and oligodendroglioma

The platelet-derived growth factor receptor (PDGFR) is expressed during mouse embryo development, by neuroepithelial cells at E8.5 [17]. Later in development, PDGF is known to function as oligodendrocyte precursor cells mitogen. PDGF is important for the regulation of oligodendrocyte precursor number and for oligodendrocyte production *in vivo* [18,19]. While most oligodendrocyte precursors go into mature oligodendrocyte differentiation early in postnatal life, in adult brain remains a slowly dividing population of oligodendrocyte precursors [20,21].

Apart from its developmental roles, PDGF signaling has been associated to the formation of brain tumors. In most cases of oligodendrogliomas and astrocytomas an activation of the pathway occur [22,23]. This activation has been observed both in low- and high-grade tumors, with similar frequency, suggesting that the overexpression of PDGF/PDGFR pathway may be important for tumor initiation.

Gliomas are a group of aggressive heterogeneous brain tumors. They are classified into astrocytomas, oligodendrogliomas, mixed oligoastrocytomas and glioblastomas, based on the expression of specific cell type markers of the central nervous system [24,25]. Due to the number of different signaling pathways and population of origin involved in the generation of gliomas, these tumors are considered very diverse [26-28]. Alteration of PDGF-B signaling is commonly observed in human gliomas of different histophatological grades PDGF-B signaling is usually altered, and previous studies demonstrate the ability of PDGF-B to induce gliomas in mouse perinatal and adult neural stem cells and progenitors [29-31].

4.2. Mouse models of oligodendroglioma

PDGF-B overexpression has been proven to be efficient in tumor induction in telencephalic mouse neural progenitor cells [32]. PDGF-B-induced tumors are a very uniform class of gliomas. All these tumors share unambiguous markers of the oligodendroglial lineage, even when displaying histopathological traits typical of glioblastoma. To create a mouse model of oligodendroglioma, a replication-deficient retroviruses expressing a PDGF-B-IRES-GFP cassette has been injected into the lateral telencephalic ventricles of mouse embryos at mid neurogenesis (E14) [32]. At this stage, precursor cells of the future telencephalon divide actively and this retrovirus can infect the cells [41-44].

5. Breast cancer and stem cells

Most vertebrate organs are patterned during embryogenesis and maintain their basic structure through adult life. In case of the mammary gland, proliferation and expansion occurs during pregnancy and it remodels to its virgin state after weaning [33]. This suggests an involvement of mammary stem cells in these processes.

While the developing mammary gland proliferates and expands, it is able to control angiogenesis to prevent premature involution; it is therefore resistant to apoptosis [34]. Interestingly, proliferation, invasion, angiogenesis, and resistance to apoptosis are all features that are abused during the etiology of breast carcinogenesis. Thus, breast cancer cells resemble mammary stem cells [35]. In addition, in a variety of human solid tumors, including breast cancer, it appears that only a subset of cells with stem cell properties is able of tumor formation. For these reasons, research focusing on identification of mammary stem cells and markers associated with them would aid in the prognosis and treatment of these breast cancers.

5.1. ErbB2 and breast cancer

ErbB2/Her2/Neu is a transmembrane tyrosine kinase receptor, member of the human epidermal growth factor receptor family. Receptor dimerization is an essential requirement for ErbB2 function and signaling activity. After receptor dimerization, transactivation of the tyrosine kinase portion of dimer occurs. Phosphorilation allows recruitment and activation of downstream proteins and the signaling cascade is initiated.

ErbB2 functions as a protooncogene [36]. Gene amplification induces protein overexpression in cell membranes and regulates signal transduction in cellular processes, including proliferation, differentiation and cell survival. Aberrant ErbB2 expression or function is associated with 15-30% of human breast cancers [37,45], and it has been implicated in gastric carcinogenesis and is evident in other cancer types [36].

5.2. Mouse models of breast cancer

The rat homolog of ErbB2, which has a substitution point mutation at residue 664 (Val to Glu) in the transmembrane domain, encodes an activated transforming form of tyrosine kinase [38]. Transgenic expression of the activated form in MMTV-ErbB2 (driven by the Mouse Mammary Tumor Virus promoter) in transgenic mice results in the development of multifocal adenocarcinomas within 8 weeks [46], and lung metastasis was observed after 15 weeks [39]. These transgenic mice have been assayed for their pattern of tissue-specific expression, and the results correspond to tissues where MMTV promoter is known to be active. [46]

SCOPE OF THE THESIS

The aim of this study is to investigate the role of Sox2 in two different kinds of tumors, an oligodendroglioma mouse model and a breast cancer mouse model. The “cancer stem cell hypothesis” suggests that pathways involved in normal stemness maintenance could play key roles in tumor formation and survival. Since Sox2 has been involved in cancer biology, we examined the consequences of genetic ablation of this transcription factor in the tumor models mentioned above. For this purpose we used a mouse model carrying a conditional mutation of the Sox2 allele ($Sox2^{flox/flox}$), where the Sox2 gene is flanked by loxP sites, allowing complete Sox2 ablation by Cre recombinase *in vivo* or *in vitro*.

We first addressed the effects of the Sox2 loss in a PDGF-B induced high-grade oligodendroglioma mouse model. Here Sox2 was excised *in vitro* from tumor reinitiating cells, using a cre encoding lentivirus. We addressed the effect of Sox2 ablation *in vitro* and *in vivo*, following transplantation into the brain.

The second line of research was addressed to understand the consequences of Sox2 deletion in a mouse model of breast cancer in mammary tissue (MMTV-ErbB2). The Cre recombinase gene is driven by the Mouse Mammary Tumor virus (MMTV) promoter, specifically active in the mammary gland [40]. Here we addressed umor development (frequency and size) following Sox2 deletion.

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CHAPTER 1

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 pHGG 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 pHGG cells, and we illustrated a Sox2-directed strategy of immunotherapy to eradicate tumor-initiating cells.

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.

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 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 Sox2flox/flox mice

$Sox2^{flox/flox}$ [13] E14.5 embryos from homozygous $Sox2^{flox/flox}$ matings were injected in the ventricular space with PDGF-BIRES-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 BstXI/SalI); this avoided GFP toxicity observed while superinfecting pHGG 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-IRESGFP lentivirus [25] by SalI/BstxI 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].

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 EdUpositive 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 nontransduced 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 Primmsrl (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 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 $\pm$ 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 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 pHGG 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 pHGG 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 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 CSCdirected 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 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 reforma 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-b 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 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 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.

Acknowledgments

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Figures

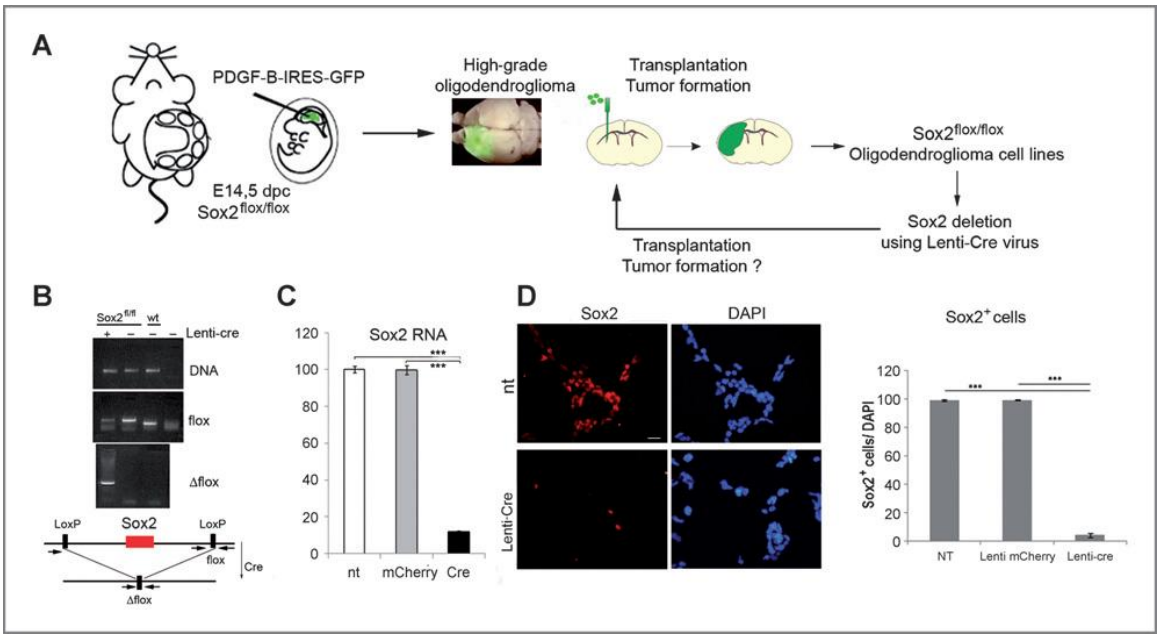


Figure 1. Obtainment of Sox2$^{flox/flox}$-induced oligodendrogliomas and Sox2 deletion via lentiviral Cre recombinase.

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 non deleted (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 Sox2mRNA in nontransduced (nt; set=100), control mCherry, or Cre lentivirus-transduced pHGGcells. **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).

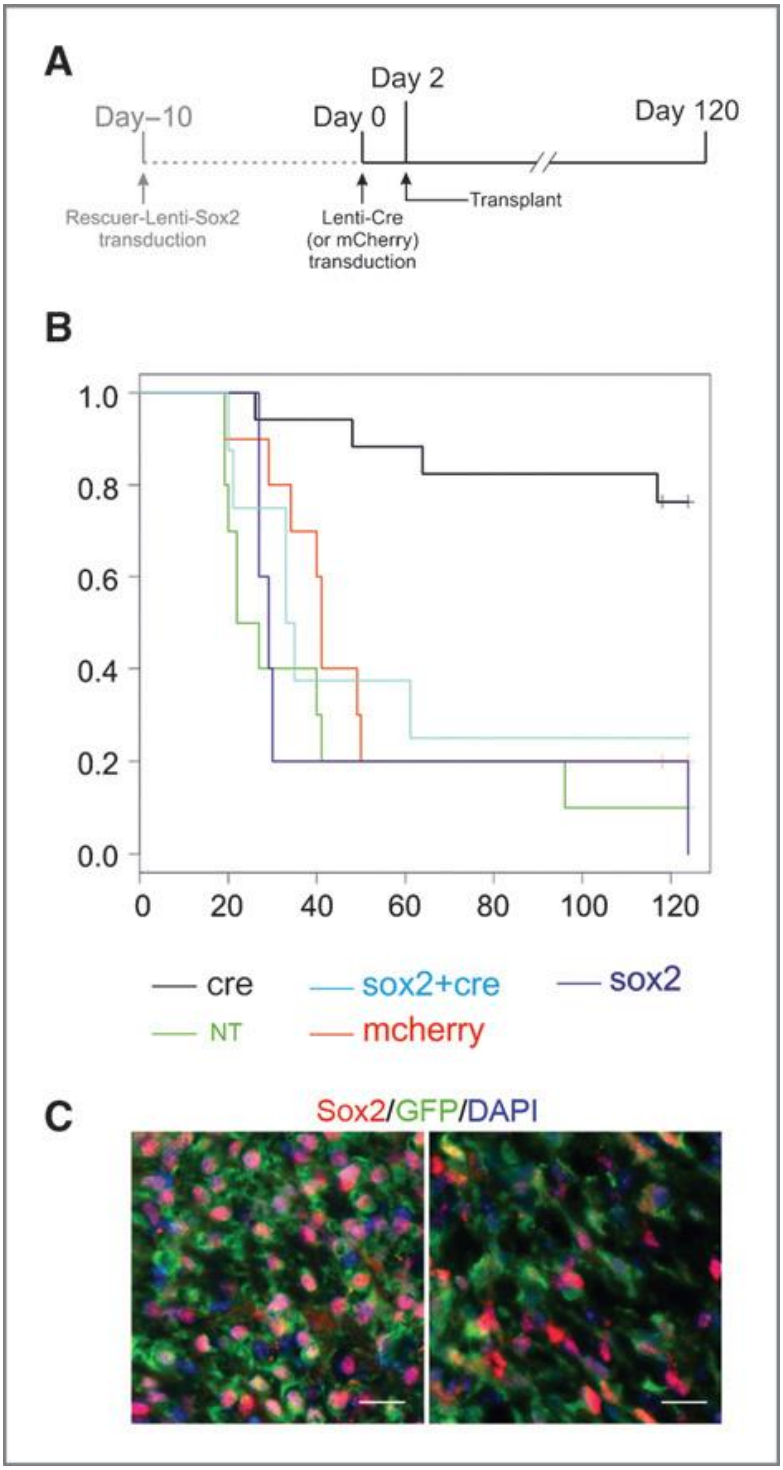


Figure 2. Sox2$^{flox/flox}$ deletion before pHGG stem cells transplantation efficiently antagonizes tumor reinitiation and tumor-caused lethality.

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.

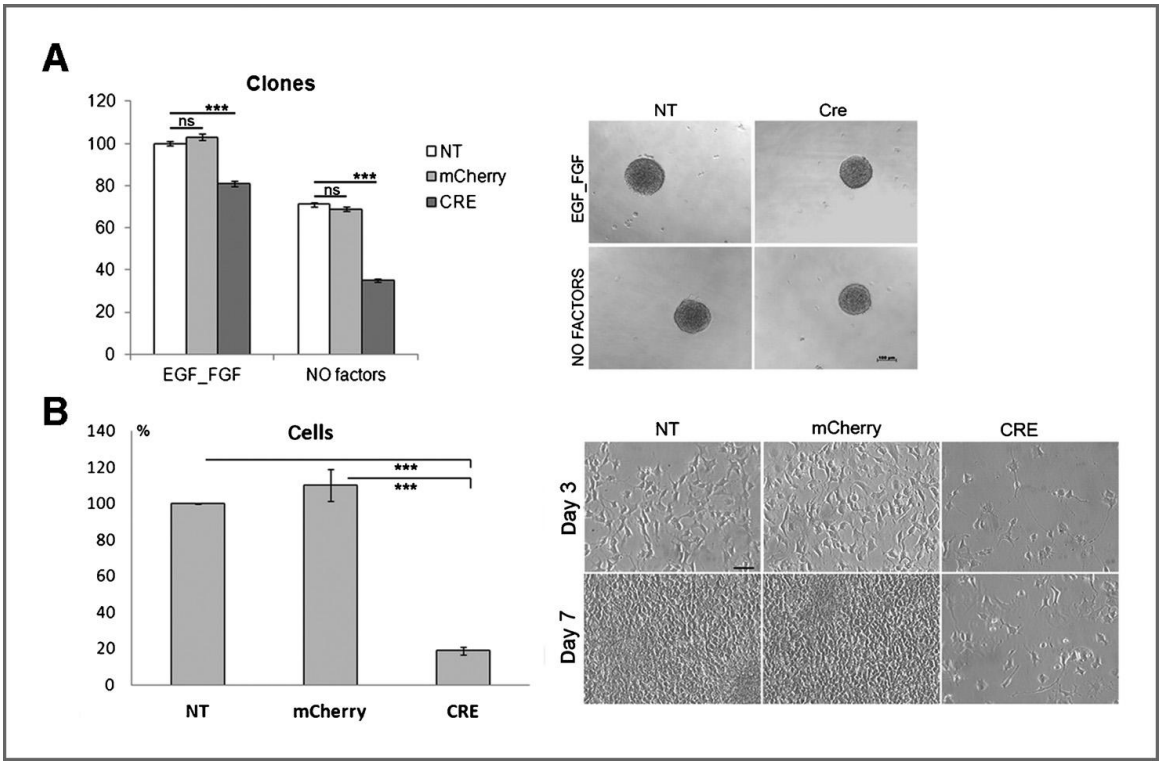


Figure 3. Sox2$^{flox/flox}$ deletion reduces *in vitro* growth of pHGG stem cells.
A, pHGG oligodendroglioma clone numbers obtained in EGF+bFGF-containing medium (EGF_FGF), or factor-free medium (NO Factors), with nontransduced cells (NT), or cells transduced with control (mCherry) or Cre-expressing (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, 100µm. **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.

