Tumor and Stem Cell Biology

Sox2 Requirement in Sonic Hedgehog-Associated Medulloblastoma

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Abstract

The transcription factor Sox2 has been shown to play essential roles during embryonic development as well as in cancer. To more precisely understand tumor biology and to identify potential therapeutic targets, we thoroughly investigated the expression and function of Sox2 in medulloblastoma, a malignant embryonic brain tumor that initiates in the posterior fossa and eventually spreads throughout the entire cerebrospinal axis. We examined a large series of tumor samples (n = 188) to show that Sox2 is specifically expressed in Sonic hedgehog (SHH)-associated medulloblastoma with an interesting preponderance in adolescent and adult cases. We further show that cerebellar granule neuron precursors (CGNP), which are believed to serve as the cell of origin for this medulloblastoma subgroup, express Sox2 in early stages. Also, Shh-associated medulloblastoma can be initiated from such Sox2-positive CGNPs in mice. Independent of their endogenous Sox2 expression, constitutive activation of Shh signaling in CGNPs resulted in significantly enhanced proliferation and ectopic expression of Sox2 in vitro and Sox2-positive medulloblastoma in vivo. Genetic ablation of Sox2 from murine medulloblastoma did not affect survival, most likely due to a compensatory overexpression of Sox3. However, acute deletion of Sox2 from primary cultures of CGNPs with constitutive Shh signaling significantly decreased proliferation, whereas overexpression of Sox2 enhanced proliferation of murine medulloblastoma cells. We conclude that Sox2 is a marker for Shh-dependent medulloblastomas where it is required and sufficient to drive tumor cell proliferation. Cancer Res; 73(12); 3796–807. ©2013 AACR.

Introduction

Medulloblastoma is the most common malignant brain tumor in childhood with an average of 5-year overall survival rate of 66% (1). State of the art treatment combines surgery, craniospinal irradiation, and multiple chemotherapeutics with severe sequelae of toxic side effects (2–4). The development of novel treatment options is therefore urgently needed. Medulloblastoma comprises 4 core subgroups that are defined by distinct gene expression signatures from global gene expression arrays. These subgroups that are named WNT medulloblastoma, SHH medulloblastoma, Group 3 medulloblastoma, and Group 4 medulloblastoma, significantly differ with respect to genetics, histology, and clinical outcome (5, 6). Consequently, diagnostic markers as well as potential therapeutic targets have to be identified in a subgroup-specific manner.

While Group 3 and 4 medulloblastomas are characterized mostly by amplifications in MYC genes and the WNT-associated group features pathway-activating mutations in the Ctnnb1 gene, Sonic hedgehog (SHH)-associated medulloblastomas are characterized by a constitutive activation of the SHH signaling pathway. The pathologic activation is caused mainly by mutations of genes encoding the SHH receptors PTCH (patched) or SMO (smoothened; refs. 7, 8). SHH medulloblastomas have recently been shown to arise from cerebellar granule neuron precursors (CGNP; ref. 9), which, during normal development, extensively proliferate due to a physiologic stimulation by the secreted protein SHH. SHH-induced proliferation continues until granule cell precursors migrate away from their mitotic niche within the external granule cell layer (EGL) and differentiate at postnatal stages to form the inner granule cell layer (10, 11). Apart from its role in cerebellar development, SHH is essential for the development of various structures in the central nervous system, including the hippocampus, by regulating proliferation of neural stem cells (12, 13). In this context, a close interaction between SHH signaling and the function of the nuclear transcription factor SOX2 [sex determining region Y (SRY)-box 2] has recently been suggested. Favaro and colleagues were able to show that loss of Sox2 in...
the murine hippocampus resembled the defects that are caused by late Shh-loss (12).

SOX2 is associated with self-renewal, and pluripotency of embryonic stem cells (14, 15) plays a key role in various stages of mammalian development (16, 17) and is crucially involved in cancer development (18, 19). For instance, amplification and overexpression of Sox2 has been shown to be correlated with increased proliferation and tumorigenicity of stem cell-like cells in a number of tumor types (19, 20). In the cerebellum, Sox2 is well known to be expressed in mature glia, but an additional population of Sox2-positive cells has recently been discovered within the white matter of the cerebellum (21). This population not only has stem cell potentials, but it was even shown to be susceptible for neoplastic transformation. *Ex vivo* deletion of p53 and Rb in such cells followed by a subcutaneous injection into immunocompromised mice resulted in the formation of highly aggressive tumors, as shown by Sutter and colleagues (21). However, the expression of SOX2 in human medulloblastomas, its functional relevance for tumorigenesis, and its interaction with SHH signaling has never been thoroughly investigated.

