SOX2 regulates self-renewal and tumorigenicity of human melanoma-initiating cells

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INTRODUCTION

Melanoma is a dynamic and heterogeneous skin cancer, with adverse prognosis in advanced stages. A large body of evidence suggests that within the heterogeneous population that constitutes a melanoma certain cell types exhibit molecular and functional features similar to stem cells. These putative melanoma-initiating cells (MICs) have unlimited self-renewal and multilineage differentiation abilities and the potential to initiate conventional therapy at advanced stages. We and others have previously shown that Hedgehog-GLI (HH-GLI) signaling is required for melanoma growth and for survival and expansion of melanoma-initiating cells (MICs). Recent reports indicate that HH-GLI signaling regulates a set of genes typically expressed in embryonic stem cells, including SOX2 (sex-determining region Y (SRY)-Box2). Here we address the function of SOX2 in human melanomas and MICs and its interaction with HH-GLI signaling. We find that SOX2 is highly expressed in melanoma stem cells. Knockdown of SOX2 sharply decreases self-renewal in melanoma spheres and in putative melanoma stem cells with high aldehyde dehydrogenase activity (ALDHhigh). Conversely, ectopic expression of SOX2 in melanoma cells enhances their self-renewal in vitro. SOX2 silencing also inhibits cell growth and induces apoptosis in melanoma cells. In addition, depletion of SOX2 progressively abrogates tumor growth and leads to a significant decrease in tumor-initiating capability of ALDHhigh MICs upon xenotransplantation, suggesting that SOX2 is required for tumor initiation and for continuous tumor growth. We show that SOX2 is regulated by HH signaling and that the transcription factors GLI1 and GLI2, the downstream effectors of HH-GLI signaling, bind to the proximal promoter region of SOX2 in primary melanoma cells. In functional studies, we find that SOX2 function is required for HH-induced melanoma cell growth and MIC self-renewal in vitro. Thus SOX2 is a critical factor for self-renewal and tumorigenicity of MICs and an important mediator of HH-GLI signaling in melanoma. These findings could provide the basis for novel therapeutic strategies based on the inhibition of SOX2 for the treatment of a subset of human melanomas.

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SOX2 is a member of the high mobility group domain family that plays a critical role in embryonic development and in maintaining pluripotency and self-renewal in embryonic stem cells and several cell lineages such as neural cells, osteoblasts and neural crest melanocytes. In adult mice, Sox2 is expressed in different epithelial compartments, in which it marks cells with self-renewal properties, and its targeted ablation lethally disrupts epithelial tissue homeostasis. Moreover, SOX2 is one of the key transcription factors capable of reprogramming differentiated somatic cells into induced pluripotent stem (iPS) cells.

Recent studies have pointed to an important role of SOX2 in cancer. SOX2 is amplified in esophageal, oral and lung squamous cell carcinomas and in small-cell lung cancer. SOX2 is involved in several types of cancer, such as glioblastoma and osteosarcoma, lung, breast, ovarian, pancreatic, prostate and gastric cancers and promotes tamoxifen resistance in breast cancer cells. SOX2 is expressed in about 50% of melanomas and only in a minority of nevi. Silencing of SOX2 has been shown to decrease A2058 melanoma cell growth in vivo but not in vitro and to reduce melanoma cell invasion. However, it is currently unknown whether SOX2 is critical in regulating MICs.

Here we have thoroughly investigated the role of SOX2 in melanoma cell proliferation, stemness and tumorigenicity in a...
panel of patient-derived MICs and its interaction with HH signaling. We show that silencing of SOX2 leads to a remarkable growth suppression and apoptosis in melanoma cells. Importantly, SOX2 abrogation impairs the ability of MICs to self-renew in vitro and to initiate and to maintain tumor growth in vivo. Furthermore, we find that SOX2 is directly regulated by HH-GLI signaling and acts as a mediator of HH in melanoma cells.

RESULTS

SOX2 silencing inhibits growth and induces apoptosis in primary melanoma cells

SOX2 expression was investigated in 19 patient-derived primary melanoma cells, in A375 melanoma cell line and in normal human epidermal melanocytes (Supplementary Table S1). Quantitative real-time PCR (qPCR) revealed variable expression of SOX2; approximately half of the primary melanoma cultures showed high/intermediate levels and half no/very low expression (Figure 1a), in line with previous immunohistochemistry studies.50–52 SOX2 expression was documented at low levels in normal human epidermal melanocytes. Immunofluorescence analysis revealed SOX2 expression in the nuclei of primary melanoma cells (Supplementary Figure S1). No significant correlation was found between SOX2 expression and tumor grade or other clinical features.