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.

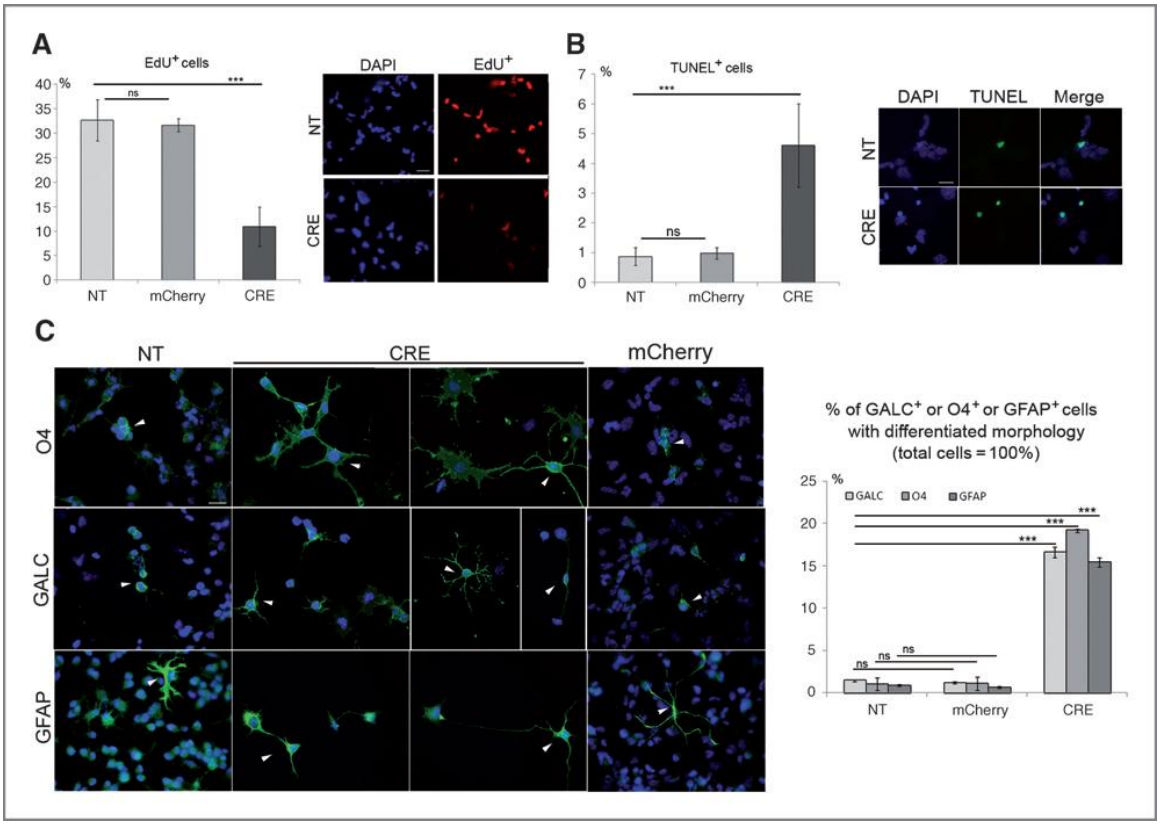
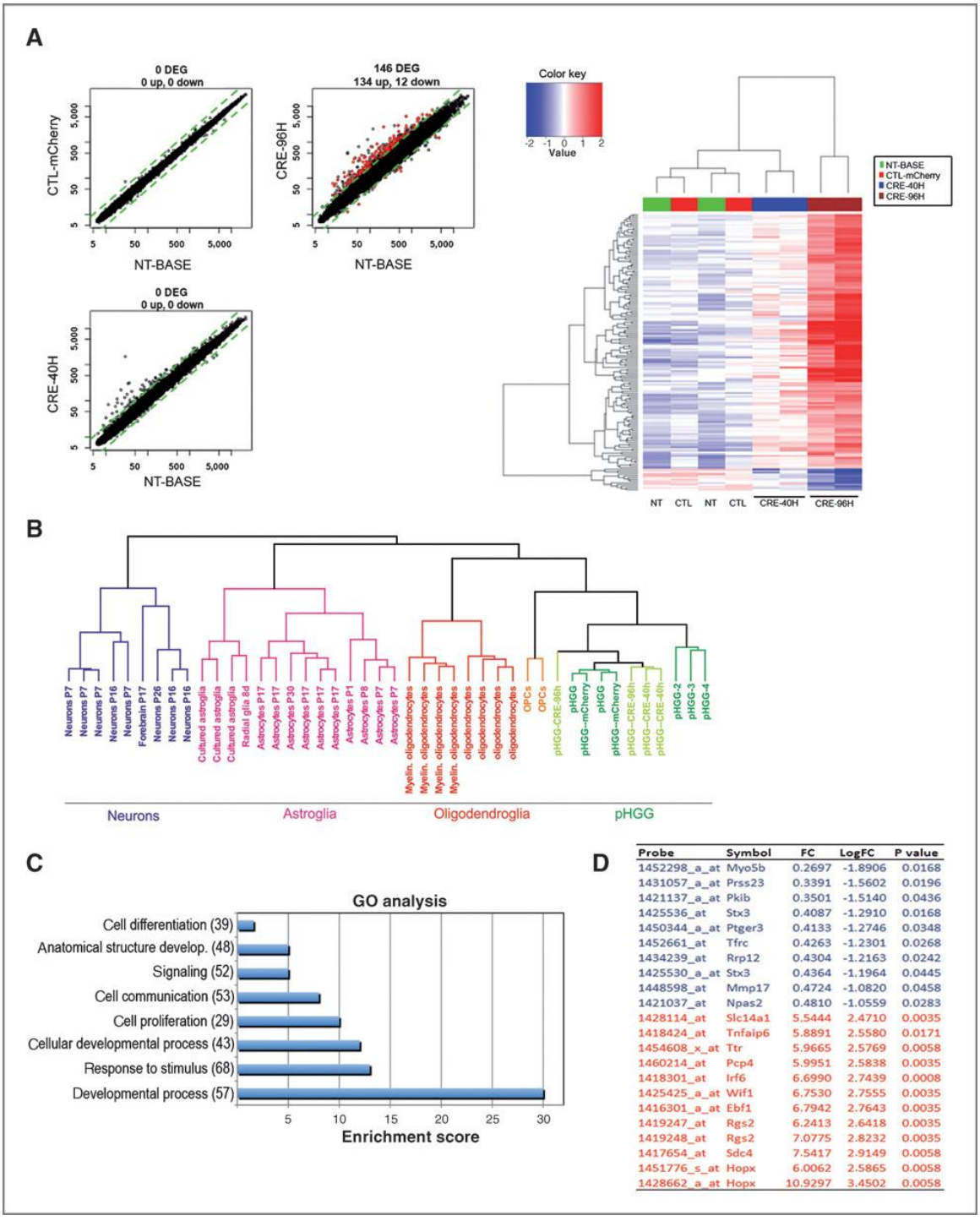


Figure 4. *In vitro* effects of Sox2$^{flox/flox}$ deletion on pHGG cells.
A, EdU incorporation obtained following transduction with control (mCherry) or Cre virus after 2 days in serum-containing medium. Histograms report the percentage of EdU-positive nuclei over the total number of (DAPI-positive) nuclei. Representative images showing EdU-positive cells are shown besides the histograms (scale bar, 20µm). **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.



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). Data represent the mean of probe expression values of the replicates samples in the considered condition. Right, heat map diagram of gene expression changes (red, increased expression; blue, reduced expression) in Cre-treated

cells, as compared with the indicated controls. Probe sets (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.

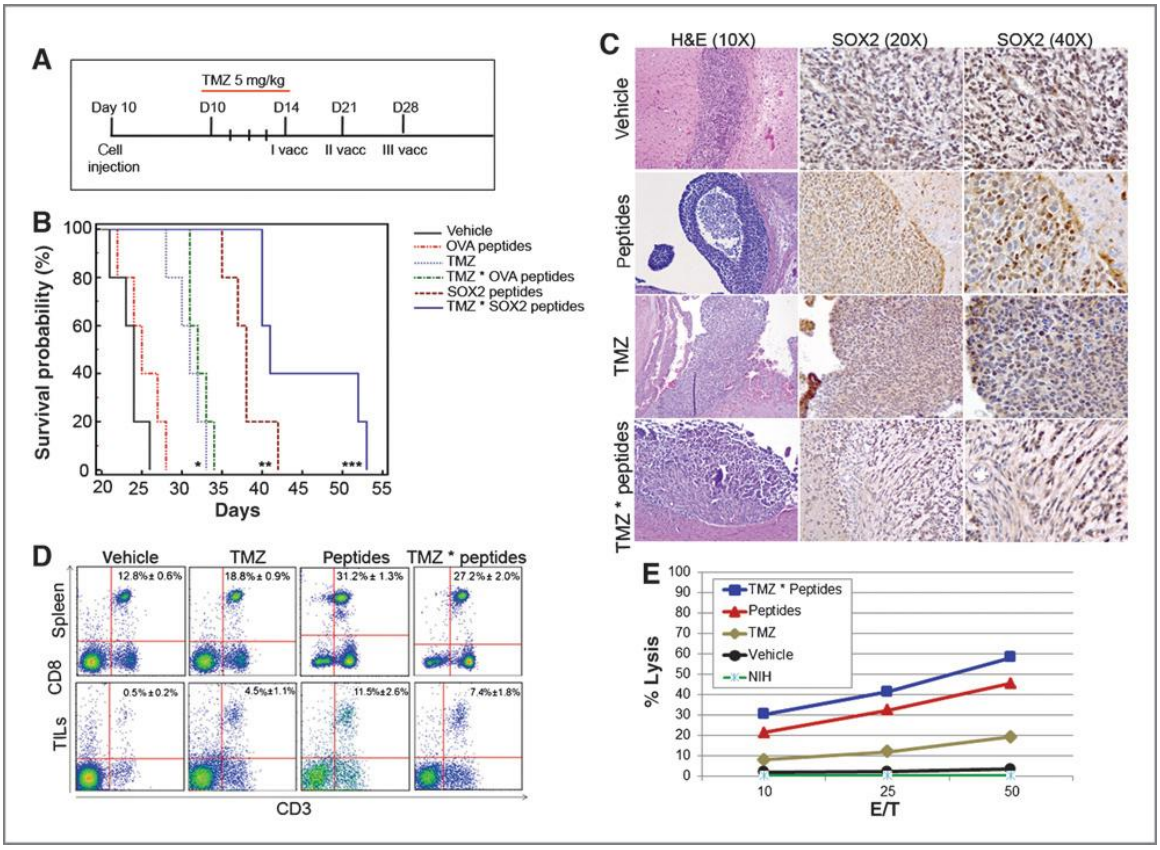


Figure 6. Vaccination with Sox2 peptides causes a significant delay in tumor development and lethality following transplantation.

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).

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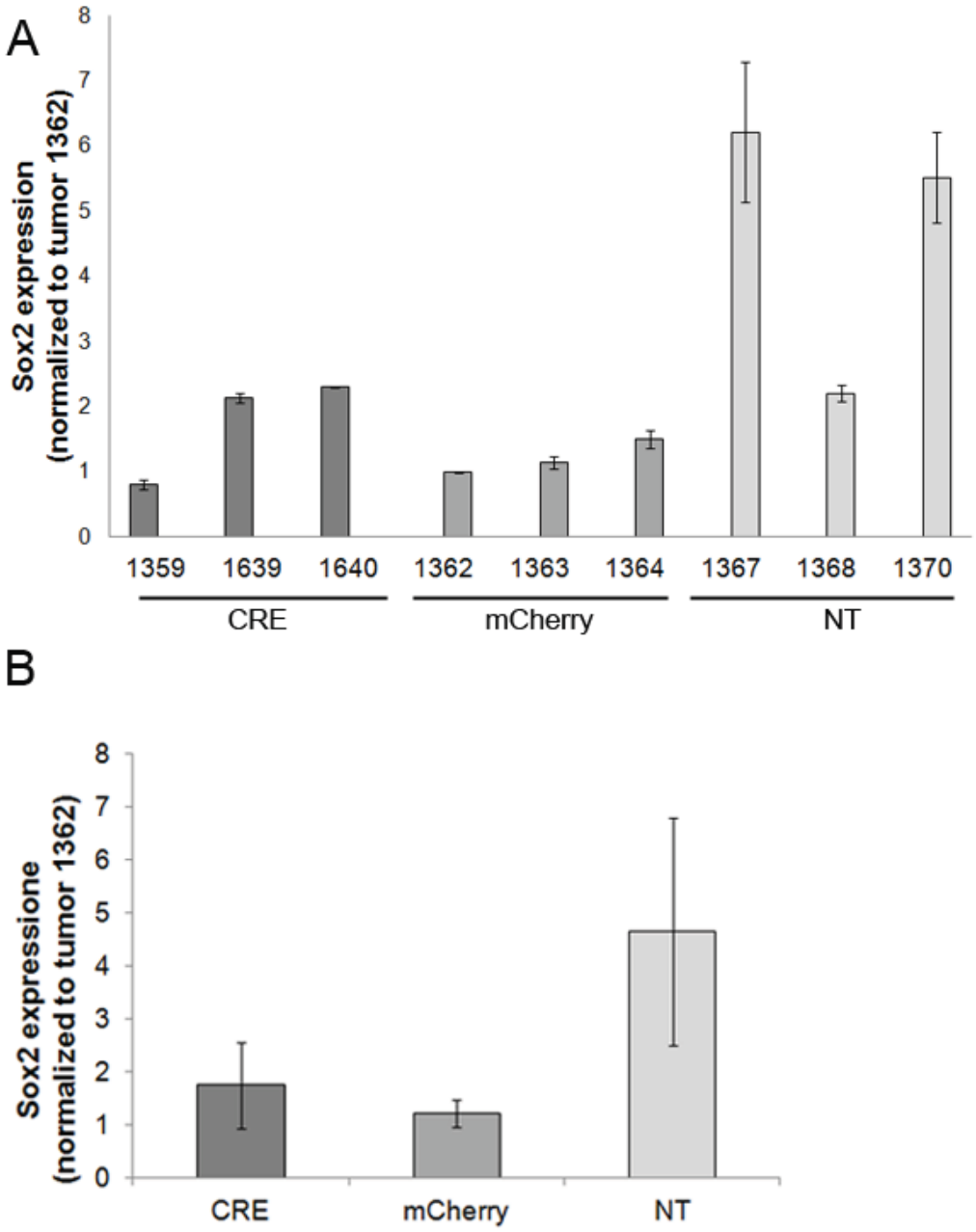
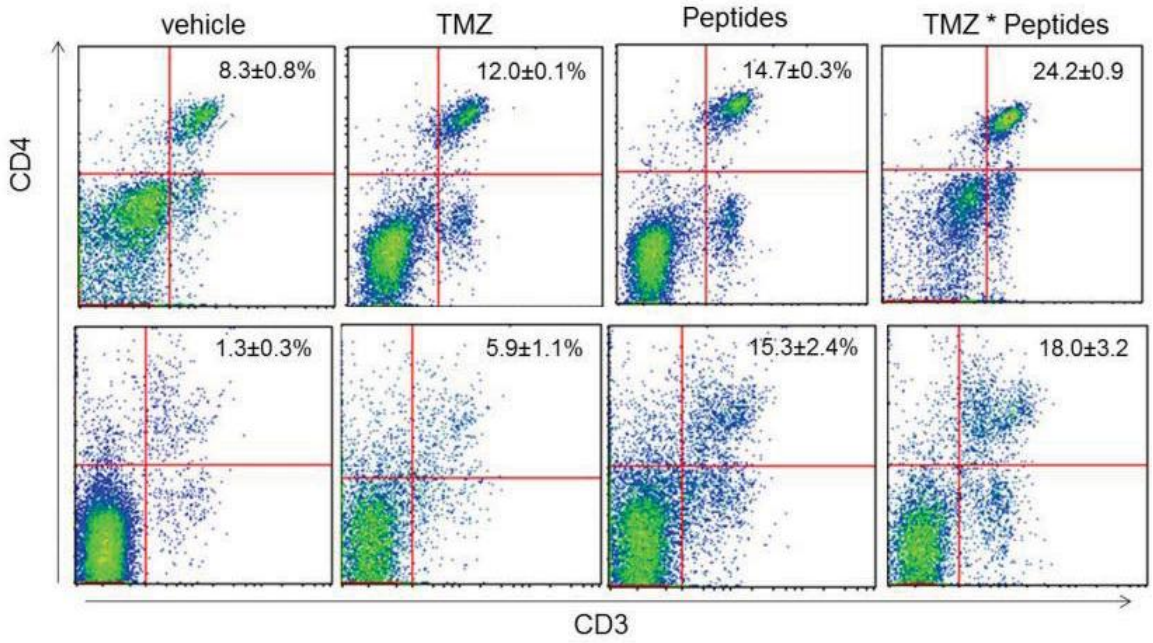
Supplementary figures