**Materials and Methods**

**Patients and tissue samples**

A total of 65 formalin-fixed, paraffin-embedded surgical tumor specimens from patients with medulloblastoma were analyzed. Inclusion of patients in the study was unbiased and only dependent on the availability of sufficient tumor material and clinical follow-up data. Patients included 36 males (55.4%) and 29 females (44.6%). They were treated in the University Hospitals of Munich, Göttingen, Bremen, Hannover, and Münster (Germany). The majority of patients was enrolled in the German Society of Pediatric Hematology and Oncology multicenter treatment studies for pediatric malignant brain tumors (HIT). The median age was 7.4 years (range, 0.5–51.8 years). The study included 33 medulloblastomas of desmoplastic histology, 7 medulloblastomas with extensive nodularity, and 25 samples of the classic histologic subtype. Detailed patient characteristics are given in Supplementary Table S1. Tumor diagnosis was established by standard light microscopic evaluation of hematoxylin and eosin (H&E)–stained sections and silver stains. Diagnoses were made independently by at least 2 neuropathologists, based on the criteria of the latest World Health Organization brain tumor classification (22). Research on clinical samples has been approved by the ethics committee of the Ludwig Maximilians University (Munich, Germany; signed letter from October 26, 2006, without reference number).

**Immunohistochemistry**

Paraffin-embedded tissue was sectioned, deparaffinized, and rehydrated before heat-induced antigen retrieval was conducted at 100°C for 20 minutes in 10 mmol/L sodium citrate buffer for all antibodies. Immunohistochemical staining was done using primary antibodies [Sox2: 1:500, Millipore AB5605; Pax6: 1:50, Developmental Study Hybridoma Bank (DSHB); Kit67: 1:25, DAKO Tec-3; Cre: 1:1,000, Covance PBB106C; Olig2: 1:100, Millipore AB9610; S100: 1:2,000, DAKO 4C4;9; NeuN: 1:300, Millipore MAB377; GFAP: 1:10, DAKO m0761-6F1; Map2: 1:40,000, Sigma M44003] and the HRP/DAB Staining System (DAKO) according to the manufacturer’s specifications. Hemalaun was used for nuclear counterstaining. All histologic photomicrographs were taken digitally using an Olympus BX50 microscope in combination with the Color view Soft imaging system. For immunofluorescent stainings, sections and fixed cells from cell cultures were washed twice with PBS/0.1% Triton X-100 and then incubated in blocking buffer (I-Block protein- based blocking reagent; Applied Biosystems) for 30 minutes. Primary antibodies (GFP: 1:200, Santa Cruz sc-8334; Sox2: 1:500, R&D Systems MAB2018; BrdU: 1:500, BioScience Products 010198; Ki67: 1:25, DAKO Tec-3; NeuN: 1:300, Millipore MAB377; Pax6: 1:50, DSHB) were diluted in blocking buffer and applied overnight at 4°C. Next, cells were washed twice with PBS/0.1% Triton X-100 and incubated for another 60 minutes with a 1:500 dilution of fluorescence-labeled secondary antibodies (goat anti-mouse Alexa546; goat anti-rabbit Alexa488, Invitrogen) in blocking buffer. The cells were washed twice with PBS/0.1% Triton X-100, counterstained with 4,6-diamidino-2-phenylindole, and mounted in Fluorescent Mounting Medium (DAKO). All images of tissue sections and cell cultures were collected on an Olympus IX50 microscope in combination with the Color view Soft imaging system.