SOX2 silencing has been shown to decrease A2058 melanoma cell growth in vitro but not in vitro.50 To clarify its function in melanoma, we knocked down SOX2 in patient-derived melanoma cells with high (SSM2c and M26c) and intermediate (M5 and the A375 melanoma cell line) SOX2 levels using two independent SOX2 shRNAs (LV-shSOX2-1 and LV-shSOX2-2). SOX2 silencing led to a near complete loss of SOX2 protein (Figure 1b) and resulted in a drastic reduction in the number of viable cells in SSM2c, M26c to a near complete loss of SOX2 protein (Figure 1c), M5 and A375 cells (Supplementary Figure S2). Analysis of the proliferation index, determined by carboxyfluorescein succinimidyl ester (CFSE) staining, indicated that SSM2c and M26c SOX2-depleted cells grew slower than control cells (Figure 1d). Cell cycle analysis confirmed a slight reduction of cells in S phase, but no changes in the fraction of cells in G0/G1 upon SOX2 knockdown (P < 0.05 in both cell types; Supplementary Figures S3a and b). SOX2 silencing significantly increased both early and late apoptosis, as shown by Annexin V/7-aminoactinomycin D (7-AAD) staining (Figures 1e and f). Consistently, SOX2 knockdown induced the expression of the apoptotic markers TP73 and NOXA and concomitant downregulation of the anti-apoptotic factor BCL-2 (Figure 1g). Transient silencing of SOX2 induced phosphorylation of γH2AX and promoted poly ADP-ribose polymerase (PARP) cleavage, confirming signs of DNA damage and apoptosis as soon as 48 h after transfection (Figure 1h). Altogether, these results indicate that interference with SOX2 function inhibits melanoma cell growth by promoting apoptosis and, partially, by reducing proliferation.

SOX2 expression is enhanced in melanoma cells with stem cell features

Because tumor sphere assay allows the enrichment of potential MICs,1,7,54–56 we compared SOX2 expression in melanoma spheres and in adherent cells by qPCR. We found that melanoma spheres obtained from SSM2c and M26c cells were greatly enriched in the expression of SOX2 when compared with the corresponding adherent cells (Figure 2a). Confocal microscopy in spheres showed SOX2 protein expression in the nucleus of SSM2c and M26c sphere-forming cells, with higher levels in a fraction of them (Figure 2b).

As an alternative approach to analyze SOX2 expression in MICs, we sorted melanoma cells with high aldehyde dehydrogenase activity (ALDHhigh), which has been shown to mark a population enriched for melanoma stem cells.6,7,57 SOX2 protein level was 2–3-fold higher in ALDHhigh cells compared with the ALDHlow population in both SSM2c and M26c cells (Figure 2c). Consistently, expression of SOX2 mRNA in ALDHhigh cells was 2.7- and 3.6-fold higher than in the ALDHlow population, respectively, in SSM2c and M26c cells (Figures 2d and e). In addition, increased expression of KLF4, NANOG and, to a lesser extent, OCT4, which have a role in the maintenance of cancer stem cells (CSCs), was observed in the ALDHhigh population (Figures 2d and e). Altogether, these data indicate that SOX2 is highly expressed in melanoma cells with stem cell-like features.

SOX2 regulates self-renewal of melanoma stem cells

Because SOX2 is highly expressed in the ALDHhigh population (Figure 2), which is enriched in CSCs, we evaluated whether SOX2 was involved in the maintenance of putative ALDHhigh MICs. To test this possibility, we measured the number of ALDHhigh melanoma cells after SOX2 silencing. Cytometric analysis revealed a drastic reduction in the fraction of ALDHhigh cells in SSM2c, M26c, M5 and A375 melanoma cells transduced with two different SOX2 shRNAs (Figure 3a and Supplementary Figure S4). Importantly, SOX2 silencing reduced self-renewal of ALDHhigh but not that of ALDHlow spheres (Figure 3b), consistent with lower levels of SOX2 expression in ALDHlow cells (Figure 2). Knockdown of SOX2 significantly decreased the size of ALDHhigh cells and, to a lesser extent, of ALDHlow spheres in both SSM2c and M26c cells (Figure 3c), suggesting an effect on proliferation and/or death of sphere-forming progenitor cells. Indeed, analysis of bromodeoxyuridine (BrdU) and Annexin V/7-AAD staining indicated that SOX2 silencing diminishes ALDHhigh sphere size by reducing proliferation and by increasing apoptosis (early and late). On the other hand, the slight decrease of ALDHlow sphere size upon SOX2 knockdown appeared to depend mostly on reduced proliferation (Figures 3d and e).