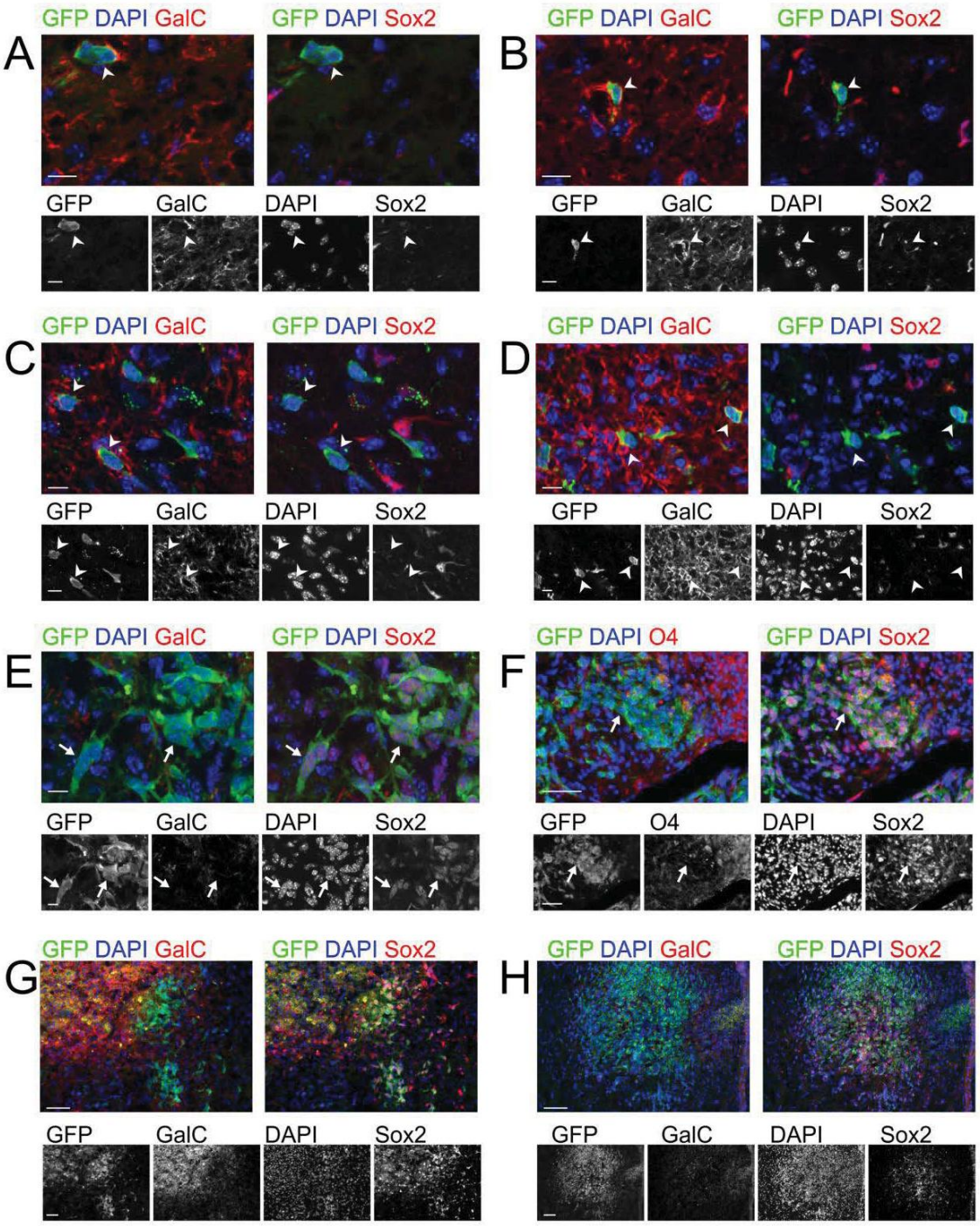


Supplementary figure 1. Sox2 expression in tumors analyzed by qRT-PCR
A, Sox2 expression in tumors obtained following transplantation of pHGG cells transduced with Cre lentivirus, or mCherry control lentivirus, or non transduced (NT). Numbers on the X axis identify individual tumors, Expression values have been normalized to GFP mRNA expression to evaluate Sox2 expression specifically in tumor (i.e. GFP-positive) cells. **B,** Average Sox2 expression in the different tumor classes, calculated from the measurements in (A). Expression values are normalized to that of tumor 1362 set=1, as in A. Mean values are indicated, +/- standard deviation.



Supplementary figure 2. SOX2 peptides are able to induce a specific anti-tumor immune response in the peripheral and local immune effector cells

Flow cytometry analysis performed on splenocytes (upper panel) and tumor infiltrating lymphocytes (lower panel) shows that the frequency of CD4 T cells increased significantly in immunized compared to vehicle mice (n=4 animals tested/group; $P < 0.001$). Data obtained from four different evaluations are reported in dot plots as the mean % $\pm$SD.



Supplementary figure 3. Sox2-deleted cells expressing differentiation markers in transplantated brain.

pHGG cells treated with lentivirus expressing CRE recombinase (A-G) or non-treated (H), 10 days after transplantation, immunostained for Sox2, GFP and GalC or O4. **A-D,** show examples of isolated pHGG cells, immunonegative for Sox2 and immunopositive for GalC (arrowheads). **E-G,** show clusters of pHGG cells which escaped recombination and are immunopositive for Sox2 and immunonegative for GalC (E,G) and O4 (F)(arrows). **H,** depicts a typical cluster formed by nontransduced (control) pHGG cells. Scale bars: 10 μm A,B,C,D,E; 50 μm F,G, 100 μm H.

Supplemental Materials and Methods

List of Primers for Sox2 PCR and qRT-PCR

For DNA analysis: Sox2 flox allele (spanning the 3' loxP site):
RF23: 5'- CAGTCCAAGCTAGGCAGGTT-3'
RF24: 5'-AGGCTGAGTCGGGTCAAT TA-3';
Δflox Sox2 allele:
RF106: 5'-TGCAAACACACATTGGAGAGGTTCAGACTA-3'
RF24: 5'-AGGCTGAGTCGGGTCAATTA-3';
Genomic sequence for normalization:
RF57: 5'-ATTCACAGCTCTTTAGCACGAAC-3',
RF58: 5'-AGCAAAGTCCAAGGAGCTAGAAC-3';
PCR conditions: 95°C 5 min, 35 cicles of 95°C 40sec, 58°C 30sec, 72°C 40sec.

qRT-PCR detection of Sox2 mRNA primers:
Sox2 coding region:
F6: 5'-GGCAGCTACAGCATGATGCAGGAGC-3';
B11: 5'-CTGGTCATGGAGTTGTACTGCAGG-3';
Sox2 3' UTR (specific for endogenous, versus viral Sox2):
RF366: 5'-ACCGTGATGCCGACTAGAAAA-3'
RF367: 5'-CAGATCTATACATGGTCCGATTCC-3'
Real time data were normalized for HPRT expression with primers:
HPRT-F: 5'-TCCTCCTCAGACCGGTTT-3';
HPRT-R: 5'-CCTGGTTCATTCATCGCTAATC-3'.
EmGFP 472-592-F: 5'-CAGAAGAACGGCATCAAG-3'
EmGFP 472-592-R: 5'-GCTCAGGTAGTGGTTGTC-3'
MSox2-F: 5'-TCGCAGACCTACATGAACG-3'
MSox2-R: 5'-CGGACTTGACCACAGAGC-3'
Data were analyzed with a 7500 System Software v1.4 Applied Biosystem as previously described [1].

Gene expression analysis

Microarray hybridization was performed using the Affymetrix GeneChip Mouse Genome 430A 2.0 Array at Consorzio Genopolis (University of Milano-Bicocca, Department of Biotechnology and Biosciences), as previously described [2]. Data Analysis handling was mainly done using AMDA software [3]. The Robust Multi-array Analysis (RMA) method was employed to calculate probe set intensity and normalization was performed by a quantile method [4]. To verify the quality of replicates, hierarchical clustering based on Pearson correlation coefficients of transcript nature and abundance patterns in the different experimental conditions was performed.

The identification of differentially expressed genes (DEG) was addressed using a linear modeling approach (Limma) [5] together with false discovery rate correction of the p-value [6]. Differentially expressed genes with p-values of <0.05 were selected. Probe sets were annotated following Affymetrix annotation files.

Gene Ontology (GO) annotation

A functional annotation of DEG was performed on the basis of a subset of the annotation provided by the Bioconductor project (mouse430a2.db 2.3.5 from ww.bioconductor.org). The annotation resource considered is Gene Ontology (GO) (www.geneontology.org). The most representative functional annotations for DEGs from each experimental condition were identified by determining the probability of random occurrence of functional terms (hyperGeometric distribution). Gene expression comparison between our dataset and those previously reported for other pHGG (Fig. 5B) were performed as described [2].

SOX2 immunohistochemistry

Tumors were fixed with 4% PFA, paraffin-embedded and sectioned. Paraffin was removed with xylene, followed by rehydration in graded alcohol. Antigen retrieval was carried out using preheated Target Retrieval Solution (Dako) (pH 6.0) for 45 minutes. Sections were blocked with 10% Fetal Bovine Serum in PBS for 90 min, incubated overnight with primary anti-SOX2 antibody (1:30, Cell Signaling) and incubated with biotinylated secondary antibodies (1:200 Vector Lab) for 1 h. Antibody binding was detected using the Vectastain Elite Avidin–Biotin Complex-Peroxidase kit according to manufacturer's instructions, followed by a diaminobenzidine chromogen reaction (Peroxidase substrate kit, DAB, SK-4100; Vector Lab). All sections were counterstained with Mayer's hematoxylin and visualized using a bright-field microscope.

Cytotoxicity assay

Splenocytes isolated from mice sacrificed after the second immunization and from control mice were tested for their ability to recognize and lyse pHGG cells *in vitro*. Splenocytes were pre-stimulated for 5 days in the presence of irradiated pHGG cells (20 Gy) in RPMI-10% serum supplemented with 10 U/mL of IL-2. Pre-stimulated lymphocytes were tested for specific cytotoxicity using 10:1, 25:1, and 50:1 effector:target (E:T) ratios. NIH 3T3 cells were used as negative controls. To quantify cell lysis, a colorimetric cytotoxic assay (MTT colorimetric assay, Millipore) was performed according to the manufacturer's instructions. Absorbance for the various cell groups were used to calculate the percentage of specific cytotoxicity according to the following equation:

$$\% C = 100 - \frac{[\text{optical density of effectors + targets}] - [\text{optical density of effectors}]}{[\text{optical density of targets}]} \times 100$$

Isolation of tumor infiltrating lymphocytes and flow cytometry.

Tumor infiltrating lymphocytes were isolated after the second immunization and from control mice using a tumor dissociation kit (mouse, Miltenyi Biotec). Brains from treated and control mice (n=4/group) were explanted, and tumor areas were cut into small pieces of 2-4 mm, and dissociated using GentleMACS (Miltenyi Biotec) according to manufacturer's instruction.

The cells were suspended in PBS/0.5% bovine serum albumin/2 mM EDTA for labelling and flow cytometry evaluation. Briefly, 1.0x106 cells were stained in PBS for 10 or 30 minutes at 4ºC with the following antibodies: anti-CD4-PE-Cy5 (BD Bioscience), anti-CD3-FITC (Miltenyi Biotec), anti-CD8-PE (Miltenyi). Flow cytometry acquisition was performed on a MACSQuant®, and data analyzed with the MACSQuantify™ Software (Miltenyi Biotec).

Supplementary Materials and Methods references

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Additional experiments:
Initial studies on Hopx, a Sox2 target in oligodendroglioma

We demonstrated that Sox2 has a crucial role in the expansion of CSC in oligodendroglioma, *in vivo* and *in vitro*. To address Sox2 target genes that could mediate its function in oligodendroglioma CSC, we performed RNA microarray analysis of *in vitro* Sox2 pHGG deleted cells compared with the non-deleted cells (Chapter 1, Figure 5D) at 48h and 96h after Sox2 deletion. We found 12 genes that are downregulated and 134 genes that are upregulated 96h after Sox2 deletion. The gene that is most upregulated in Sox2-deleted cells (about 10–fold upregulation) is Homeodomain only protein homeobox (Hopx), a small protein with chromosomal structural function.

We wish to test individual Sox2 target genes for their contribution to CSC maintenance. Because Hopx is upregulated following Sox2 loss, we aimed at overexpressing Hopx (instead of deleting Sox2), and address the effect on *in vitro* growth to see if any of the changes observed following Sox2 loss were observed, at least in part, following Hopx overexpression. To do this experiment we cloned Hopx coding region within a lentiviral vector to be used for transduction of pHGG cells.

Hopx

Hopx has three spliced transcript variants, HOPX-α, -β, and -γ, that encode the same protein, which contains a putative homeodomain motif that acts as an adapter protein to mediate transcription [1]. Hopx does not directly bind DNA because its homeodomain lacks specific conserved amino acid residues that are required for protein-DNA interactions in Hox proteins. Nevertheless, Hopx is a nuclear protein that can function to modulate transcription. Hopx interacts with serum response factor (SRF) and modulates SRF-dependent cardiac-specific gene expression and cardiac development [2]. It is also described to have a function in heart [3], lung development [4], and has an important role in hippocampus stem cell maintenance. Importantly, Hopx was shown to be a tumor suppressor gene in human lung cancer, choriocarcinoma and glioma [5-7]. Its decreased expression level (when compared to the normal tissue) was found in other human malignant tissues, including colorectal cancer or esophageal squamous cell carcinoma [5-10]. Thus, Hopx increase following Sox2 deletion may have an important role in the loss of tumorigenesis that we observed in Sox2-deleted cells.

Hopx expression is upregulated in the $Sox2^{flox/flox}$ model of oligodendroglioma 96h after Sox2 deletion (Chapter1, Figure 5D), however, there is no change in its expression 40h after Sox2 deletion, even if the Sox2 protein is already not anymore present in most of the cells, suggesting that Hopx could be an indirect target of Sox2.

Bioinformatic analysis of Sox2 ChIP data obtained from neural stem cells (neurospheres derived by postnatal day 0 from telencephalon) in the Hopx, locus revealed that Sox2 can bind a previously described Hopx enhancer described in Mühlfriedel et al.

[13], and the putative promoter of the isoform 1 of the Hopx gene (Figure 1). Sox2 binding to the regulatory regions of Hopx could have a role in determining its specific expression mediating its repression in a tissue/cell specific way. It is known that one of the effects of "staminal genes", such as Sox2, Oct4 and Nanog, is to repress the expression of genes that induce cell differentiation. The fact that the transplanted Sox2-deleted pHGG cells are not able to form tumors *in vivo* and the fact that these cells show enhanced ability to differentiate *in vitro* (Chapter 1), could indicate a role for Hopx in blocking the tumorigenesis of the cells *via* the commitment of a pathway that induce differentiation.