**Cell culture**

Cerebellar granule neuron precursor cultures were generated as described previously (23). Briefly, cerebella of postnatal day 7 (P7) pups were taken out and prepared in Hank’s buffered saline solution (HBSS; Gibco) with glucose (6 g/L). Meninges and plexus tissue were carefully removed. Dissociation of pooled cerebella was triggered by trypsin-E-DTA-DNase (Sigma). HBSS was replaced by culture medium containing Dulbecco’s Modified Eagle Medium (DMEM)-F12 (Gibco), 25 mmol/L KCl, N2 supplement (Gibco), penicillin–streptomycin (Pen-strep), and 10% heat-inactivated fetal calf serum (FCS; Sigma). After centrifugation, cells were plated at a concentration of 3 million/mL in poly-l-ornithine (Sigma)-precoated wells and incubated at 37°C for 6 hours to recover. Then, medium was changed to serum-free culture medium containing Shh protein at a concentration of 3 μg/mL. After 24 hours, the cells were transfected with virus supernatant for 2 hours, followed by another 24 hours of incubation in serum-free medium with Shh. The cells were pulsed with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdUrd) 2 hours before fixation with 4% paraformaldehyde. BrdUrd is incorporated into the DNA of dividing cells by DNA polymerase as a substitute for thymidine. Thus, proliferating cells can be detected by immunohistochemical staining for BrdUrd.

**MSCV-ires-GFP and MSCV-Cre-IRES-GFP** plasmids were kindly provided by Dr. David Rowitch (University of California, San Francisco, San Francisco, CA). The **MSCV-Sox2-IRES-GFP** vector was generated by ligating a 980 bp Sox2 sequence into the **MSCV-IRES-GFP** backbone (T4 DNA-Ligase, Promega). Production of lentiviral particles was carried out as previously...
published (23). Briefly, HEK293T cells (American Type Culture Collection) were grown in DMEM containing 10% FCS and 1% glutamine at 37°C and 5% CO2 in a humidified atmosphere and transfected with 10 μg of each virus construct, as well as rsv-g and gag-pol plasmids containing sequences encoding for packaging and envelope proteins, using the XtremeGene HP Transfection Reagent (Roche). Virus-containing medium was collected every 24 hours for 3 consecutive days after replacement of transfection mix. Supernatant was pooled, sterile filtered, and stored at −80°C until use.

**DNA and RNA extraction and real-time PCR**
For cDNA from snap-frozen cerebellar tumor tissue (hgFAP-cre::Sox2Fl+/−; hgFAP-cre::Sox2Fl+/−; SmoM2Fl+/−), total RNA was extracted from snap-frozen tissues using TRizol reagent (Invitrogen). DNase digestion with RQ1 RNase-Free DNase (Promega) was conducted on RNA to avoid contamination with gDNA fragments. Random hexamer primers, oligo dTs, and Superscript II reverse transcriptase (Invitrogen) were used to run reverse transcription (RT) for tumor samples. For quantitative real-time RT-PCR, the LightCycler480 system (Roche) and the corresponding SYBR Green detection format were used as a housekeeper, as it proved to be consistently expressed in medulloblastomas.

**Results**

SOX2 is expressed in SHH-associated medulloblastoma
To investigate the expression profile of SOX2 in human medulloblastomas, we first examined a cohort of 65 medulloblastoma samples for SOX2 protein expression. Using specific antibodies against SOX2, we detected no or weak expression in 27 of 65 cases (−, 41.5%), intermediate expression in 20 of 65 cases (+, 30.8%), and strong expression of SOX2 in 18 of 65 medulloblastomas (+++, 27.7%; Supplementary Table S1). Interestingly, correlation of the expression of SOX2 with the histologic subtype of the tumor revealed a significantly higher SOX2 expression in tissue samples of the desmoplastic and extensively nodular subtype (n = 40, Fig. 1C−E) when compared with the classic subtype (n = 25, P = 0.002; Fig. 1A, B, and E). This distribution was confirmed by the analysis of recently published global gene expression arrays of an independent cohort of 152 medulloblastoma samples (P < 0.0003, Fig. 1F; ref. 28). Acknowledging that medulloblastomas may also be subdivided into 4 molecular subgroups (6), we looked at a cohort of 188 molecularly profiled medulloblastoma samples (including the 152 cases from Fig. 1F) to determine the expression of SOX2 in relation to the molecular biology of the tumor. We detected significantly higher levels of SOX2 expression in the group of SHH-associated medulloblastomas than in the other subgroups (P < 0.0001, Fig. 1G). This is in line with the observation that most, if not all, desmoplastic medulloblastomas molecularly belong to the SHH group (6). It is further known that SHH medulloblastomas themselves divide into diverse clinically and molecularly distinct subpopulations, namely infantile, childhood, and adult cases (29). When dividing our cohort into these 3 age groups, we found that SOX2 is of the approval for animal research was 55.2-1-54-2531-21-09 (Government of Upper Bavaria, Germany).