As an alternative approach to test the role of SOX2 in MICs, we evaluated whether the modulation of SOX2 could affect the behavior of melanoma spheres, which are enriched in CSCs and are able to self-renew. Interestingly, SOX2 expression correlated with their ability to form primary spheres in limiting dilution assays (Supplementary Figures S5a-c). All melanoma spheres used in this study could form tumors with high penetrance when injected into nude mice (data not shown) and express variable levels of melanoma stem cell markers, including ABCB5, JARID1B and CD271 (Supplementary Figure S5d). To test SOX2 function in melanoma spheres, we first silenced it. In both SSM2c and M26c spheres, LV-shSOX2-1 and LV-shSOX2-2 reduced SOX2 protein levels by 90–95% compared with LV-c control (Figure 4a). SOX2 silencing inhibited self-renewal of SSM2c, M26c, M5 and A375 spheres (Figure 4b). Moreover, SOX2-depleted melanoma spheres were significantly smaller than controls (Figures 4c and d). Knockdown of SOX2 in SSM2c and M26c melanoma spheres slightly reduced cell proliferation, as determined by BrdU incorporation and CFSE analysis (Figure 4e and Supplementary Figure S6a), and increased apoptosis (Figure 4f and Supplementary Figure S6b). To further confirm the effect of SOX2 in melanoma spheres, we overexpressed it in M33c cells, which show low levels of SOX2 (Figure 1a), and in SSM2c cells. SOX2 overexpression significantly increased the number of secondary spheres (Figures 5a and b) and slightly increased melanoma sphere size (Figures 5c and d). Moreover, SOX2 marginally increased cell proliferation, as determined by CFSE analysis (Supplementary Figure S7a), and decreased apoptosis (Supplementary Figure S7b) of SSM2c and M33c melanoma spheres. qPCR analysis showed that SOX2 induced the expression of OCT4 and KLF4 only in SSM2c spheres (Figures 5e and f). SOX2 overexpression induced the expression of BCL-2 and, to a lesser extent,
Figure 1. SOX2 silencing suppresses cell growth and induces apoptosis in primary melanoma cells. (a) qPCR analysis of SOX2 in a panel of 19 patient-derived melanoma cells, A375 melanoma cells and normal human epidermal melanocytes. qPCR values reflect Ct values after normalization with two housekeeping genes (GAPDH and β-ACTIN). (b) Western blotting analysis of SSM2c and M26c adherent cells stably transduced with LV-c, LV-shSOX2-1 and LV-shSOX2-2. HSP90 was used as a loading control. (c) Growth curves of SSM2c and M26c melanoma cells stably transduced with LV-c, LV-shSOX2-1 or LV-shSOX2-2. Three to five thousands transduced cells/well were plated in 12-well plates, and cells were counted on days 3, 5 and 7. (d) Proliferation index measured by CFSE staining in SSM2c and M26c melanoma cells stably transduced with LV-c, LV-shSOX2-1 or LV-shSOX2-2. (e, f) SSM2c and M26c cells transduced with LV-c, LV-shSOX2-1 or LV-shSOX2-2 were analyzed by flow cytometry for Annexin V+/7-AAD− (early apoptosis) and Annexin V+/7-AAD+ cells (late apoptosis). (g) qPCR analysis of NOXA, TP73, BCL2 and GADD45A in M26c cells transduced with LV-c and LV-shSOX2-1. qPCR values reflect Ct values after normalization with two housekeeping genes (GAPDH and β-ACTIN). (h) Western blotting analysis of p-γH2AX, p-Chk2 and poly ADP-ribose polymerase (PARP) in M26c cells, showing increased phosphorylation of γH2AX at Ser139 and PARP cleavage in cells transfected with shSOX2 for 48 h. HSP90 was used as loading control. Data shown are the mean ± s.e.m. of at least three independent experiments. *P ≤ 0.05; **P ≤ 0.01 vs LV-c.
SOX2 regulates melanoma-initiating cells

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SOX2 regulating self-renewal and promotes survival of MICs.