Methods

To create the Hopx overespressing lentivirus, we excided the HA-Hopx fragment from pGex-HA-Hopx vector [8] and cloned into blunted BamHI site in pHR SIN BXIR/EMW vector, derived from pHR SIN CSGW vector [12], deleted of the Emerald-GFP reporter gene. This vector harbor a ΔnGFR reporter. As controls we used the non-transduced cells (NT), cells transduced with the empty pHR SIN BXIR/EMW vector (from now on ΔnGFR) and as positive control we used the Cre-encoding lentivirus (from now on Cre) used for the previous experiments.

Results

We infected $Sox2^{flox/flox}$ pHGG cells with a multiplicity of infection (MOI) of 7 (as in the previous experiments) and cultured the cells in medium for maintenance of stem cell properties (with EGF/bFGF and without serum) for 96h after infection; then we changed the growth conditions to a differentiation inducing medium (no factors and 2% serum).

To test infection efficency and understand how Hopx and Sox2 levels variate in the Hopx overespressing pHGG cells, we performed a qRT-PCR for Sox2 and Hopx (isoform 1) in pHGG cells harvested 96h after infection and also after 7 days in differentiation induction medium (Figure 2). We found that Hopx lentivirus infection enhances Hopx levels 96h after transduction, compared to controls (non transduced cells and the cells infected with the ΔnGFR); and the transcript accumulate after 7 days in differentiation medium, as expected. The Hopx increase we saw here after 96h, is comparable to what we saw following Sox2 loss (Chapter 1, Figure 5D). qRT-PCR for Sox2 revealed that cells infected with ΔnGFR and Hopx overexpression lentiviruses showed decreased levels of Sox2 after 96h, but this effect could be due to the infection itself, since after 7 days in differentiation medium, Sox2 levels return comparable with the untrasduced cells. We could not evaluate any potentially effect due to this transient downregulation of Sox2 in ΔnGFR and Hopx infected cells. We confirmed the deletion of the Sox2 gene in the Cre infected cells, and after 7 days in differentiation medium they still show low levels of Sox2 transcript, indicating that there is no significative selection of Sox2 positive cells (that escaped Cre infection) in the cell population. We observed that Hopx levels after 7 days nearly disappeared in Cre infected cells, meaning that Hopx expression is transient in Sox2-deleted cells.

Since the lentiviral infection does not reach 100% efficiency, we analyzed the cells *via* FACS to quantify the percentage of infected cells (ΔnGFR positive, see Methods) and to discriminate pHGG cells (that are GFP positive, see Chapter 1) from the ones that had lost PDGF-B expression (and are not tumorigenic). The results indicate nearly 80% of ΔnGFR positive cells (Figure 3A) 96h after the infection. After 7 days in differentiation medium the Hopx infected cells and the control cells (infected with ΔnGFR virus) show the same percentage of infected cells. This means that in these conditions of growth, Hopx overexpression does not give any advantage or disadvantage to the infected cell in this pHGG cell population.

We also tested the *in vitro* growth of these cells by a proliferation assay in the differentiation medium. We plated 40.000 cells per well and counted them every 2 days (Figure 3B). While Sox2-deleted cells stop growing, as observed in previous experiments, the Hopx overexpressing cells has a growth rate similar to the one of control cells (NT and ΔnGFR) (Figure 3C). Thus, Hopx does not affect the capacity of the tumorigenic cells to grow in these conditions.

Since the Sox2-deleted cells undergo glial differentiation, we tested if the Hopx infected cells show a higher rate of differentiation. Immunofluorescence analysis using typical oligodendrocyte differentiation markers as galactosylceramidase (GalC) or astrocyte marker glial fibrillary acidic protein (GFAP) were performed together with the anti-Hopx antibody (Figure 4).

We could not detect GalC or GFAP positive cell in Hopx overespressing cells or in control cells whereas positive cells were present in Sox2-deleted population as described previously. These results suggest that Hopx has no role in differentiation in these conditions in the presence of Sox2 transcript.

Thus, in the presence of Sox2 expression, we cannot find any difference in the rate of growth or in the ability to differentiate of Hopx transduced cells. The pure overexpression of Hopx cannot reproduce, not even in part, the effect of Sox2 deletion *in vitro* (differentiation, reduction of cell growth, increase of cell death). However, other research groups reported no changes in differentiation rate due to Hopx, instead, they saw a role for Hopx in methastasis and aggressiveness [15,16]. It is also possible that, in the presence of endogenous Sox2, Hopx overexpression is not sufficient to overcome the effect of Sox2 presence. A different experiment to test this point would be, to delete Sox2, and simultaneously antagonize Hopx increase, by shRNA depletion. This experiment would address more precisely the specific contribution of Hopx to the effects of Sox2 deletion.

We did not test the Hopx overexpressing cells *in vivo* in mouse brain; the possibility remains that Hopx overexpression has an anti-tumorigenic effect *in vivo*, that is not reflected in altered properties *in vitro*.

A role for Sox2 regulation of Hopx in normal brain development?

The published study of Hopx role in brain development, in particular in hippocampus and the description of specific enhancer of this gene [13] suggests that Sox2 can regulate Hopx expression in this particular tissue; this idea is supported by the phenotypical aspect of the Sox2 mutant in hippocampus described in Favaro et al. [14]. Our lab is studying a $Sox2^{flox/flox}$ mutant mouse line where the Cre recombinase is driven by the BF1 promoter, so, Sox2 is excised at early embryonic stages, at day 8.5 (E8.5). Preliminary data suggest that this mouse has a severe phenotype in the hippocampus. So we wanted to see if Sox2 loss in this area affects Hopx expression. We isolated RNA from the telencephalon of these mice at different stages of embryonic development, E15.5 and E18.5, and analyzed it by qRT-PCR (Figure 5). Interestingly, the results show that when Sox2 levels decrease – due to the Sox2 allele excision – Hopx levels increase slightly, suggesting that Sox2 may also negatively regulate Hopx in the hippocampus. These data encourage a deeper investigation of this point.

Additional results Figures

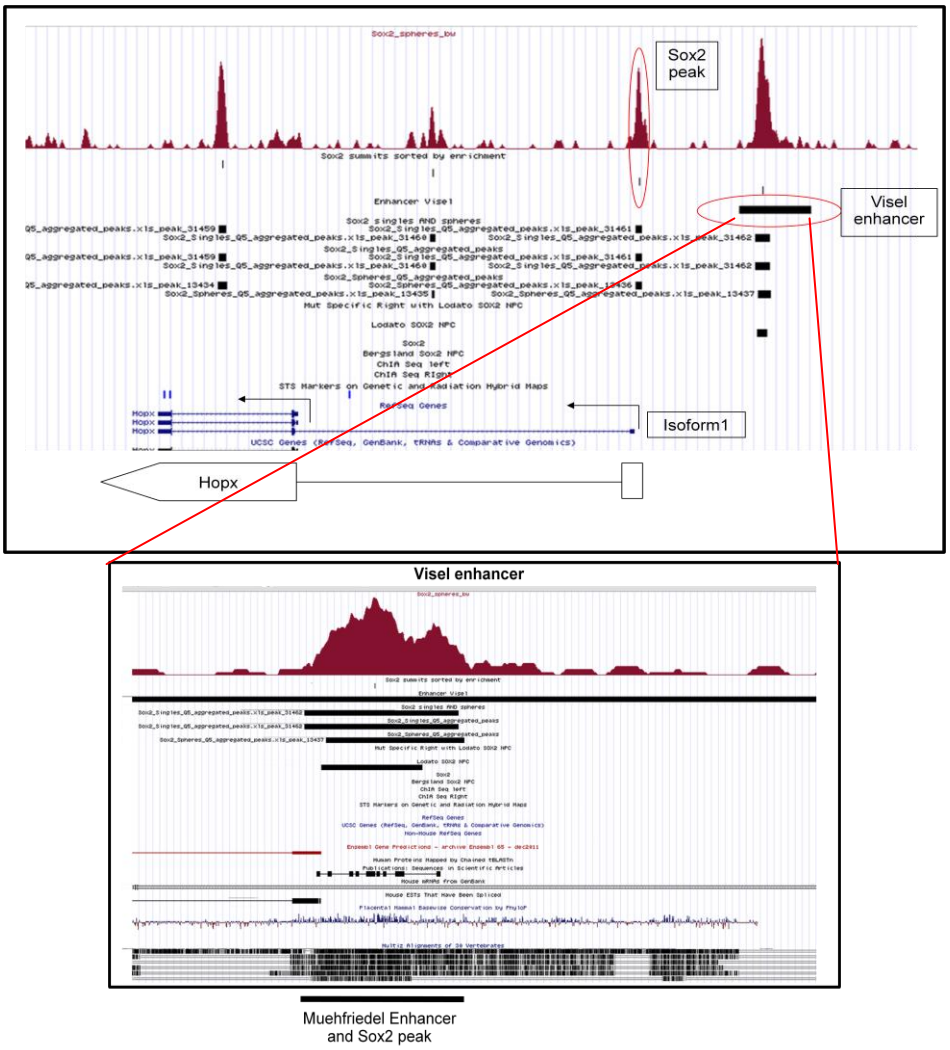

Figure 1. Bioinformatic analysis of the Sox2 ChIP data for Hopx locus obtained from neural stem cells. A Visel enhanser has been found near the Hopx isoform 1, with a high Sox2 peak. Another Sox2 peak is located on the promoter of Hopx isoform 1. From these data Sox2 appears to regulate Hopx.

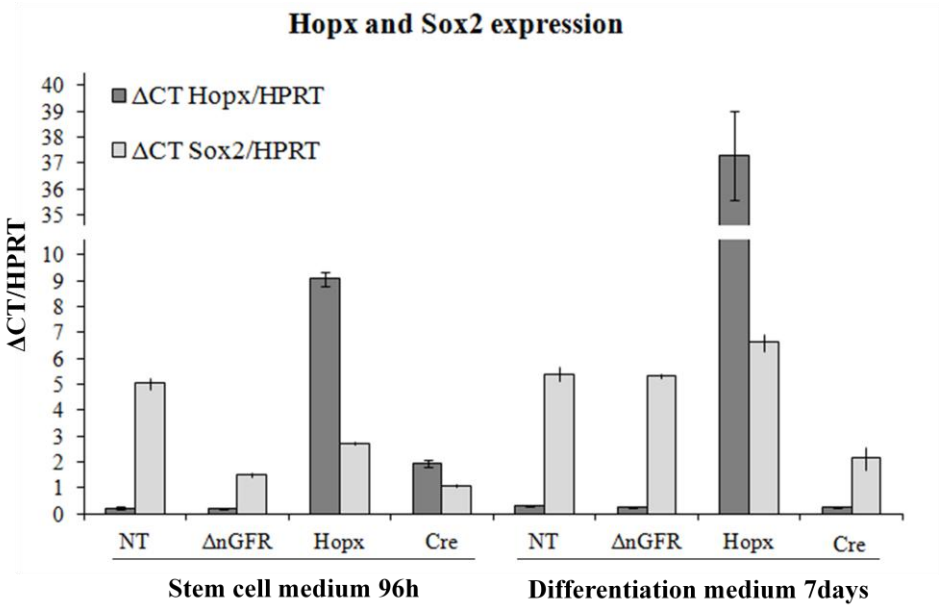

Figure 2. qRT-PCR analysis of pHGG cells infected with Hopx or control lentiviruses. RNA levels of Hopx are shown in dark gray and Sox2 mRNA levels are shown in light gray. The expression was calculated as ΔdCT referred to HPRT. The histograms report average values of two independent experiments performed in triplicate. The vertical bars indicate the standard deviation. NT: non-treated; ΔnGFR: control empty lentivirus; Hopx: lentivirus overexpressing Hopx; Cre: lentivirus espressing Cre recombinase.

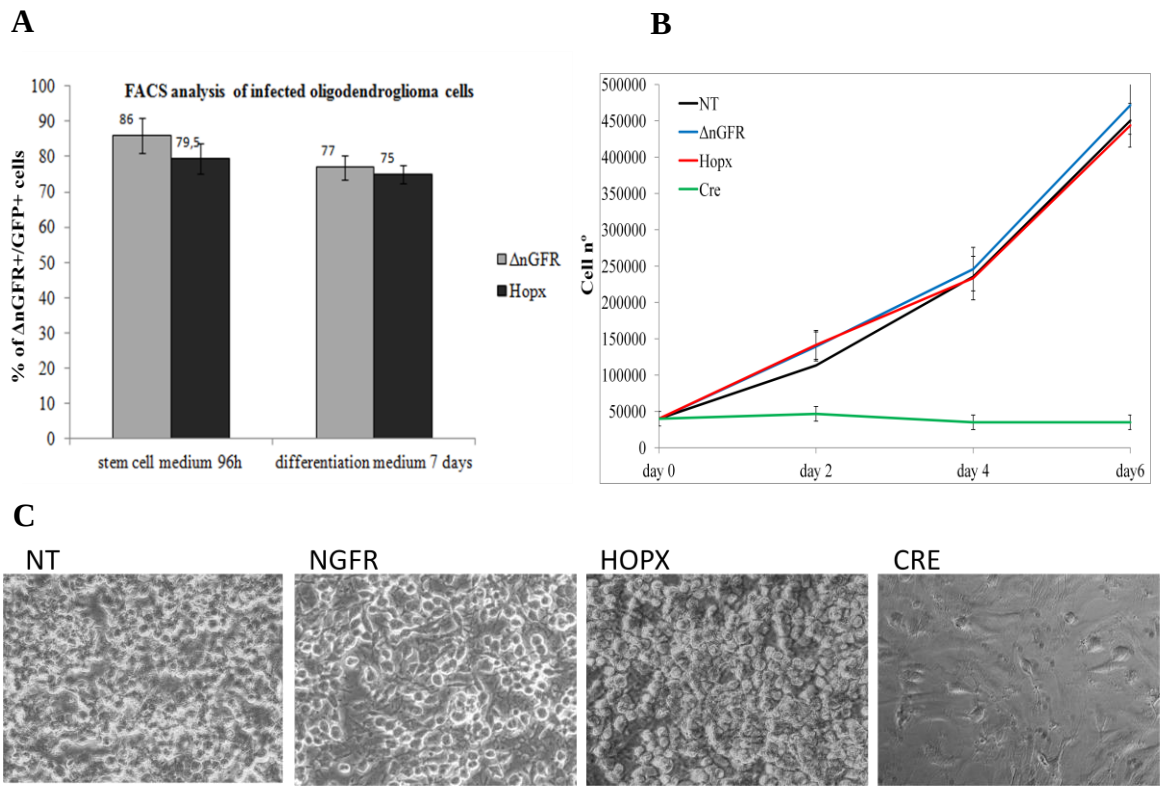

Figure 3. Evaluation of *in vitro* growth of pHGG cells infected with different lentiviruses.

A, FACS analysis of Sox2$^{flox/flox}$ pHGG cells infected with ΔnGFR and Hopx lentivirus. The pHGG cells (expressing PDGF-B-IRES-GFP) are GFP^{+}, the percentage of ΔnGFR^{+} of this GFP^{+}population is indicative of the efficiency of transduction. Both the control empty virus and the Hopx overexpressing virus express ΔnGFR. The histograms report average values of two independent experiments. The bar indicates the standard deviation. **B,** Proliferation assay of transduced pHGG cells. 96h after infection with the indicated viruses the cells were plated at 40.000cells/well on matrigel in differentiation medium and counted every 2 days. The experiment was done twice in triplicate. **C,** Images show examples of cells after 7 days in serum-containing medium.