**Quantification and statistical analysis**
All results were analyzed using the Prism4 software (GraphPad). Survival of mice was analyzed using Kaplan–Meier survival curves, and the log-rank test was used to examine the significance of results. P values less than 0.05 were considered significant. For analysis of the expression data, the Mann–Whitney U test was used to compare the median of 2 groups. Correlation of 2 paired data sets was done with the Spearman correlation with r as correlation coefficient and displayed with a linear regression curve. To calculate the fraction of tumor cells labeled with antibody against SOX2, at least 500 tumor cells were counted for each tumor. The unpaired t test was applied to compare the means of 2 groups without assumed Gaussian distribution and equal variances. Proliferative activity in virus-treated granule neuron precursor cultures was measured in at least 3 independent culture preparations with 3 wells of a 24-well plate and at least 300 GFP+ cells counted in each well of each condition in each preparation. Results were analyzed using the Fisher exact test. For LightCycler assessments, all experiments were done with at least 3 mice per genotype, each in triplicate. All respective histograms illustrate the SEM (error bars).
SOX2 is expressed in human desmoplastic and SHH-associated medulloblastoma (MB). In human samples, SOX2 expression is significantly higher in desmoplastic medulloblastoma (dMB) or in medulloblastoma with extensive nodularity (MBEN) than in classic medulloblastomas (cMB; A–F). Analysis of mRNA expression data for SOX2 within the 4 major molecular subgroups of medulloblastoma reveals strong SOX2 expression within the SHH subgroup (G). SOX2 is expressed in an age-dependent distribution. Both by analysis of protein expression in dMBs and MBENs (n = 40; H) as well as by looking at mRNA expression in SHH-associated medulloblastomas (n = 50; I), SOX2 was significantly more abundant in juvenile and adult tumors than in the infantile cases. Scale bars in low magnification images, 200 mm; scale bars in high magnification, 50 mm. n. s., not significant. –, no or weak expression; +, intermediate expression; ++, strong expression.

Figure 1. SOX2 is expressed in human desmoplastic and SHH-associated medulloblastoma. In human samples, SOX2 expression is significantly higher in desmoplastic medulloblastoma (dMB) or in medulloblastoma with extensive nodularity (MBEN) than in classic medulloblastomas (cMB; A–F). Analysis of mRNA expression data for SOX2 within the 4 major molecular subgroups of medulloblastoma reveals strong SOX2 expression within the SHH subgroup (G). SOX2 is expressed in an age-dependent distribution. Both by analysis of protein expression in dMBs and MBENs (n = 40; H) as well as by looking at mRNA expression in SHH-associated medulloblastomas (n = 50; I), SOX2 was significantly more abundant in juvenile and adult tumors than in the infantile cases. Scale bars in low magnification images, 200 mm; scale bars in high magnification, 50 mm. n. s., not significant. –, no or weak expression; +, intermediate expression; ++, strong expression.

expressed in an age-dependent manner. As shown by the analysis of protein expression (Fig. 1H) as well as mRNA expression data (Fig. 1I), we show that SOX2 is significantly more abundant in adult cases (≥16 years) than in infantile cases (≤3 years; P = 0.0013 for protein and P = 0.0129 for mRNA expression).
**Sox2 is expressed in granule cell precursors that have the ability to form medulloblastoma**

To determine the role of Sox2 in early cerebellar development, we first conducted immunohistochemical studies on wild-type mice. The cerebellar anlage, which is the primordium of the developing cerebellum (30), does not express Sox2 at E11.5 (Fig. 2A). However, a specific signal was found at E13.5 both in the ventricular zone, which contains multipotent neural stem cells that give rise to the majority of cerebellar neurons and glia, as well as in the EGL of the cerebellum that exists of committed precursors of the granule cell lineage (Fig. 2B). At E18.5, expression of Sox2 in granule cell precursors was hardly detectable (Fig. 2C). Several analyzed stages beyond E18.5 displayed a strong Sox2 signal in Bergmann glia as well as in the cerebellar white matter, but not in premature or mature granule neurons (data not shown). We concluded from these findings that Sox2 was transiently expressed in the EGL at early stages of embryonic development. To investigate the nature of

![Image of Sox2 expression in cerebellar development](image_url)