SOX2 silencing reduces tumor-initiating capability of ALDH\textsuperscript{high} melanoma cells

To address whether SOX2 silencing affects the ability of ALDH\textsuperscript{high} putative MICs to maintain tumor growth, we performed serial transplantation assays using 10,000 ALDH\textsuperscript{high} SSM2c and M26c cells transduced with LV-c, LV-shSOX2-1 or LV-shSOX2-2. In the first passage, SOX2 knockdown significantly reduced tumor growth in SSM2c and M26c xenografts compared with LV-c (Figures 6a–c). In the subsequent in vivo passage, SOX2-depleted cells lost their potential to grow (Figures 6a and b). Western blotting analysis confirmed the efficacy of SOX2 downregulation in LV-shSOX2-1 xenografts compared with control tumors (Figure 6d). Consistently, immunocytochemistry of sections obtained from tumors dissected 40 days after injection showed a reduction of SOX2 protein expression in LV-shSOX2-1 tumors compared with control sections (LV-c) (Figure 6e, left panels). SOX2-depleted tumors showed an increase in the number of apoptotic cells, as revealed by activated caspase-3 immunostaining (Figure 6e, right panels), consistent with in vitro data (Figures 3 and 4). However, no changes were observed in the number of Ki-67-positive cells (data not shown). To further assess whether SOX2 knockdown could impact on tumor-initiation ability, we performed limiting dilution experiments by subcutaneous injection of 1000, 100 or 10 cells from ALDH\textsuperscript{high} SSM2c and M26c cells transduced with LV-c or LV-shSOX2-1. SOX2-depleted cells at limiting dilution showed greatly reduced tumor take rates compared with control cells in both SSM2c and M26c xenografts (Figures 6f and g). Calculation of tumor-initiation frequency revealed 100- to 20-fold reduction in the fraction of tumor-initiating cells, respectively, in M26c and SSM2c xenografts upon SOX2 silencing (Figure 6h). Altogether, these data suggest that SOX2 is required to initiate and maintain tumor growth.

SOX2 expression is regulated by HH-GLI signaling in melanoma cells

HH-GLI signaling has been shown to modulate SOX2 expression in several contexts. Yet, it is not known whether HH can regulate SOX2 in melanoma cells. qPCR analysis in 19 patient-derived primary melanoma cells revealed a positive correlation between the expression of SOX2 and of the two HH targets GLI1 and PATCHED1 (PTCH1), the best read-out of an active HH pathway (Figure 7a). To investigate whether HH might regulate SOX2 in melanoma cells, we modulated HH signaling by silencing the transmembrane receptor SMOOTHENED (SMO) or the downstream transcription factor GLI1 nearly abolished SOX2 protein expression in SSM2c cells and reduced SOX2 mRNA in melanoma cells (Figures 7b and c), suggesting a regulation of SOX2 at transcriptional level. Consistently, activation of HH signaling by silencing the negative regulator PTCH1 increased SOX2 mRNA levels, with a stronger induction in cells with higher endogenous SOX2 expression (Supplementary Figures S8a and b).

To investigate whether the transcriptional regulation of SOX2 by HH-GLI was direct or indirect, we applied a web-based bioinformatic approach to identify putative GLI-binding sites in
SOX2 promoter. We detected a possible site with the consensus GLI-binding sites at about 300 bp upstream to SOX2 transcription start site (TSS), within the core proximal promoter region of SOX2 (−528 and +238 from the TSS). Chromatin immunoprecipitation (ChIP) in M26c melanoma cells showed that both endogenous GLI1 and GLI2 transcription factors bound to SOX2 promoter at about 300 bp upstream of the TSS (Figures 7d and e).

SOX2 acts as a mediator of HH-GLI signaling in regulating melanoma cell proliferation and MIC self-renewal. Our findings suggest that HH-GLI signaling directly regulates SOX2 expression in melanoma cells. To investigate whether SOX2 acts as a downstream mediator of HH signaling in regulating melanoma cell growth, we activated HH pathway by silencing PTCH1 and knocked down SOX2 in melanoma cells. We transduced SSM2c and M26c cells with LV-shPTCH1,60,61 alone or in combination with LV-shSOX2, and we assessed proliferation. In both the cell types, shPTCH1 increased cell number compared with LV-c, as already reported.61 Silencing of SOX2 drastically reduced the number of cells compared with LV-c, and it suppressed the increase of cell proliferation induced by HH pathway activation (Figure 7f). These results support the hypothesis that endogenous SOX2 might mediate the effect of HH pathway activation in regulating melanoma cell proliferation.