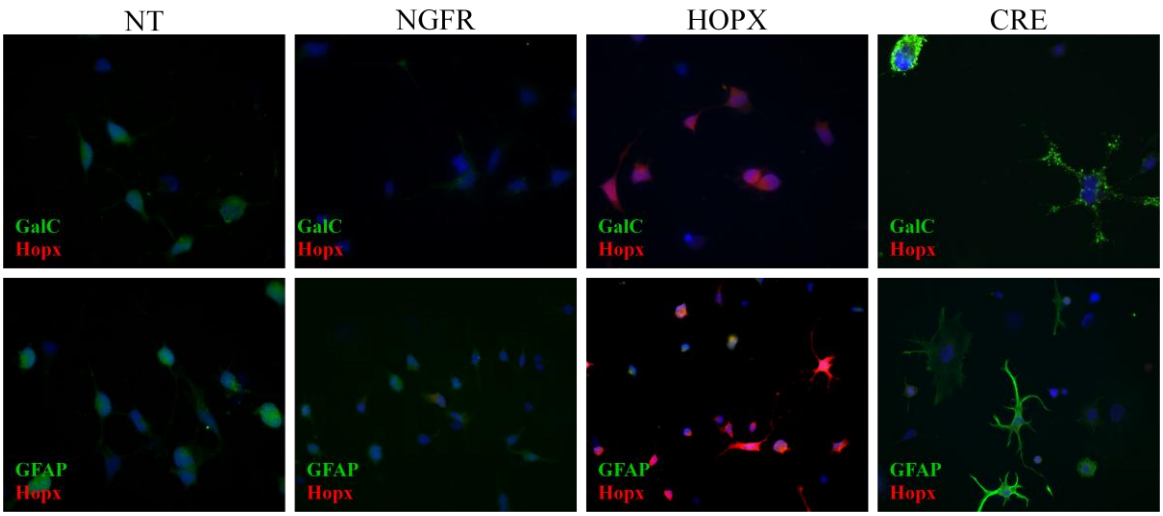

Figure 4. Immunostainig for Hopx and differentiation markers.
Top row: immunostaining for GalC in green and Hopx in red. Bottom row: immunostaiining for GFAP and Hopx. Nuclei are stained with DAPI. The light green in NT, NGFR and HOPX samples is due to the GFP expressed by pHGG cells (PDGF-B-IRES-GFP). It is clearly different from GalC and GFAP staining. Some clearly Hopx positive sells are visible in cells transduced with the Hopx virus. However they are not positive for differentiation markers GalC or GFAP, and do not show the "differentiated" morphologies observed in cells transduced with the Cre virus.

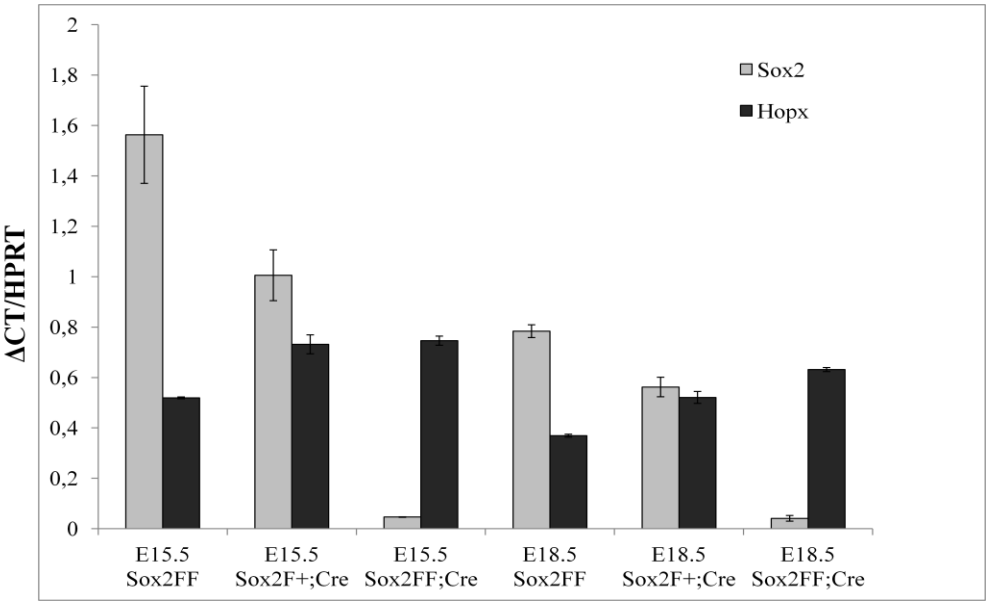

Figure 5. qRT-PCR from Sox2flox/flox;Bf1Cre embryonic telencephalon at different stages (E15.5 and E18.5). Sox2FF (Sox2flox/flox): control mouse with both Sox2 intact alleles. Sox2F+;Cre: heterozygote mouse with only one intact copy of Sox2. Sox2FF;Cre: homozygote mouse without intact Sox2 alleles. The experiment was made once in triplicate. The data are calculated on HPRT. The bar indicates the standard deviation.

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CHAPTER 2

Understanding the role of Sox2 in an ErbB2-induced breast cancer mouse model

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(Submission: PLoS ONE)

Abstract

The Sox2 transcription factor is active in normal stem cells within different tissues, and in cancer stem cells of different tumor types. We previously demonstrated, by Sox2 conditional deletion, a requirement for Sox2 in the maintenance of normal neural stem cells, and of cancer stem cells within a mouse model of neural tumor (high-grade oligodendroglioma). Sox2 has been described to be active in mammary tumors and in tumor-derived cell lines, including stem-cell-enriched "mammospheres" with cancer stem cell properties, and functional experiments had pointed to a requirement for Sox2 for the maintenance of pro-tumorigenic properties of some of these cell lines. We used Sox2 conditional deletion by an MMTV-Cre transgene (active in mammary tissue) to address Sox2 requirement within a widely studied mouse model of mammary tumor, produced by expression of a transgene encoding a mutated ErbB2/Neu oncogene, driven to mammary tissue by the MMTV promoter. Sox2 was efficiently deleted in tumors; yet, we found that Sox2-deleted tumors developed with very similar timing and frequency than tumors carrying one or two intact Sox2 copies. Average tumor size was slightly reduced. The development of normal mammary tissue was not notably affected in MMTV-Cre Sox2-deleted mice. Sox2 expression was not detected, by qRT-PCR nor immunofluorescence, in MMTV-ErbB2 tumors, nor in mammospheres grown from them. Our results point to a heterogeneity within mammary tumors regarding Sox2 expression and function, in particular within ErbB2/Neu-positive tumors, that it will be important to consider when hypothesizing therapy approaches.

Introduction

Breast cancer is one of the most common malignancies and a leading cause of mortality in women [1]. Death, and most of the complications associated with breast cancer, are due to the wide variety of pathologic entities with different outcomes and responses to therapy that this kind of tumor has [2]. Several solid tumors, including breast cancer, exhibit a functional hierarchy of cells of which only a small subpopulation of stem-like cells give rise to the differentiated cells that make the bulk of the tumor [3]. The

cancer stem cell (CSC) hypothesis states that a small population of cancer cells, also termed tumor-initiating cells (TICs), share many biological properties similar to those of embryonic stem cells and are responsible for tumor progression, metastasis, recurrence, and resistance to therapy [4,5]. Therefore, eradicating CSCs could be a therapeutic avenue for overcoming chemoresistance.

Sox2 is a transcription factor that plays an important role in normal stem cells, including pluripotent and tissue-specific stem cell types [6-10]. In recent years, Sox2 has been found in cancers, including those of the lungs, brain, ovaries, bone, colon, skin and breasts [11-18]. In breast cancer cell lines originally derived from human tumors, Sox2 was found in the cancer stem-like cell population [14,19-22]. Studies using various experimental models have demonstrated that Sox2 promotes key tumorigenic properties in cancer cells, including proliferation, invasion, migration, colony formation, non-adherent stem cell associated sphere formations *in vitro* and tumorigenicity *in vivo* [14,18-23]. In breast cancer, expression of Sox2 has been observed in both primary tumors and in tumor derived CSC-enriched non-adherent cultures, "mammospheres", and cell lines [17,18, 21, 22]. These suggest that Sox2 plays a role in breast cancer tumorigenesis [24-26]. While the biological importance of Sox2 in stem cell biology has been well established, in particular we miss a complete understanding in how affects *in vivo* tumor development in a physiological situation. There is, however, increasing evidence that Sox2 is expressed in human breast cancer. Although little is known about the role of Sox2, seems to be expressed in normal breast tissue, at low levels [17,18,21].

Activation of the ErbB family of growth factor receptors and subsequent stimulation of their associated intracellular signaling pathways is a significant factor in the genesis of several human cancers [27]. Amplification of the ErbB2 gene and consequent protein overexpression occurs in 15–30% of primary human breast tumors [28]. ErbB2 stimulates proliferation of breast cancer cells, and overexpression of this protein is strongly associated with poor prognosis [27], as well as resistance to endocrine and conventional chemotherapy [29-32]. A substitution point mutation at residue 664 (Val3Glu) in the transmembrane domain of rat Neu (from now on referred as ErbB2) encodes an activated transforming tyrosine kinase [33]. Overexpression of the mutated ErbB2 in transgenic mice under transcriptional control of the mouse mammary tumor virus (MMTV) promoter, induces mammary adenocarcinoma with high frequency [34,35]. This mouse model serves as an excellent tool for deciphering the molecular pathways responsible for ErbB2-induced tumorigenesis and identifying novel targets for both chemoprevention and chemotherapy [36-41].

In this study, we ask whether Sox2 is required for the *in vivo* development of ErbB2-caused cancer in a mouse model. Here, we used our $Sox2^{flox}$ conditional mutation [42], in combination with the MMTV-ErbB2 mouse model [22], and MMTV-Cre mouse model [43] to address the effects of Sox2 ablation on tumor initiation and maintenance, monitoring the onset of breast tumors in mice and analyzing cell growth *in vitro*.

Materials and methods

Mouse strains

FVB/N mice were purchased from Charles River Laboratories, MMTV-ErbB2 mice [35] and MMTV-Cre mice [43] where purchased from Jackson laboratories (stock number 005038 and 003551, respectively). $Sox2^{flox/flox}$ mice [42] are generated in our lab. All mouse strains were backcrossed to the FVB/N background (as required to obtain full tumorigenicity by the MMTV-ErbB2 oncogene [35]) and then bred to generate $Sox2^{flox/flox}$;MMTV-ErbB2;MMTV-Cre mice and control mice derived from the breedings.

PCR-based genotyping

Primers for PCR-based genotyping of tail DNA were as follows:
$Sox2^{flox}$
RF460 5'- GCAGGTTCCCCTCTAATTAATGC -3'
RF24 5'- AGGCTGAGTCGGGTCAATTA -3'
PCR conditions: 95ºC 5min, 95ºC 40s, 61ºC 30s, 72ºC 45s, for 40 cycles
MMTV-Cre
RF202 5'- GCGGTCTGGCAGTAAAAACTATC -3'
RF203 5'- GTGAAACAGCATTGCTGTCACTT -3'
PCR conditions: 95ºC 5min, 95ºC 30s, 57ºC 30s, 72ºC 40s, for 40 cycles
MMTV-ErbB2
RF275 5'-ATCGGTGATGTCGGCGATAT-3'
RF276 5'-GTAACACAGGCAGATGTAGG-3'
PCR conditions: 95ºC 15min, 94ºC 30s, 48ºC 30s, 72ºC 1min, for 45 cycles
ErbB2 mutation region
RF478 5'-TCCTGTGTGGATCTGGATGA-3'
RF479 5'-GTCAGCGGCTCCACTAACTC-3'
PCR conditions: 95ºC 5min, 95ºC 40s, 60ºC 20s, 72ºC 35s, for 40 cycles

Tumor detection and harvesting

Mice were observed weekly to detect tumors. At 1–2 weeks after initial tumor detection, mice were sacrificed and part of the tumors were immediately dissected, the volume was measured (by immersion in a know volume of PBS in a 15ml Falcon or Eppendorf tube, and annotating the volume increase), stored and fixed in 4% paraformaldehyde in PBS at 4ºC overnight, then put through sucrose gradient (15% sucrose in PBS for 24h at 4ºC and then 30% sucrose in PBS for 24h at 4ºC) and OCT-embedded.

The remainder of the tumor was treated as in Cicalese et al. [66]. Briefly, the tumor was minced into paste with sterile scissors, and a part was kept for DNA and RNA analysis. The other part was washed in PBS, and digested with Enzyme Digestion Mix (DMEM (Gibco), 200units/ml collagenase (Sigma), 100 units/ml hyaluronidase (Sigma), 1% penicillin/streptomycin (EuroClone), 1% glutamine (EuroClone)) for 3h at 37ºC with occasional mixing. The samples were washed with PBS and centrifuged at 600rpm for 5min. The pellet was dissociated with PBS and passed through cell strainer (BD Falcon) of 100um, 70µm, 40µm and 20µm to obtain single-cell suspension. Then it was again centrifuged at 1500rpm for 5 min and the pellet was resuspended in ACK lysing buffer to lyse red blood cells for 1min at 4ºC. Finally washed with PBS, centrifuged at 1200rpm and resuspended in mammosphere medium (MEBM, Lonza)

Tumor mammosphere in vitro culture

Mammosphere culture was essentially as in Cicalese et al. [66]. Briefly, single-cell suspension of tumors were plated in 6 low-adhesion wells (SIGMA) coated with polihema (SIGMA) in stem cell medium (MEBM medium (Lonza), 1% penicillin/streptomycin (EuroClone), 1% glutamine (EuroClone), 5µg/ml insulin (Life Tech), 0,5µg/ml hydrocortisone (Life Tech), 20 ng/ml bFGF (TEBU BIO), 20 ng/ml EGF (TEBU BIO), B-27 supplement (1:50 dilution, GIBCO), and heparin (1:10000 dilution SIGMA)) and cultured at 37ºC, 5% CO2. Spheres were mechanically dissociated every 2 weeks followed by passing through 20µm cell strainers.

Immunofluorescence on sections

OCT-embedded tissue samples were sectioned (20 µm) using a cryostat. For immunofluorescence staining, antigen retrieval was done by boiling samples in 0,01M citric acid and 0,01M sodium citrate (pH 6.0) for 3 min in microwave, followed by gradual cooling at room temperature. The slides were incubated with Blocking buffer and permeabilized (10% normal goat serum (NGS), 0,2% triton x100 in PBS). The primary antibodies used are: anti-Sox2 made in mouse (1:50 dilution, R&D systems), keratin-14 made in rabbit (1:100 dilution, Covance), keratin-18 made in mouse (1:20 dilution, Progen), diluted in Blocking buffer, incubated in a humidified chamber at 4ºC overnight. The samples were washed with PBS and incubated with secondary antibodies (goat anti-mouse Alexa 488 (1:500 dilution, Life Tech) and goat anti-rabbit Alexa 568 (1:500 dilution, Life Tech)) for 45min at room temperature. Nuclei were visualized with 4',6'-diamidino-2-phenylindole (DAPI). The samples were mounted in FluorSave reagent (Calbiochem).

RNA extraction, cDNA synthesis, quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA extraction was performed with Trizol (Life Tech) according to the manufacturer's protocol. Then, 500ng of RNA was reverse transcribed using the High capacity cDNA reverse transcription kit (Applied Biosystem) according to the

manufacturer's protocol. 1:5 dilution of the resulting cDNA mixture was added to the MESA GREEN qPCR MasterMix Plus for SYBR® Assay Low ROX (Biosense) and amplified with target gene specific primers.
Primers sequences:
Sox2
B11 5'-CAGCTGGGCTACCCGCAGCA-3'
F6 5'-CAGCTGGGCTACCCGCAGCA-3'
Oct4
RF472 5'-TTGGGCTAGAGAAGGATGTGGTT-3'
RF473 5'-GGAAAAGGGACTGAGTAGAGTGTGG-3'
MMP3
RF474 5'-TGTCCCGTTTCCATCTCTCTC-3'
RF475 5'-TGGTGATGTCTCAGGTTCCAG-3'
MMP9
RF476 5'-TGCCCATTTCGACGACGAC-3'
RF477 5'-GTGCAGGCCGAATAGGAGC-3'
All genes of interest are normalized to HPRT transcript expression levels. Primers:
HPRT-FW 5'- TCCTCCTCAGACCGGTTT-3'
HPRT-RV 5'- CCTGGTTCATTCATCGCTAATC-3'
qRT-PCR conditions: 95ºC 10min, 95ºC 40s, 62ºC 15s, 60ºC 40s, for 40 cycles

Data were analyzed with a 7500 System Software v1.4 Applied Biosystem

Results

ErbB2 tumor development in breast cancer transgenic mouse lines

We initially wished to test if deletion of Sox2 in mammary epithelium would affect the growth of mammary tumors induced by the expression of the mutated active form of ErbB2, similar to what has been reported for other kind of tumors [44-46] and for some human breast cancer cell lines [21,47,48].