**Figure 2.** Sox2 is expressed in early cerebellar granule neuron precursors. Sox2 expression in CGNPs starts at around E13.5 (arrows in B) and was lost at around E18.5 when we only sporadically detected weakly Sox2-positive cells (A–C). Immunofluorescent colabeling was conducted to show neuronal (Pax6; D–F) and proliferative (Ki67; G–I) properties of Sox2-expressing cells in the EGL at E14.5 (arrows). As compared with controls (J), constitutive activation of Shh signaling in such cells by tamoxifen-induced (1 mg at E12.5 and E13.5) Cre and subsequent SmoM2 expression resulted in inadequate proliferation and tumor formation at P31 (arrows in K). Scale bars in low magnification images, 100 μm; scale bars in high magnification insets, as well as images (D–K), 50 μm. EGL, external granular layer; RL, rhombic lip; IV, fourth ventricle.
Sox2-positive cells in the cerebellar EGL, immunofluorescent colabeling was conducted with antibodies against Sox2 and the granule cell marker Pax6 as well as proliferation marker Ki67. Our findings confirm that Sox2-positive cells within the developing EGL were of neuronal lineage and harbored the potential to proliferate (arrows point to Sox2/Pax6 double-positive cells in Fig. 2D–F and to Sox2/Ki67-positive cells in Fig. 2G–I).

Granule cell precursors within the developing EGL of the cerebellum proliferate in response to Shh (10) and may give rise to medulloblastoma once Shh signaling is constitutively active (9). To show that this holds true for the subset of Sox2-positive granule cell precursors, we used Sox2-creERT2 mice (26) and crossed them with a lox-stop-lox-SmoM2-eYFP allele (SmoM2fl/flSmoM2fl) that constitutively activates Shh signaling after exposure to Cre-recombinase (31). As shown in Fig. 2J and K, tamoxifen-mediated induction of Cre at E12.5 and E13.5 resulted in inadequate proliferation and tumor formation originating from the EGL of Sox2CreERT2:SmoM2fl/fl (Fig. 2K), but not of SmoM2fl/fl mice (Fig. 2J). This suggested that Sox2-positive granule cell precursors give rise to Shh-induced medulloblastoma, and this is in line with our findings of SOX2 expression in human SHH-associated medulloblastomas (Fig. 1).

Constitutive activation of Shh signaling drives expression of Sox2 in vitro and in vivo

We established that granule cell precursors of the early rhombic lip express Sox2. As such precursors are crucially dependent on Shh signaling (10), we asked whether the expression of Sox2 was a consequence of Shh signaling rather than an independent event. We used primary granule neuron cultures as a versatile in vitro system. First, we confirmed that exogenous administration of Shh protein leads to a dose-dependent increase of granule cell proliferation in vitro. As shown and evaluated by quantification of BrdUrd-incorporation assays, 3 μg/mL Shh result in a more than 10-fold increase of proliferation compared with conditions without Shh protein (Fig. 3A–D). Interestingly, Sox2 expression similarly increased in cultured granule cell precursors after the application of higher levels of Shh protein in the culture medium. While less than 0.5% of Pax6-positive granule cell precursors simultaneously expressed Sox2 in conditions without Shh, Sox2 is expressed in approximately 3 times more Pax6-positive granule cell precursors, if the concentration of Shh in the medium raises to 3 μg/mL (P < 0.0001, Fig. 3E–H). To confirm these results by an independent strategy, we used primary cultures of granule neuron precursors from SmoM2fl/fl mice. Transduction of such cells with retroviruses carrying an IRES-GFP sequence merely resulted in a BrdUrd incorporation in approximately 5% of the transduced GFP+ cells (Fig. 3I–L). However, transduction of cells with a Cre-IRES-GFP virus, hence activating the Shh pathway, was followed by an incorporation of BrdUrd in approximately 25% of the transduced GFP+ cells (P < 0.0001, Fig. 3L–O).

More importantly, the activation of SmoM2 by Cre resulted in a significantly increased expression of Sox2 in granule neurons (P = 0.01, Fig. 3P–S), and this confirmed the results obtained by addition of exogenous Shh protein (Fig. 3E–H). So, even though granule cells had lost their expression of Sox2 by the time we started cell cultures (P5–P7), it was possible to reinduce Sox2 expression and increase proliferation via activation of Shh signaling. In vivo, conditional expression of the SmoM2 mutation under control of the human GFAP promoter results in the formation of Shh-associated medulloblastoma (9). If permanent Shh signaling induced expression of Sox2 as suggested by our in vitro experiments, such tumors should appear Sox2 positive. This was indeed the case as shown in Fig. 3W and X and is well in line with Sox2 being expressed in human medulloblastomas (see Fig. 1). Interestingly, we found more than 30% Sox2-positive cells in tumor prone mice at P1, but the fraction of cells positive for the transcription factor significantly decreased with age and reached a level of 3.1% at P31 (Fig. 3Y).