We next tested for a possible role of SOX2 in controlling HH-mediated MIC self-renewal, using SSM2c and M26c melanoma spheres transduced with LV-shPTCH1 and/or LV-shSOX2. Silencing of PTCH1 increased by two-fold the number of melanoma spheres compared with LV-c, as expected.61 Silencing of SOX2 decreased the number and size of melanoma spheres, and more importantly, it reverted the increase in self-renewal and...
size induced by shPTCH1 (Figures 7g and h, Supplementary Figure S9a), SOX2 silencing slightly decreased sphere proliferation and abolished the effect of HH pathway activation, as shown by proliferation index analysis (Figure 7i and Supplementary Figure S9b). Analysis of Annexin V/7-AAD staining indicated that SOX2 knockdown increased early and late apoptosis, whereas shPTCH1 increased only early apoptosis in one line (Figure 7j) and Supplementary Figure S9c), suggesting sign of proliferative stress. These data suggest that SOX2 mediates, at least in part, the effect of HH pathway activation on the regulation of MIC self-renewal.

**DISCUSSION**

In this study, we describe for the first time an important role for SOX2 in MICS. We show that depletion of SOX2 reduces MIC self-renewal in vitro and progressively abrogates tumor growth, leading to significant decrease in tumor-initiating capability in vivo. Our study strongly suggests that in melanoma SOX2 is essential for tumor initiation and for tumor maintenance. Furthermore, we provide evidence that SOX2 is directly regulated by the transcription factors GLI1 and GLI2 and that SOX2 functionally acts as a mediator of HH signaling in controlling melanoma cell growth and MIC self-renewal.

The major finding of our investigation is the essential role of SOX2 in the maintenance of melanoma stem cells. This result is in line with the previously described role of SOX2 in other types of cancer. Our data suggest that SOX2 expression is not only restricted to melanoma stem cells but is also relevant in non-stem cancer cells, where it is required to sustain the proliferative rate of melanoma cells that constitute the tumor bulk. However, not all melanomas are SOX2-positive; indeed, we find that SOX2 is expressed in about half of melanomas, consistently with previous immunohistochemical studies.

In addition, we show that the expression of SOX2 correlates with activation of the HH pathway, which is required for melanoma cell proliferation and melanoma stem cell self-renewal. Together, our findings suggest that in this subset of melanomas a HH-SOX2 axis is crucial for the maintenance and survival of both tumor bulk and CSC compartments.

The molecular mechanisms underlying SOX2-mediated stemness remain largely unknown. In this study, we observe induction of NANOG, OCT4 and partly KLF4 upon SOX2 expression. This could be a direct transcriptional effect induced by SOX2 or mediated by the fact that SOX2 induces a stem cell state characterized by expression of these factors. The latter hypothesis is consistent with the findings that SOX2 can reprogram differentiated somatic cells to become iPSC cells and that SOX2 is required in several types of CSCs.

Our data indicate that silencing of SOX2 induces apoptosis and signs of DNA damage in melanoma cells and MICS. These data suggest that SOX2 modulates the balance between survival and pro-apoptotic factors. Further studies are needed to explore in detail the mechanisms of apoptosis induced by loss of SOX2 and to investigate whether BCL-2, NOXA or GADD45A are direct transcriptional targets of SOX2 in melanoma. In support of our data, a recent study identified SOX2-binding regions in BCL-2, NOXA/PAIP1 and GADD45A genes using a ChiP-sequencing approach in glioblastoma cells. In previous experiments, it was found that SOX2 knockdown decreases tumorigenicity of A2058 melanoma cells. Our xenotransplantation experiments using...
patient-derived primary melanoma cells fully confirm this finding. In addition, in our hands SOX2 knockdown also inhibits in vitro growth of primary melanoma cells and of A375 cell line.

HH-GLI pathway has been shown to regulate stemness in several contexts. For instance, ectopic expression of GLI1 in mouse Nestin+ progenitors drives neural stem cell self-renewal and induces the expression of several stemness genes, including Sox2.60 Nanog, which has been identified as a direct Hh target in neural stem cells,68 is also a mediator of HH in glioblastoma cells.69 Here we present evidence that HH signaling regulates SOX2 expression at transcriptional level and that this regulation might be direct, as suggested by the binding of GLI1 and GLI2 to SOX2 promoter. Interestingly, our results indicate that SOX2 function is required for the maintenance of melanoma cell proliferation and MIC self-renewal induced by the activation of HH signaling, suggesting that SOX2 acts as downstream mediator of HH in melanoma cells. In line with our results, SOX2 has recently been shown to be required in Sonic Hedgehog-associated medulloblastomas.70 In basal cell carcinoma and tumor-initiating pancreatic cancer cells, HH-GLI and epidermal growth factor receptor signaling integration synergistically activates a number of cooperation response genes, including SOX2 and SOX9.25