To evaluate the role of Sox2 in tumor initiation and maintenance, we generated mice carrying the MMTV-ErbB2 transgene, two *flox* Sox2 alleles ($Sox2^{flox/flox}$), and the MMTV-Cre (see Materials and Methods). The control mice – retaining Sox2 – harbor different combinations of the wild type (WT) Sox2 allele, the $Sox2^{flox}$ allele and the MMTV-Cre transgene (Figure 1). Based on the published results, expression of MMTV-Cre recombinase starts between P6 and P22 [49], and expression of MMTV-ErbB2 is detectable from postnatal week 6 [66]; thus, Sox2 excision is expected to occur before ErbB2 activation. Mice harboring the MMTV-ErbB2 transgene were checked weekly for tumor appearance. The mice were sacrificed within 2 week after the first tumors were clearly detectable. The dissection usually revealed multiple tumors in each mouse, of

different size. We classified the tumors in large, medium and small depending on the volume (Table 1, Figure 2A).

All MMTV-ErbB2-positive mice developed visible tumors within 6-8 months regardless of the Sox2 genotype; in particular, mice that were expected to be Sox2-deleted in the mammary gland ($Sox2^{flox/flox}$;MMTV-ErbB2;MMTV-Cre) developed tumors within the same time frame as controls (Table 1).

Interestingly, the average tumor size was significantly reduced in Sox2-deleted mice (Table 1, Figure 2B). The overall tumor number was somewhat increased (Table 1, Figure 2C); however, these increase was observed specifically in the small tumors (Table 1, Figure 2D). We found no difference in the tumor growth site nor in the average degree of vascularization or necrosis (supplementary Table 1).

Next, to address Sox2 deletion in tumors, we analyzed the DNA of all tumors from mice expected to be Sox2-deleted, and from various controls by PCR (Table 2, Figure 3). Tumors from mice carrying the Cre transgene and $Sox2^{flox}$ alleles ($Sox2^{flox/flox}$ and $Sox2^{flox/+}$) showed no detectable band corresponding to the undeleted $Sox2^{flox}$ allele; instead, the PCR product corresponding to the Sox2-deleted allele was clearly present (Figure 3B). This indicated efficient excision of $Sox2^{flox}$ both in heterozygous and homozygous tumors.

These results indicate that Sox2-deleted tumors can develop in MMTV-ErbB2 transgenic mice within the same time frame as seen with control mice. The increased fraction of small tumors and the reduction in average tumor size observed following Sox2 deletion suggest a role in the kinetic of tumor growth.

Sox2 expression in MMTV-ErbB2 derived breast tumors

Sox2 is expressed in a consistent percentage of luminal A and B human tumors which include ErbB2-expressing tumors [51] and pre-neoplastic tissue [62]. However, the Sox2 expression pattern in mouse MMTV-ErbB2 induced tumors has never been specifically addressed. Thus, we addressed Sox2 expression in MMTV-ErbB2 tumors by qRT-PCR.

We isolated total RNA and performed qRT-PCR for Sox2 on all the tumors shown in Table 2. We also investigated if Sox2 RNA was present in Sox2-deleted tumors, to rule out the possibility that the tumor could be derived from a subpopulation of cells in which the Sox2 gene was still present. All samples (controls and Sox2-deleted) showed non-detectable levels of Sox2; as a reference for Sox2 expression levels, we used serial dilutions of RNA from a Sox2-expressing oligodendroglioma (see Chapter 1). Sox2 expression in mammary tumors was lower than that observed in a 1:5000 dilution of oligodendroglioma RNA (Figure 4).

Sox2 expression in MMTV-ErbB2 derived tumor mammospheres

In most of the described mammospheres cultures derived from human breast tumors Sox2 is reported to be expressed [21,58,59]. We hypothesized that Sox2 might be expressed also in "mammospheres" derived from our tumors, since *in vitro* culture is a stem cell enriched model. We thus generated mammospheres cultures from control tumors, retaining both copies of Sox2, expanded them for two passages *in vitro* to enrich for the stem cell component, and analyzed them for Sox2 expression by qRT-PCR. Also in this case Sox2 was undetectable (Figure 4) (levels lower than 1:5000 of Sox2 levels in oligodendroglioma).

Sox2 expression by immunofluorescence in tumor and mammary gland tissue

Sox2 is a stem cells marker, and in breast cancer tissue, the mammary tumor-initiating cells were identified at a frequency of 1 every 300 tumor cells [52]; hence, hypothesizing that Sox2 might be expressed only or preferentially in tumor-initiating cells, the overall Sox2 levels expected in qRT-PCR experiments in tumor tissue might be very low. For that reason, we performed an immunohistochemical evaluation of Sox2 protein by immunofluorescence in tumor sections allowing single cell resolution, to allow the identification of rare Sox2-expressing cells, if they were present. We analyzed various control tumors and Sox2-deleted tumors; in particular we analyzed several small tumors because of published observations reporting Sox2 expression specifically in early tumors [53]. We also included some large tumors in our analysis because other studies had reported that Sox2 was expressed more frequently in tumors of large size and higher histological grade [54]. All of the tumors we analyzed were negative for Sox2 (Figure 5A).

The reported existence of Sox2 positive mammary tumors (see Introduction), raises the question of Sox2 expression in normal mammary tissue. Indeed, contrasting reports exist in the literature regarding this point; Chen et al. report absence of Sox2 expression from the mammary epithelium [18], whereas Vazquez-Martin et al. report low level expression [48]. In our experiment, the WT mammary gland (taken from an FVB female) also shows no detectable staining for Sox2 by immunohistochemistry (Figure 5B). As a positive control for Sox2 expression we used a P0 mouse brain where Sox2 is known to be expressed [42].

The normal development of the mammary gland is not impaired in MMTV-Cre Sox2-deleted mice

Prior to tumor development, mice carrying mammary Sox2-deletion have no problem with lactation – all the females fed their pups as well as controls, and no abnormality was detected in the mammary gland (data not shown). This observation supports the notion that Sox2 is not necessary for normal mammary gland development, at least postnatally – when our transgenic model loses Sox2.

Tumor marker analysis of MMTV-ErbB2 induced tumors

It has been reported that Sox2 is expressed in tumors that maintain ductal structure [48], so we asked if the ducts were detectable in these MMTV-ErbB2 derived tumors. For this purpose we analyzed the expression of two markers of the ductal system, cytokeratin 14 (K14) for basal/mesenchimal cells and cytokeratin 18 (K18) for luminal cells (Figure 6). Only 2 out of 14 analyzed tumors of different size and genotype, show some intact ducts. Sox2 was not detectable in those tumors, as in the duct-negative tumors. On the other hand, a correlation was apparent between cytokeratin expression and tumor size: the small tumors are K18-positive, but negative for K14; the medium size tumors show lower K18 positivity compared with the small ones, and show little K14 positivity; the large tumors have few K18-positive cells and more K14-positive cells compared to medium and small size tumors (Figure 6). Thus, the MMTV-ErbB2 induced tumors appear to have luminal tumor features (expression of K18) at early stages, as observed also by other research groups [65]; however, in their subsequent development (bigger tumors) they may develop basal/mesenchimal features (K14 expression).

We also analyzed the tumors to confirm that they have gene expression characteristics previously described for the MMTV-ErbB2 induced tumors [51]. We first confirmed that ErbB2 transgene transcribed in the tumors carries the mutation 664 V-E [33], as expected, by PCR amplification and sequencing (not shown). Then, we analyzed if other genes usually expressed in these breast tumors were expressed in the tumors we were studying.

Octamer4 (Oct4), another early stem cell marker like Sox2, is reported to be expressed in some of the MMTV-ErbB2 derived tumors [51], and also the matrix metallopeptidase 3 (MMP3) and matrix metallopeptidase 9 (MMP9) have been detected in this and other breast cancers [51, 55, 56]. We analyzed the RNA of various tumors for these three markers with a qRT-PCR and we found out that all of them were expressed, regardless of tumor genotype. In particular, we tested tumor samples with two, one or no copies of Sox2, and with or without the Cre transgene ($Sox2^{flox/+}$,MMTV-ErbB2,MMTV-Cre or $Sox2^{flox/+}$, MMTV-ErbB2 or $Sox2^{+/+}$, MMTV-ErbB2, MMTV-Cre or $Sox2^{flox/flox}$, MMTV-ErbB2, MMTV-cre). The analyzed markers were expressed in all samples, although at different levels, whereas Sox2 was never detected (Figure 7).

Discussion

In this work, we have addressed the consequences of Sox2 deletion on the development of mouse breast tumors caused by expression in mammary tissue of a transgene expressing the ErbB2 oncogene carrying a 664 (Val to Glu) mutation [33].

Sox2 loss does not prevent MMTV-ErbB2-determined tumorigenesis, though Sox2-deleted tumors are on average smaller

We observed MMTV-ErbB2 induced breast tumor development in all the analyzed mice, including those carrying the MMTV-Cre mediated Sox2 deletion. Thus, Sox2 presence is not necessary for the development of MMTV-ErbB2 induced tumors. The Sox2 gene in the $Sox2^{flox/flox}$;MMTV-ErbB2;MMTV-Cre model mouse is deleted early in mammary gland development (around P6) [43]; thus, our data suggest that tumor initiation (induced by MMTV-ErbB2 expression which starts around 6 weeks) [66] can happen in the absence of Sox2.

Although the average number of tumors per mouse was not reduced by Sox2 deletion, the mean tumor size of the Sox2-deleted tumors was smaller compared to the control genotypes (Table 1; Figure 2). A similar effect was previously described in mice inoculated with tumorigenic MCF-7 cells, a human derived breast cancer cell line that does express Sox2, following Sox2 silencing by siRNA [48]. The fact that the size of the tumor is reduced when Sox2 is depleted, suggests a possible quantitative role for Sox2 in tumor development. In the case of our tumors, it could be an indirect role of Sox2 since the tumors do not express detectable levels of this transcription factor. On the other hand, it is possible that few cells initially expressing Sox2 give rise to the tumor and at some point, early in tumor development, they stop expressing Sox2.

While average tumor size is smaller following Sox2 deletion, the absolute number of very small tumors is somewhat increased (Table 1, Figure 2). The fact that we observe an increased number of small tumors might be due to a possible role of Sox2 to control the origin of the cells that can be forced by ErbB2 mechanism to form tumors. Perhaps, the fact that without Sox2, MMTV-ErbB2 is sufficient for mammary tumorigenesis initiation [34] but not for full size tumor development, somehow favours an increased development of new tumors. This hypothesis needs further investigation to really understand the mechanism behind this event.

A comparison of Sox2 function in human breast tumor-derived cell lines and in MMTV-ErbB2-derived mouse breast tumors

Sox2 was previously described to be expressed and functionally important within human breast cancer-derived cell lines grown *in vitro,* including ErbB2 expressing cell lines [21]. However Sox2 does not appear to be required for *in vivo* tumorigenesis in our breast tumor mouse model generated by ErbB2 overexpression. How can we reconcile these observations?

In our mouse model, high ErbB2 expression is driven by a trangene driven by the MMTV promoter, which is the primary cause of tumorigenesis. In human tumor cell lines, ErbB2 expression must depend on different mechanisms; this include endogenous ErbB2 amplification, and other mechanisms still to be understood (in those cell lines

which do not have ErbB2 amplification). It is possible that in patients' tumor cells, but not in mouse tumor cells, Sox2 activity is upstream of ErbB2, and contributes to maintain its expression; in our mouse model, the high expression of ErbB2 driven by the strong MMTV promoter my override the need for Sox2.

MMTV-ErbB2 tumors do not express detectable Sox2

The fact that we could not find Sox2 expression in the MMTV-ErbB2 breast tumor model (Figures 4,5 and 7) was somewhat surprising since we were expecting that in some stage of tumor development Sox2 would be expressed.

In humans, Sox2 expression was described in both early (small) and larger, more developed, breast tumors [21,48,54]; thus, we analyzed all tumor sizes in our model system for Sox2 expression without detecting any (Figures 4, 5 and 7). As discussed above, whether Sox2 is expressed in very early stages of this tumor has still to be thoroughly examined for example by analyzing pre-tumorigenic cells [66]. The use of a YFP or GFP reporter driven by Sox2 regulatory sequences, could be of help to enhance sensitivity if expression is too low [67]; alternatively the use of Cre-ERT2 transgene knock-in at the Sox2 locus [68]) in combination with a YFP reporter of Cre activity may be used to label Sox2 expressing cells and their progeny in the developing tumors, if they exist.

Sox2 expression was detected by many groups in all breast cancer tumor subtypes in humans, where it is expressed in a variable proportion of cells; however, diverse, sometimes contrasting data exist in the literature as to the proportion of tumors expressing Sox2 within these different subtypes [18,21,48,54,60,61,63]. Some reports observed Sox2 expression particularly in early stages of tumor development [21,48]. Other reports also find Sox2 expression in patients with different human breast tumor subtypes; here, however, Sox2 overexpression (in comparison to normal mammary tissue, showing no or weak expression) was observed more pronouncedly in tumors of higher grade [18,54.

The mouse model we are studying is described in literature as a tumor model that has features of human luminal B tumor type, where Sox2 expression has been described. Human luminal B tumors are ErbB2 and K18 (a marker for luminal epithelial cells) positive [57]. We indeed saw the luminal signature (K18 expression) in our tumors, as expected (Figure 6); however, in tumors of bigger size, the luminal marker K18 was reduced, and the basal marker K14 appeared in a set of cells. This result suggests that when our tumors develop and grow, they may evolve a different identity with a different gene expression signature from the cell that initiated the tumor. Thus, our mouse tumor may represent a more advanced state of some luminal B tumors that no longer expresses Sox2. As discussed above, it will be interesting to look for a possible Sox2 expression at very early stages of tumor development in our model, using more sensitive tools.

In conclusion, a diversity exists within human tumors with respect to Sox2 expression and it is likely that MMTV-ErbB2 mouse model is representative of some human tumor subclasses, and or developmental stages, but not others.

The greatest part of the studies on Sox2 function in human breast tumors have been performed in tumor derived cell lines that do express Sox2, for example MCF-7 [61, 47, 48] and ZR751 [59]. In particular some functional studies have manipulated Sox2 levels in such cell lines and found that Sox2 levels were important for *in vitro* growth. These human cell lines are derived from luminal type tumors, but, differently from the cells of the MMTV-ErbB2 mouse tumor, are ER^+ and ErbB2 negative. Thus, our mouse tumor may not be representative of the human tumors from which these cell lines were derived. However, a different explanation may lie in the fact that these human cells have been expanded in culture extensively over many passages. It is thus possible that they evolved through this long term *in vitro* culture, characteristics different from the original cancer stem cells *in vivo*. These characteristics may involve Sox2 expression that it is known to give advantage to cells *in vitro*.

Contrasting results have been reported in literature with respect to Sox2 expression in the MCF-7 line: Piva et al. [47], found it difficult to detect Sox2 expression. This data further emphasize the fact that *in vitro* culture is an important factor in determining gene expression patterns. This is one of the reasons why we focused our research on *in vivo* assays, because it is important to determinate the Sox2 necessity for the tumor development, in physiological conditions.

Trying to characterize the tumors developed from our breast cancer transgenic model, we confirmed the expression of markers previously seen in the MMTV-ErbB2 induced tumors, including Oct4, MMP3 and MMP9 [51] (Figure 7).