Early loss of Sox2 in murine Shh-associated medulloblastoma is accompanied by upregulation of Sox3

After having accumulated evidence that Sox2 is expressed in early granule cell precursors that go on to form the EGL of the cerebellum, or in the pathologic situation Shh-associated medulloblastoma, we wanted to examine whether Sox2 is vital to cerebellar or tumor development.

To test whether a loss of Sox2 will affect cerebellar development, we generated hGFAP-cre:Sox2fl/fl mice. In such mice, Sox2 was deleted from hGFAP-positive neural precursors that give rise to cerebellar granule neurons, Bergmann glia, and various other cell types. However, gross morphology was neither altered at E13.5 nor at P42, although Cre was sufficiently expressed and expression of Sox2 was lost as expected from E12.5 on, when the human GFAP-promoter becomes active (Supplementary Fig. S1A–L). To further analyze and compare the structure of the cerebellum with and without loss of Sox2, we also conducted various immunohistochemical stains for oligodendrogial (Olig2; Supplementary Fig. S1M and S1N), proliferative (Ki67; Supplementary Fig. S1O and S1P), glial (S100; Supplementary Fig. S1Q and S1R), and neuronal (NeuN; Supplementary Fig. S1S and S1T) markers to confirm that there is no difference between the 2 genotypes. We therefore concluded at this point that expression of Sox2 in hGFAP-positive neural precursors is not crucial for cerebellar development. However, this did not exclude a fundamental role of Sox2 for medulloblastoma formation. In fact, we had shown massive expression of Sox2 in mouse and human medulloblastoma that arise from hGFAP-positive granule cell precursors (Fig. 3W). To decipher a potential function of Sox2 during tumor development, we bred Sox2fl/fl mice with our hGFAP-cre:SmoM2fl/fl model for Shh-associated medulloblastoma, which should clarify whether the generation of medulloblastoma is possible despite a deficiency for Sox2. Figure 4A–C and D–F show representative images of hGFAP-cre:Sox2fl/fl, SmoM2fl/fl and hGFAP-cre:Sox2fl/flSmoM2fl/fl cerebellar sections. The lack of staining for Sox2 in the homozygous knockout (Fig. 4F) provided proof of an efficient and complete loss of Sox2 expression. However, development and overall morphology of the tumor were not grossly altered. Furthermore, Kaplan–Meier survival analysis of a total of 25 mice showed no significant difference in overall survival time of the 2
genotypes ($P = 0.6039$; Fig. 4G). Finally, immunophenotyping of Sox2-deficient tumors and controls using antibodies against GFAP, Map2, Ki67, and NeuN did not reveal any obvious differences (Supplementary Fig. S2). At first glance, this suggested that Sox2 might be dispensable for the formation of Shh-associated medulloblastoma. However, given the fact that Sox2 belongs to a large family of functionally similar or redundant proteins, we aimed to screen for compensatory mechanisms that potentially hid relevant functions of Sox2. We conducted quantitative real-time PCR experiments with cDNA isolated from tumors of P0 (Fig. 4H) and P6 (Fig. 4I) animals harboring a homozygous (clear bars in Fig. 4H, I) or a heterozygous (shaded bars in Fig. 4H, I) Sox2 knockout. As expected, we found that Sox2 expression was significantly reduced to almost zero in the $hGFAP$-cre::$Sox2^{Fl/Fl}$$SmoM2^{+/+}$ situation (P0: $P = 0.049$; P6: $P = 0.043$), and we did not detect a significant change in $Sox1$ levels.
Sox2 in Medulloblastoma

However, when analyzing the expression level of Sox3, we identified a significant increase in Sox3-deficient tumors that reached from a 2-fold relative increase at P0 ($P = 0.029$) up to more than 3-fold increase at P6 when normalized to the heterozygous control ($P = 0.031$). Interestingly enough and pointing to potentially redundant functions, the expression pattern of SOX3 in human medulloblastoma cases was strikingly similar to the expression of SOX2 with a preponderance in SHH medulloblastomas (Supplementary Fig. S3A) and a highly significant correlation between the expression of SOX3 and SOX2 in medulloblastoma in general ($P < 0.0001$, Supplementary Fig. S3B).