Figure 5. Enhanced SOX2 function increases stemness in melanoma cells. (a) Western blotting analysis shows SOX2 expression levels in control (LV-c) and SOX2-stably transfected (LV-SOX2) SSM2c and M33c melanoma cells. HSP90 was used as loading control. (b) Increase in the number of secondary spheres upon SOX2 expression. (c) Measurement of the size of secondary melanoma spheres in LV-c and LV-SOX2 transfected SSM2c and M33c spheres. (d) Representative images of secondary melanoma spheres as indicated in panel (c). Scale bar = 100 μm. (e–h) Gene expression analysis of SOX2, KLF4, NANOG, OCT4 (e, f) and BCL-2 and BCL-XL (g, h) in LV-c and LV-SOX2 stably transfected melanoma spheres, as measured by qPCR. Ct values were normalized with two housekeeping genes, with the values in control spheres equated to 1. Data shown are the mean ± s.e.m. of at least three independent experiments. *P ≤ 0.05; **P ≤ 0.01 vs LV-c.

In addition to being controlled by HH, SOX2 might also directly regulate HH signaling, contributing to create a HH-GLI-SOX2 regulatory loop, as suggested by the binding of Sox2 to the promoter of Shh in mouse developing brain70 and of GLI2, GLI3 and SHH in glioblastoma cells.67 and neural stem cells.71 Although the possibility to selectively target SOX2 or other oncogenic transcription factors remains a challenge, recent studies began to evaluate new strategies to inhibit SOX2. For instance, a zinc finger-based artificial transcription factor approach has been used in breast cancer cells to selectively suppress SOX2 function72 and a novel inhibitor of Sox2 DNA binding has been identified.73 However, these strategies have yet limited use in vivo, and further studies are needed to explore novel approaches to effectively and safely target SOX2 in patients. Our study provides several pieces of evidence for the relevance of SOX2 as a therapeutic target in a subset of melanomas. First, we show that SOX2 silencing reduces proliferation and induces apoptosis in melanoma cells (Figure 1). Second, we demonstrate that SOX2 knockdown inhibits self-renewal and tumorigenicity of MICs (Figures 3, 4 and 6). Third, the HH-GLI signaling, which is active in melanomas and MICs,7,15 induces SOX2 expression (Figure 7 and Supplementary Figure S8).
MATERIALS AND METHODS

Patient samples and cell lines
A375 melanoma cells (CRL-1619) were obtained from ATCC (Manassas, VA, USA) and normal human epidermal melanocytes from PromoCell (Heidelberg, Germany). Human melanoma samples (Supplementary Table S1) were obtained after approved protocols by the Ethics Committee. Fresh tissue samples were digested enzymatically using 1 mg/ml collagenase A and 20 μg/ml DNase I (Roche Applied Science, Basel, Switzerland) in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Euroclone, Milan, Italy) and filtered, and cells were grown in DMEM/F12 with 10% fetal bovine serum and epidermal growth factor (5 ng/ml) (Life Technologies, Paisley, UK). The identity of melanoma cells was verified by immunocytochemistry using anti-Melan A, anti-S100 and anti-Vimentin antibodies.7 For melanoma sphere cultures, SSM2c, M5, M26c, M33c and A375 cells were seeded at a concentration of 5000 cells/ml either in DMEM/F12 serum-free medium with N2 supplement, 20 μg/ml insulin, 10 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor (Life Technologies) or in human embryonic stem cell medium, as previously described.1,7 Puromycin (Sigma, St Louis, MO, USA) was used at 2 μg/ml.

RNA interference and lentivectors
Lentiviruses were produced in HEK-293T cells as previously reported.60 Lentiviral vectors were pLKO.1-puro (LV-c) (Open Biosystems, Lafayette, CO, USA), pLKO.1-puro-shSOX2-1 (LV-shSOX2-1) targeting the 3′ untranslated region of SOX2 (targeting sequence 5′-CTGCCGAGAATCCATGTAAT-3′), pLKO.1-puro-shSOX2-2 (LV-shSOX2-2) targeting the coding region of SOX2 (targeting sequence 5′-CAGCTCGCAGACCTACATGAA-3′) and pLV-CTH-shPTCH1 (LV-shPTCH1)60,61 were previously described.