Sox2 requirement in normal mammary gland development

Low levels of Sox2 expression have been described in the normal mammary gland by some reports [48], but not others [62], we could not detect any significant Sox2 expression by immunofluorescence (Figure 6). Consistently, Sox2-deletion does not appear to affect normal mammary gland development, since no problem is seen in the mammary gland following Sox2 loss and our mice fed pups normally. Thus, it seems that there is not a major requirement of Sox2 in the mammary gland development.

Sox2 is undetectable in tumor mammospheres in vitro

We analyzed Sox2 expression in MMTV-ErB2 tumor derived mammospheres due to the fact that they are described to be enriched in CSC; it was thus possible that Sox2 expression would be enriched in these cells. Sox2 was previously detected, in tumor derived mammospheres, but only in two out of three cultures analyzed, derived from

different tumors [21]; this again indicates a possible heterogeneity with respect of Sox2 expression present also at the level of cancer stem cells enriched cultures (mammospheres). However, our analysis was done at passage 2; it is possible that some increase in Sox2 may occur with further passaging, in fact Leis et al. showed that Sox2 expression was at early passages only within very few cells (2,9%) but in late passages it increased to a higher subset of cells (32%) [21]. We can also consider to use more sensitive tools for Sox2 expression detection (immunofluorescence, Sox2-GFP constructs) as described above for tumor studies.

Even in normal mammary gland derived mammospheres we did not detect Sox2 (Figure 4). This supports the notion that the expression of Sox2 seen in some mammary tumors and tumor derived mammospheres is a feature acquired during tumorigenesis and not originally present in the normal mammary stem cell.

In conclusion, our studies highlight that Sox2 expression and function are likely diverse within different breast cancers. This needs to be taken into account when hypothesizing approaches for therapy.

Figures

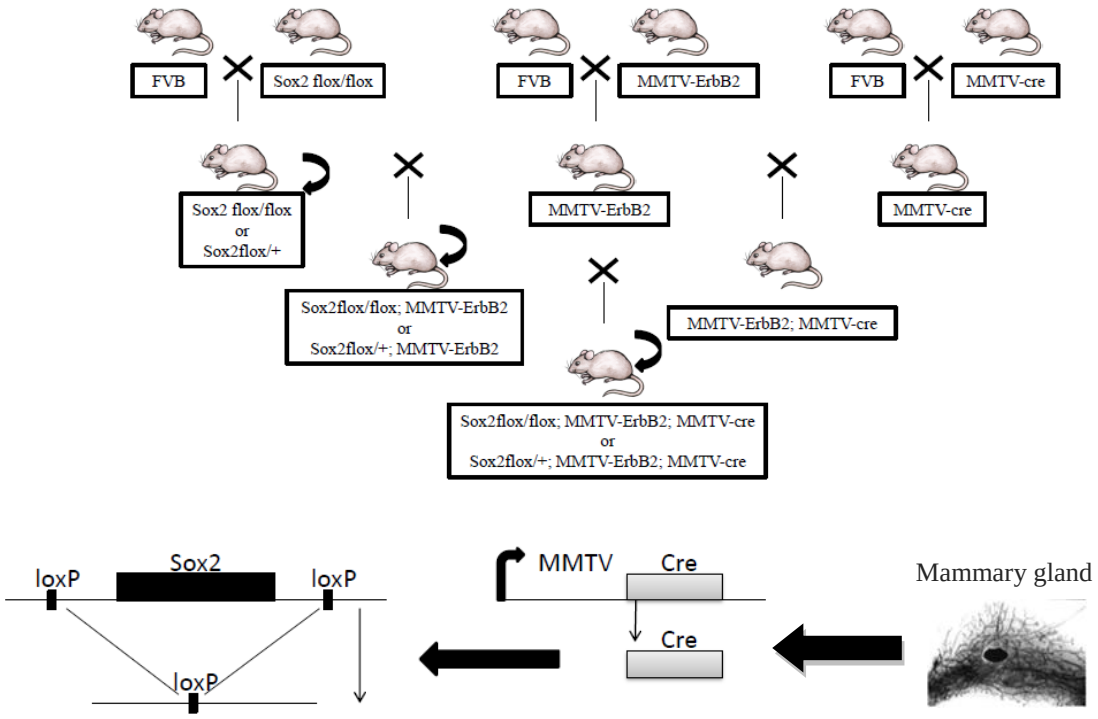
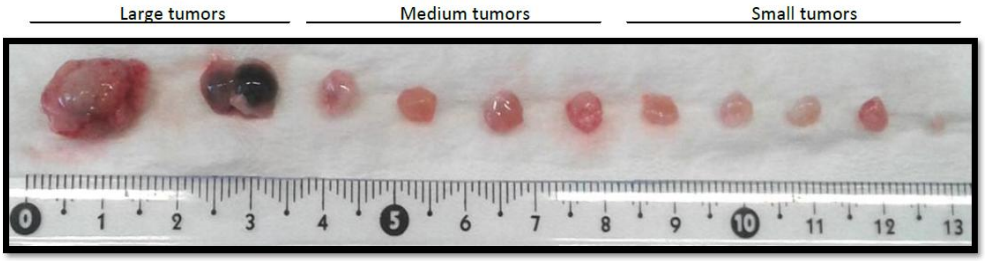


Figure 1. Diagram of the experiment.
All the transgenic mice used are enriched in FVB background. The MMTV-ErbB2 and MMTV-Cre genes are transcribed in the mammary gland. Sox2 is excised only where the cre transgene is active, in the mammary gland.

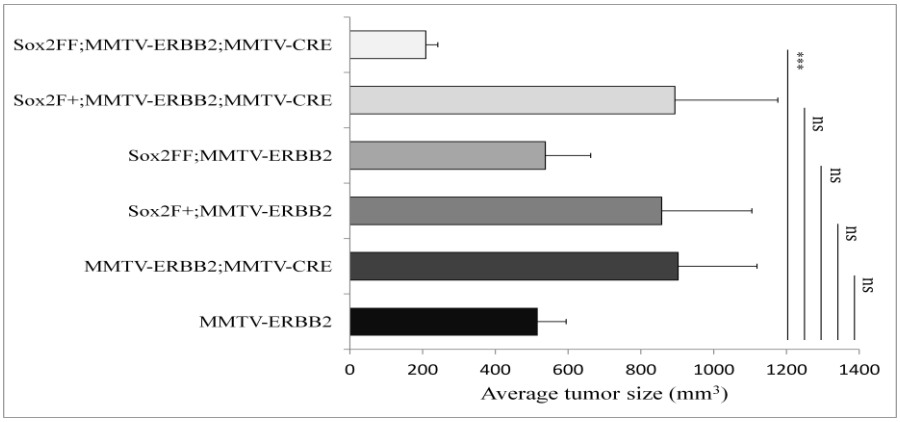
Genotype	Total mice (nº)	Average age of tumor (months)	Total tumors (nº)	Average tumor nº/ mouse	Tumor size			
					Average tumor size (mm^3)	L (nº and %)	M (nº and %)	S (nº and %)
MMTV-ErbB2	11	7.3	36	3.27	515,9	11 (30,55%)	15 (41,67%)	10 (27,78%)
MMTV-ErbB2, MMTV-cre	8	8.2	25	3.13	902	10 (40%)	9 (36%)	6 (24%)
$Sox2^{flox/+}$, MMTV-ErbB2	14	7.4	43	3.07	856,9	14 (32,56%)	20 (46,51%)	9 (20,93%)
$Sox2^{flox/flox}$, MMTV-ErbB2	9	7.6	31	3.44	537,1	10 (32,26%)	13 (41,93%)	8 (25,80%)
$Sox2^{flox/+}$, MMTV-ErbB2, MMTV-cre	9	7.4	20	2.22	893,5	6 (30%)	10 (50%)	4 (20%)
$Sox2^{flox/flox}$, MMTV-ErbB2, MMTV-cre	8	6.7	46	5.75	208,4	5 (10,87%)	12 (26,09%)	29 (63,04%)

Table 1. Frequency and size of MMTV-ErB2 induced tumors in different genotypes.
Spontaneous tumors of female mice of different genotypes were dissected and analyzed. L=large >500mm^3; M=medium 100-500mm^3; S=small ≤100mm^3.

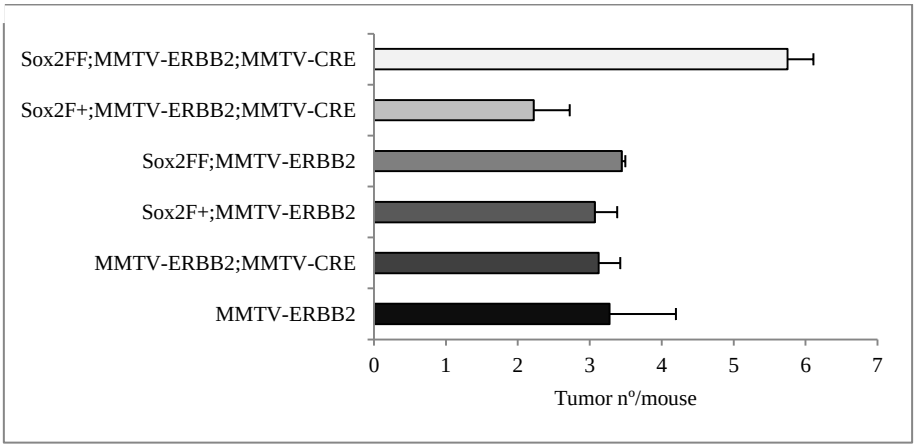
A


Large tumors
Medium tumors
Small tumors

B


Sox2FF;MMTV-ERBB2;MMTV-CRE
Sox2F+;MMTV-ERBB2;MMTV-CRE
Sox2FF;MMTV-ERBB2
Sox2F+;MMTV-ERBB2
MMTV-ERBB2;MMTV-CRE
MMTV-ERBB2
***
ns
ns
ns
ns
0
200
400
600
800
1000
1200
1400
Average tumor size (mm³)

C


Sox2FF;MMTV-ERBB2;MMTV-CRE
Sox2F+;MMTV-ERBB2;MMTV-CRE
Sox2FF;MMTV-ERBB2
Sox2F+;MMTV-ERBB2
MMTV-ERBB2;MMTV-CRE
MMTV-ERBB2
0
1
2
3
4
5
6
7
Tumor n°/mouse

D

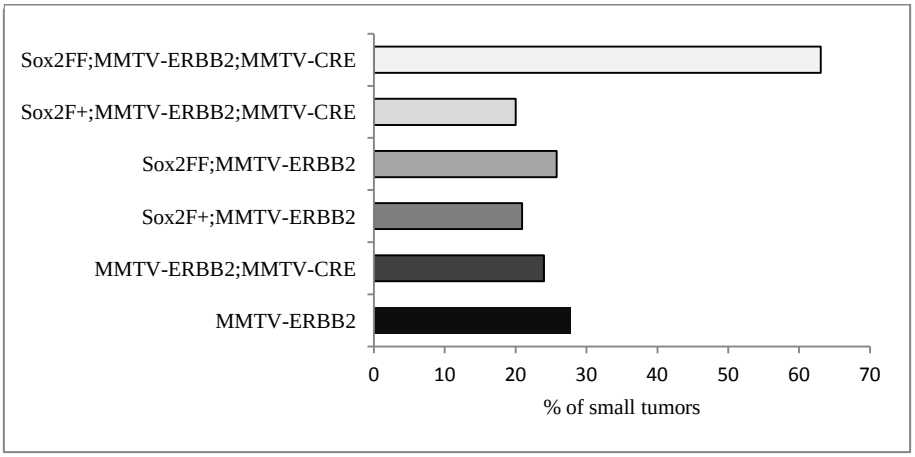

Sox2FF;MMTV-ERBB2;MMTV-CRE
Sox2F+;MMTV-ERBB2;MMTV-CRE
Sox2FF;MMTV-ERBB2
Sox2F+;MMTV-ERBB2
MMTV-ERBB2;MMTV-CRE
MMTV-ERBB2
0
10
20
30
40
50
60
70
% of small tumors

Figure 2. Tumor development in mice of different Sox2 genotypes.
A, Examples of dissected tumors of different size. From left, the first four tumors are from control mice (Sox2flox/+;MMTV-ErbB2;MMTV-cre). The others are from Sox2-deleted mice (Sox2flox/+;MMTV-ErbB2;MMTV-cre). **C**, Average tumor number per mouse for every genotype. **B,** Mean tumor size per genotype. The Sox2-deleted mice tumors are significantly smaller (***P<0,05, Student's t-test comparing MMTV-ErbB2 coupled with all the other genotypes). than the tumors of the other genotypes. The error bar indicates mean error. **D,** Percentage of small tumors per genotype. The Sox2-deleted mice present more small tumors than the control genotypes.

Genotype	Total mice (nº)	Total tumors analyzed (nº)	Tumor size		
			L (nº)	M (nº)	S (nº)
MMTV-ErbB2,MMTV-cre	1	2	-	1	1
$Sox2^{flox/+}$, MMTV-ErbB2	1	2	1	-	1
$Sox2^{flox/flox}$,MMTV-ErbB2	1	3	1	1	1
$Sox2^{flox/+}$,MMTV-ErbB2,MMTV-cre	7	16	3	9	4
$Sox2^{flox/flox}$,MMTV-ErbB2,MMTV-cre	8	46	5	12	29

Table 2. Tumors analyzed at DNA (Sox2 deletion) and RNA (Sox2 expression) level
We analyzed all tumors from all mice of the Sox2-deleted genotype (Sox2flox/flox;MMTV-ErbB2; MMTV-cre) available; and a representative sample of control mice.

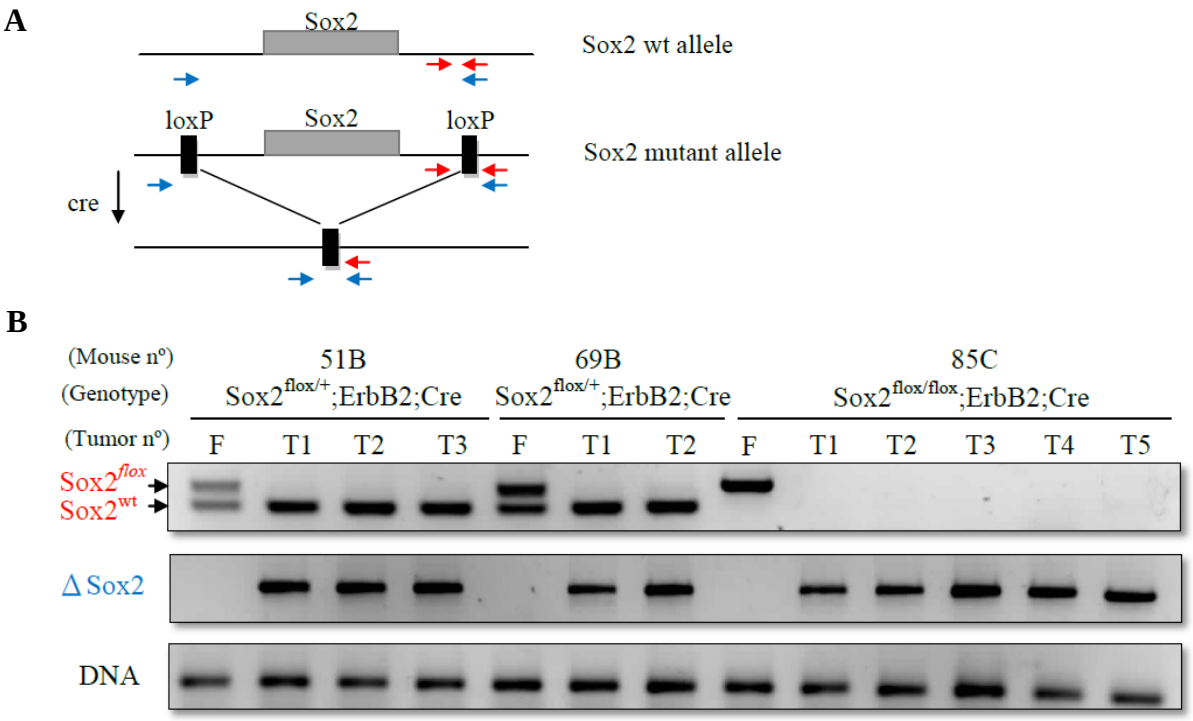


Figure 3. Sox2 deletion in tumor DNA.