**Sox2 is required and sufficient for proliferation of medulloblastoma cells**

Our data suggest that potential functions of Sox2 during medulloblastoma formation in hGFAP-cre::Sox2$^{fl/+}$SmoM2$^{fl/+}$ mice may be hidden by a compensatory upregulation of Sox3. To avoid time frames that allow for such compensatory mechanisms in response to a loss of Sox2 expression, we conducted primary granule cell culture from Sox2$^{fl/fl}$SmoM2$^{fl/+}$ and Sox2$^{fl/+}$SmoM2$^{fl/fl}$ cerebella and treated the cells with a MSCV-Cre-IREX-GFP virus for Shh activation via SmoM2 together with an acute homozygous or heterozygous loss of Sox2. Twenty-four hours after virus application, we first verified the efficiency of the retrovirally induced Sox2 knockout. Figure 5A–C show the control situation with some transduced cells that also express Sox2, whereas the expression is significantly reduced ($P = 0.005$) to almost nothing in the homozygous knockout situation (Fig. 5D–F and M). To determine the proliferation rate of cells with and without Sox2, we used the BrdUrd incorporation assay. As shown in Fig. 5G–I, by immunofluorescent staining for viral GFP and incorporated BrdUrd, we found a significant decrease in proliferation in the homozygous Sox2 knockout situation (Fig. 5J–L) when compared with the heterozygous situation (Fig. 5G–I). Quantification of 3 independent experiments showed a decrease of proliferation in transduced granule cells lacking Sox2 of more than 30% (Fig. 5N; $P = 0.0002$) as compared with the heterozygous knockout. To assure that the population of transduced cells consisted of granule neurons rather than glia, we conducted immunofluorescent colabelings for Pax6 and NeuN, both of which label granule neurons. In Supplementary Fig. S4, we show that transduced cells in our primary cultures are almost exclusively of neuronal origin (Supplementary Fig. S4Y). Furthermore, we made sure that the increase of Sox2-positive cells after SmoM2 activation is due to an increase of Sox2-positive neuronal precursors rather than to a proliferation of Sox2-positive glia. Supplementary Fig. S5 displays costaining for Sox2 and markers of neuronal lineage Pax6 and...
NeuN, which confirmed the neuronal properties of Sox2-expressing cells (Supplementary Fig. S5Y). Together, we concluded that the loss of Sox2 within the first 24 hours significantly inhibited the SmoM2-induced proliferation of CGNPs.

Finally, we aimed to examine the effect of a gain of Sox2 function in Shh-activated granule cells. We therefore conducted primary tumor cell cultures and transduced the cells with a MSCV-IRES-GFP virus as a control experiment or with a MSCV-Sox2-IRES-GFP virus. Figure 5O–T and Fig. 5AA show the efficiency of the Sox2 expression virus. Although only 20% of hGFAP-cre::SmoM2Fl/+ tumor cells normally expressed Sox2 in vitro, we significantly increased Sox2 expression in transduced...
cells to almost 100% (P < 0.0001, Fig. 5A). More importantly, we found that the proliferation of tumor cells increased significantly by 34% within the transduced population (P = 0.0022, Fig. 5U–Z, AB). These data suggested that expression of Sox2 in medulloblastoma cells is sufficient to significantly enhance tumor cell proliferation.

Discussion

We report here that Sox2 is highly expressed the molecular subgroup of medulloblastoma that is associated with pathologic activation of the Shh pathway. Developmentally, we show that in CGNPs Sox2 is only expressed until E18.5, whereas it is still abundantly found in Shh-associated murine medulloblastoma up until adolescent postnatal stages. Functionally, we show that, independent of their endogenous Sox2 expression, constitutive activation of the Shh pathway in CGNPs results in ectopic expression of Sox2 and enhanced proliferation in vitro and in vivo (Fig. 2). Furthermore, the level of Sox2 expression has an effect on the proliferation rate of Shh-activated CGNPs, as shown by loss- and gain-of-function experiments in vitro (Fig. 5). Expression studies on human tumor samples as well as in vivo mouse experiments finally indicate that Sox3 is similarly expressed and may compensate for a loss of Sox2 in medulloblastoma (Fig. 4).