Figure 6. Silencing of SOX2 impairs in vivo tumor initiation and growth of ALDHhigh melanoma stem cells. (a, b) In vivo tumor growth after two passages of serial xenotransplantation of FACS-sorted ALDHhigh SSM2c (a) and M26c (b) cells transduced with LV-c, LV-shSOX2-1 and LV-shSOX2-2. For each group, 10 tumors were analyzed. (c) Representative images of subcutaneous xenografts in athymic nude mice taken at the same time for each group. (d) Western blotting for SOX2 in SSM2c and M26c xenografts. Note the decrease in SOX2 immunoreactivity in LV-shSOX2 xenografts compared with control LV-c, confirming the efficacy of SOX2 knockdown. HSP90 was used as loading control. (e) Expression of SOX2 protein and activated Caspase-3 in paraffin sections of M26c xenografts analyzed by immunocytochemistry. Sections were counterstained with hematoxylin (blue nuclei). (f, g) In vivo tumor growth after subcutaneous injection of 1000, 100 and 10 FACS-sorted ALDHhigh SSM2c and M26c cells transduced with LV-c and LV-shSOX2-1. (h) Estimated tumor-initiation frequency after four independent serial dilution (10 000, 1000, 100 and 10 cells) transplantation experiments of FACS-sorted ALDHhigh SSM2c and M26c cells transduced with LV-c and LV-shSOX2-1. Scale bar = 10 mm (c) and 80 μm (e). Data shown are mean ± s.e.m.

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Figure 7. SOX2 is regulated by HH signaling and is required for melanoma cell growth and MIC self-renewal induced by HH activation. (a) Linear correlation analysis of SOX2 with GLI1 and PTCH1 expression, as measured by qPCR, in the melanoma cell line A375 and in 19 patient-derived melanoma cultures, 1 of which is from a primary melanoma (Prim) and 18 are from metastases (Met). Each sample is represented by a dot. Axes in each graph represent expression ratio of gene/(GAPDH and \(\beta\)-ACTIN). The extent of the correlation is indicated by \(R\) coefficient. (b) Western blotting analysis of SSM2c cells transduced with LV-c, LV-shGLI1 and LV-shS98O shows the dramatic reduction of GLI1 and complete loss of SOX2 protein upon inhibition of HH signaling. HSP90 was used as loading control. (c) qPCR analysis of SSM2c cells transduced with LV-c and LV-shGLI1, showing the drastic reduction of GLI1 and SOX2 mRNA. qPCR values reflect \(C_t\) values after normalization with two housekeeping genes, with the values in control equalled to 1. (d) qPCR analysis of ChIP experiments shows binding of GLI1 and GLI2 at SOX2 promoter (−363 bp to −232 bp) in M26c melanoma cells. (e) Location of the putative GLI-binding site on SOX2 promoter. Numbers indicate the position of the binding site relative to the TSS. (f) Growth assay in M26c and SSM2c cells transduced with LV-c, shPTCH1 or shPTCH1/shSOX2-1, showing that SOX2 silencing reduces the increase in melanoma cell proliferation induced by shPTCH1. (g) Self-renewal assay showing that SOX2 ablation prevents the increase in self-renewal induced by shPTCH1 in M26c and SSM2c melanoma spheres. (h) Representative images of panel (g). (i) Proliferation index measured by CellTrace Violet staining in SSM2c spheres stably transduced with LV-c, LV-shSOX2-2, LV-shPTCH1 or LV-shSOX2-2/LV-shPTCH1. (j) Quantification of the fraction of early (Annexin V\(^+\)/7-AAD\(^−\)) and late (Annexin V\(^+\)/7-AAD\(^+\)) apoptotic cells in SSM2c spheres stably transduced with LV-c, LV-shSOX2-2, LV-shPTCH1 or LV-shSOX2-2/LV-shPTCH1. Scale bar = 100 \(\mu\)m (h). Data represent mean ± s.e.m. values. At least three independent experiments were performed. *\(P\) ≤ 0.05; **\(P\) ≤ 0.01.
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Self-renewal assay
For self-renewal assay, cells were transduced with LV-c, LV-shSOX2-1, LV-shSOX2-2 and LV-shPTCH1 lentiviruses or stably transfected with LV-SOX2 and let to form spheres. Primary melanoma spheres were dissociated and plated either in 96-well plates at 1 cell/well or in 12-well plates at 1 cell/μl dilutions. After 1 or 2 weeks, secondary spheres were counted.2,21 All the experiments were performed in triplicate and repeated at least three times.