A, Skin of the PCR assay for Sox2 deletion. Primers for detecting the undeleted (red arrows) or the deleted (blue arrows) Sox2flox allele are shown. The red primer pair amplifies a smaller band in wild type (Sox2wt) as compared to Sox2flox carrying loxP insert. **B**, Representative PCRs on genomic DNA from tumors of different genotypes (T1, T2…). Top row: PCR with red primers detecting undeleted Sox2 (Sox2flox and Sox2wt). Middle row: PCR with blue primers detecting deleted Sox2 (ΔSox2). Bottom row: PCR with non related genomic locus (interleukin 2) for normalization. F: finger DNA (control).

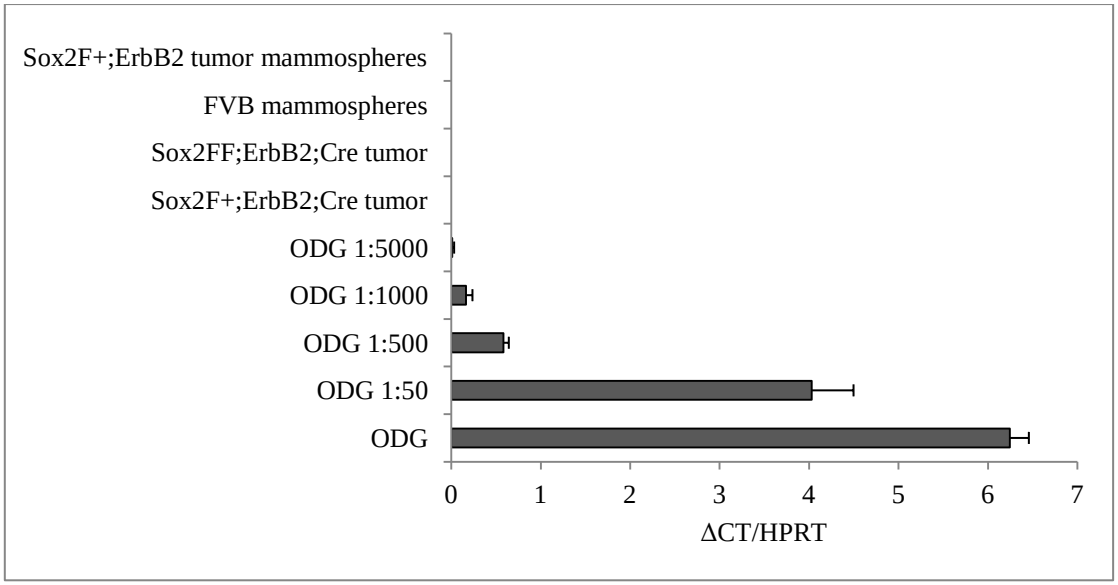


Figure 4. qRT-PCR of representative samples of breast tumors and mammospheres. ODG: pHGG cells used as positive control in sequential dilutions in a Sox2 non-expressing RNA. A control breast tumor (Sox2F+;ErbB2;Cre tumor) and a Sox2-deleted tumor (Sox2FF;ErbB2;Cre tumor) were analyzed together with *in vitro* cultured cells derived from WT mouse mammary gland (FVB mammospheres) and from a control tumor (Sox2F+;ErbB2 tumor mammospheres). Sox2 was undetectable in all of the breast cancer or tissue derived RNA.

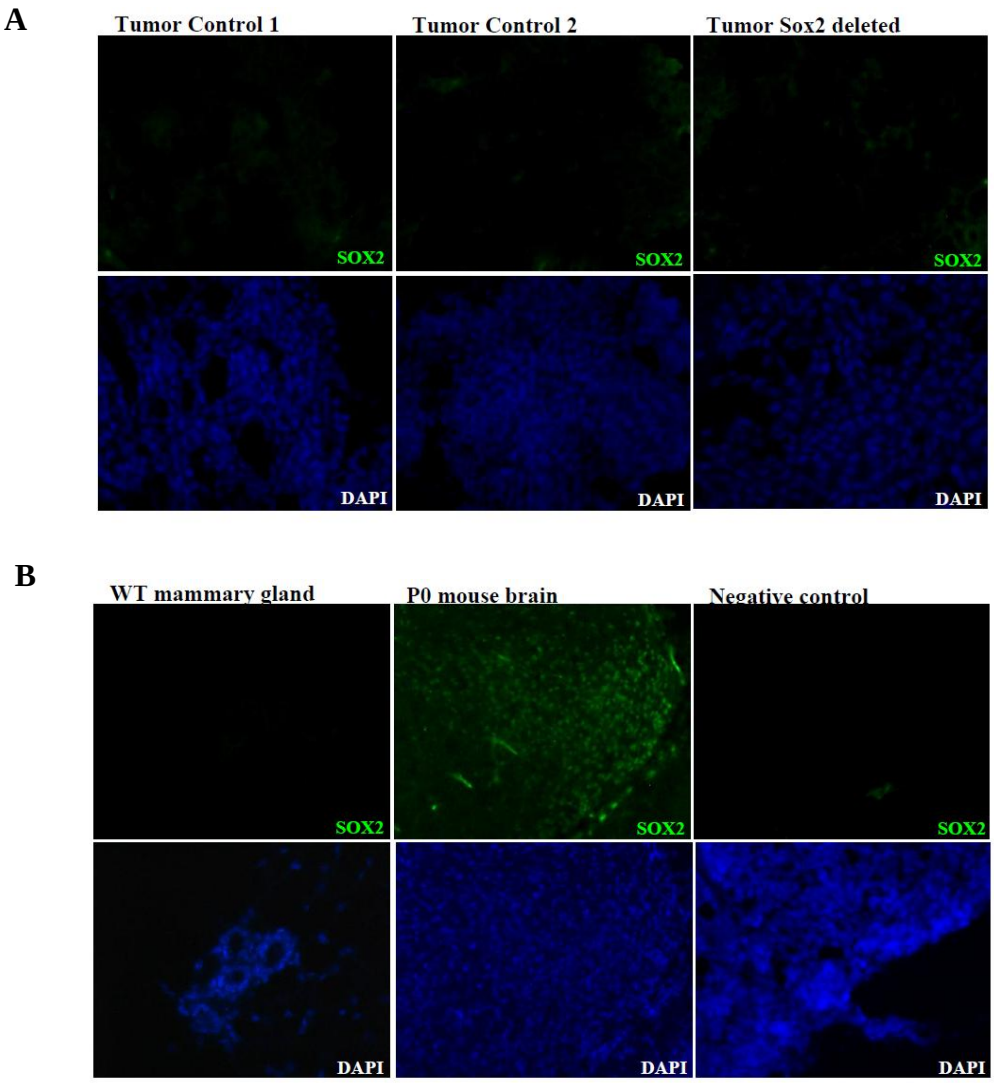


Figure 5. Sox2 immunofluorescence of tumor samples and wt mammary gland.
A, Top row: Sox2 immunofluorescence (green). Bottom row: corresponding DAPI staining. The light green in the background also appears in negative control. **B**, WT mammary gland (left), P0 mouse brain (middle) as positive control, and negative control (right) staining. Top row: Sox2 staining (green). Bottom row: corresponding DAPI staining. Many Sox2 positive cells are visible in the brain positive control. No clear positivity is detected within the tumors, nor in the mammary gland.

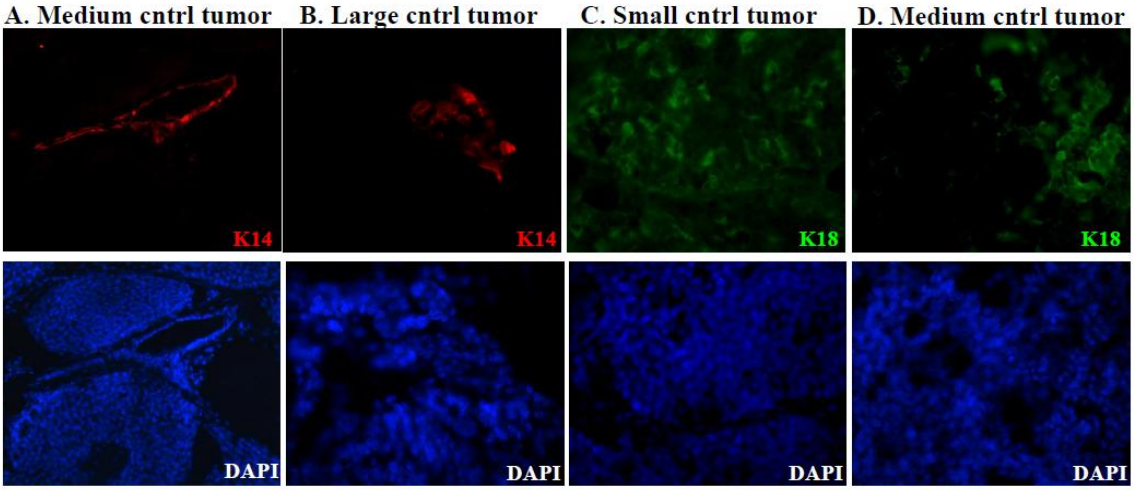


Figure 6. Immunofluorescence for characteristic markers of luminal or basal mammary cells.

Upper row: staining for keratin 14 (K14) in red and keratin 18 (K18) in different tumors (A-D). Bottom row: corresponding DAPI staining. Medium tumors show K14 positivity only in the ducts (when they are present), while large tumors have more K14 positive regions. Small tumors are extensively K18 positive, while this positivity decrease in medium size tumors.

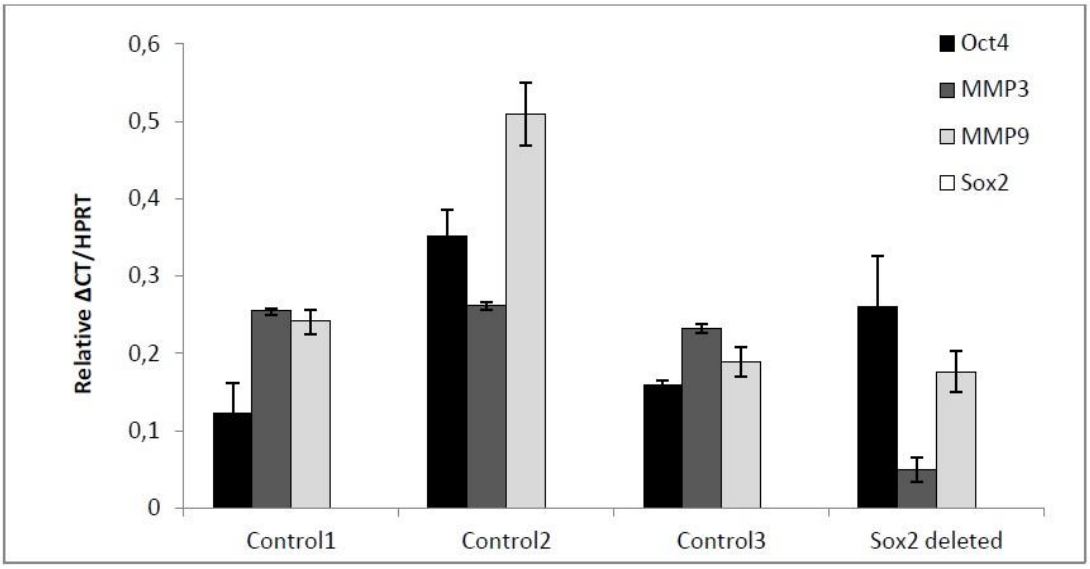


Figure 7. Sox2-deleted breast tumors to detect mRNA for Sox2 and for Oct4,MMP3 and MMP9 normally expressed in MMTV-ErbB2 tumors. Genotypes are: Control1 ($Sox2^{flox/flox}$,MMTV-erbB2), Control2 ($Sox2^{+/+}$,MMTV-erbB2,MMTV-cre), Control3 ($Sox2^{flox/+}$,MMTV-erbB2,MMTV-cre) and Sox2-deleted ($Sox2^{flox/flox}$,MMTV-erbB2,MMTV-cre). The histogram show mean expression values obtained from triplicate qRT-PCR assays, $\pm$ standard deviation, relative to HPRT, on representative tumor of indicated genotypes.

Supplementary figures

Line	Total tumor (n°)	Tumor position			Vasc.		Necrosis	
		A-L (n°)	P-R (n°)	P-L (n°)	Yes (n°)	No (n°)	Yes (n°)	No (n°)
MMTV-ErbB2	36	9	3	15	14	22	9	27
MMTV-ErbB2, MMTV-cre	25	4	5	10	12	13	6	19
$Sox2^{flox/+}$, MMTV-ErbB2	43	14	7	10	13	30	4	39
$Sox2^{flox/flox}$, MMTV-ErbB2	31	5	6	11	15	16	5	26
$Sox2^{flox/+}$, MMTV-ErbB2, MMTV-cre	20	5	5	3	3	17	1	19
$Sox2^{flox/flox}$, MMTV-ErbB2, MMTV-cre	46	17	9	14	25	21	6	40

Supplementary Table 1. Comparison of tumors in different mouse genotypes.
Tumor position was annotated (A-R= anterior-right; A-L= anterior-left; P-R= posterior-right; P-L= posterior-left). Other characteristics such as vascularization (vasc.) and necrosis are reported.

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DISCUSSION AND FUTURE PERSPECTIVES

Focusing on Sox2

Sox2 is one of the most important transcription factor for stem cells and therefore, for the development. In fact, its ablation causes early embryonic lethality [1]. In adult life, it is expressed mainly in the central nervous system, specifically in the subventricular zone and hippocampus [2]. Interestingly, there is a lot of evidence from researches that Sox2 is aberrantly reactivated in a huge amount tumors, for example brain, lung and breast, suggesting that Sox2 has an important role in cancer and might be a good candidate for cancer therapy.

Tumors have been regarded as homogeneous populations of cells with high proliferating activity, but this view is changing and nowadays it is accepted that a small population of cells with stem cell properties is responsible for the maintenance and growth of the tumor [3]. Thus, normal stem cells could be the origin of cancer stem cells or alternatively a more differentiated progeny may revert to a stem like status and give rise to cancer stem cells [4]. The notion of CSCs has important implications for cancer treatment.

Genes originally identified as important for normal stem cells may be essential also to support cancer stem cells. Several stem cell genes are found to be overexpressed in tumors even if they are not mutated or amplified, suggesting that these genes may be important for the generation of cancer stem cells from more differentiated precursors, or for cancer stem cell maintenance. Sox2 represents an example of these stem cell markers genes that is overexpressed, but not mutated, in tumors. In this regard, the Sox2 conditional knock-out mouse model developed in our laboratory, could provide a useful tool to enlighten the requirement of Sox2 in cancer.

Under appropriate conditions cancer stem cells efficiently differentiate *in vitro*, losing their stem cell properties and tumorigenicity, together with repression of Sox2 and other stem cell genes [5,6]. Furthermore we have demonstrated that the expression of Sox2 is necessary for oligodendroglioma onset. In many breast cancers Sox2 aberrant expression is reported, but the complexity of this type of cancer is huge and the causes for the onset of the tumor are not all known and well understood. Thus, suggesting Sox2 as a master gene in this case is not really appropriate. The cancer stem cell specific biological and molecular mechanisms that sustain their existence are still poor known. Further studies on stem cell genes and on signaling pathways are extremely important and may provide useful information to identify genes and molecules helpful for differentiation therapy of cancer stem cells or for apoptosis induction of tumor cells [7].

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nata a Ondarroa il 26/01/1986,
autrice della tesi di dottorato dal titolo:

Study of the role of the Sox2 transcription factor in neural and mammary cancer stem cells using Sox2 conditional knock-out in mouse

AUTORIZZA

La consultazione della tesi stessa, fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto.

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