It is known that there is a striking overlap of medulloblastoma of the SHH-associated subgroup and tumors with desmoplastic histology (6). By examining both, human medulloblastoma tissue sections and mRNA expression data, we found that SOX2 expression also coincides with both classification systems of medulloblastoma. SHH-associated and desmoplastic medulloblastoma show a biphasic distribution concerning patient age (29). Keeping this in mind, the particularly high expression of SOX2 in adult cases of SHH-associated medulloblastoma may indicate a distinct role for the formation of adult medulloblastoma. This functional relationship is further supported when considering that medulloblastoma with extensive nodularity, which tend to show a lower SOX2 expression profile within the group of SHH medulloblastomas (Fig. 1E), are exclusively found in early childhood (32).

Granule neuron precursors of the rhombic lip have been described as the origin for the SHH-associated subgroup of medulloblastoma (9). We show here that Sox2 is expressed within the population of neuronal precursors in exactly the time frame that CGNPs use to migrate and form the EGL. We have further shown that this specific Sox2-positive precursor population is responsive to an activation of the Shh pathway via SmoM2 and may give rise to a hyperproliferative progeny that is unable to exit the cell cycle. Ongoing research will have to clarify whether the expression of SOX2 in adult medulloblastoma is due to (i) ectopic Sox2-positive CGNPs that were maintained until adulthood and then give rise to tumors, due to (ii) mature neurons that have been dedifferentiated into Sox2-positive precursors before tumor formation or due to (iii) an ectopic expression of Sox2 in tumors that are not derived from Sox2-positive precursors.

With the increase in proliferation in response to constitutive Shh activation, we also detected an increase in Sox2 expression, apparently as a response to Shh signaling. This finding is in line with the results we obtained from mouse models for Shh-associated medulloblastoma, where we find postnatal Sox2 expression within the tumor, whereas Sox2 is not found in normal granule cells after E18.5. Interestingly, the fraction of tumor cells expressing Sox2 decreased with the age of the mouse. This might hint at an initiating function of Sox2 in tumor development, rather than a maintaining one, and previous findings have already suggested that Sox2 is more abundant in preneoplastic lesions that lead to tumor formation than in tumors itself (33).

A Sox2 knockout under the control of the hGFAP promoter leads to a total loss of Sox2 expression in the cerebellum from developmental day E12.5 onward as well as in murine medulloblastoma. Even though Sox2 is said to be an important developmental regulator, we did not find any apparent alterations in cerebellar development. One possible explanation is that the hGFAP promoter is only becoming active 2 to 3 days after the Nestin promoter that has been used by Favaro and colleagues who had seen a distinct phenotype due to the loss of Sox2 (12). So, we may have deleted Sox2 after its critical period in CNS development. This argument, however, does not seem valid for the development of medulloblastoma, where Sox2 had been deleted from early on (i.e., simultaneously with the recombination of SmoM2). In this case, redundancies that are well known for Sox genes in general and members of the SoxB1 family in particular (34) may be responsible for the missing phenotype. In this context, it has been shown in cell culture experiments that Sox3 expression was upregulated in Sox2-deficient cells (35), and this is perfectly in line with the findings of our qRT-PCR analysis from tumor tissue (Fig. 4). Although not definitively proven, it seems possible from these experiments that Sox3 compensates for the early loss of Sox2 in vivo and thus for all subsequent defects that would result from the acute loss of this important developmental regulator.

The finding of a compensatory mechanism for early Sox2 loss in vivo raised the question whether an acute loss of Sox2 would have a functional effect on medulloblastoma cells. The fact that an acute knockout of Sox2 resulted in a significant decrease in proliferation indeed suggested that Sox2 is required for proliferation in Shh-activated cells, as shown in our loss-of-function cell culture experiments (Fig. 5). Furthermore, the specific overexpression of Sox2 in tumor cells led to an increase in proliferation, suggesting that Sox2 is also sufficient for proliferation of medulloblastoma cells. This all fits the data we obtained on increasing Sox2 levels in response to Shh activation (Fig. 3). The apparent interaction between Sox2 and the Shh pathway will be subject to further investigations. By finding the mechanism of interaction and the level at which Sox2 exerts its effects within the pathway, we hope to identify new targets for therapeutic intervention of pathologic activation of the Shh pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: J. Ahlfield, H.A. Kretzschmar, S. Nicolis, U. Schüller

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