Aldefluor assay, proliferation index and apoptosis
The separation of cells with high and low ALDH activity (ALDHhigh and ALDHlow) was performed using the Aldefluor kit (Stem Cell Technologies, Vancouver, BC, Canada) as already described.7 For proliferation index experiments, cells were labeled with 10 μM CFSE or CellTrace Violet (Life Technologies), seeded and allowed to proliferate for 48, 72 and 96 h and analyzed using flow cytometry. Data were normalized to controls arrested at the parent generation with 1 μg/ml mitomycin C (t=0 h), and proliferation index was calculated using the ModFit LT software (Verity Software House, Topsham, ME, USA). For apoptosis analysis, cells were exposed to serum-deprived conditions, and apoptosis was measured after 48 h using an Annexin V-phycocerythrin/7-AAD apoptosis kit (BD Biosciences, San Diego, CA, USA).

qPCR
Total RNA was isolated as already described.61 Reverse transcription was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). qPCR amplifications were carried out at 60 °C using Power SYBR Green PCR Master Mix (Applied Biosystems) and analyzed by delta-ΔCt method using GAPDH and β-ACTIN as housekeeping genes. Primer sequences are listed in Supplementary Table S2.

Western blotting
Cells were lysed as previously described.61 The following antibodies were used: rabbit polyclonal anti-SOX2 (AB5603; Millipore), rabbit polyclonal anti-GLI1 (Ab49314) (Abcam, Cambridge, MA, USA), mouse anti-GLI2 (R&D Systems, Minneapolis, MN, USA) and mouse anti-HSP90 (Cell Signaling Technologies, Danvers, MA, USA), followed by an anti-rabbit fluoroscein isothiocyanate-conjugated secondary antibody. For SOX2 immunofluorescence, spheres were immunolabeled with a rabbit anti-SOX2 antibody (AB5603; Millipore, Billerica, MA, USA) and with an anti-rabbit fluoroscein isothiocyanate-conjugated secondary antibody. Formalin-fixed paraffin-embedded sections were subjected to antigen retrieval (with citrate buffer pH 6.0), incubated with rabbit anti-SOX2 antibody (AB5603; Millipore) and rabbit anti-cleaved Caspase3 (Cell Signaling Technologies) and visualized using UltraVision Detection System (Lab Vision, Fremont, CA, USA) and diaminobenzidine (Dako, Carpinteria, CA, USA).

ChIP
M26c cells were fixed with 1% formaldehyde and lysed. DNA was sonicated and diluted with ChIP Dilution Buffer, and Input Material (IM) (5%) was collected. Chromatin was incubated overnight at 4 °C with Dynabeads Protein G (Life Technologies) pre-conjugated with anti-GLI1 antibody (N-16) (Santa Cruz Biotechnology), anti-GLI2 (R&D Systems, Minneapolis, MN, USA) or a non-specific IgG control. DNA was purified, and qPCRs was carried out at 60 °C using Power SYBR Green PCR Master Mix (Life Technologies). ChIP primers are displayed in Supplementary Table S2.

Mouse xenograph assay
Six- to 8-week-old female athymic-nude mice (Foxn1 nu/nu) (Harlan Laboratories, Udine, Italy) were injected subcutaneously in lateral flanks with 10,000, 1000, 100 and 10 FACS (fluorescent-activated cell sorter)-sorted ALDEFlow patient-derived SSM2c and M26c melanoma cells transduced with LV-c, LV-shSOX2-1 or LV-shSOX2-2. For serial transplantation, primary tumors were dissociated, dissociated and FACS-sorted ALDEFlow cells were re-injected. Cells were suspended in Matrigel (BD Biosciences)/DMEM(1/1) before inoculation. Animals were monitored daily, subcutaneous tumor size was measured every 2–3 days by a caliper (2Biological Instruments, Varese, Italy), and tumor volumes were calculated using the formula V = W² × L × 0.5, where W and L are, respectively, tumor width and length. Statistical analysis of tumor size was performed using the ELDA Web-based tool (http://bioinf.wehi.edu.au/software/elda/).7,4

Statistical analysis
Data are presented as mean ± S.E.M. for at least three independent experiments, unless otherwise stated. Statistical analysis of the data was performed by a two-tailed Student’s t-test. *P<0.05; **P<0.01.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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