SOX2 Expression Associates with Stem Cell State in Human Ovarian Carcinoma

Petra M. Bareiss1, Anna Paczulla1, Hui Wang1, Rebekka Schairer1, Stefan Wiehr2, Ursula Kohlhofer3, Oliver C. Rothfuss4, Anna Fischer3, Sven Perner3, Annette Staebler3, Diethelm Wallwiener5, Falko Fend3, Tanja Fehm5, Bernd Pichler2, Lothar Kanz1, Leticia Quintanilla-Martinez3, Klaus Schulze-Osthoff4,7, Frank Essmann4, and Claudia Lengerke1

Abstract
The SRY-related HMG-box family of transcription factors member SOX2 regulates stemness and pluripotency in embryonic stem cells and plays important roles during early embryogenesis. More recently, SOX2 expression was documented in several tumor types including ovarian carcinoma, suggesting an involvement of SOX2 in regulation of cancer stem cells (CSC). Intriguingly, however, studies exploring the predictive value of SOX2 protein expression with respect to histopathologic and clinical parameters report contradictory results in individual tumors, indicating that SOX2 may play tumor-specific roles. In this report, we analyze the functional relevance of SOX2 expression in human ovarian carcinoma. We report that in human serous ovarian carcinoma (SOC) cells, SOX2 expression increases the expression of CSC markers, the potential to form tumor spheres, and the in vivo tumor-initiating capacity, while leaving cellular proliferation unaltered. Moreover, SOX2-expressing cells display enhanced apoptosis resistance in response to conventional chemotherapies and TRAIL. Hence, our data show that SOX2 associates with stem cell state in ovarian carcinoma and induction of SOX2 suppresses CSC properties on SOC cells. We propose the existence of SOX2-expressing ovarian CSCs as a mechanism of tumor aggressiveness and therapy resistance in human SOC. Cancer Res; 73(17); 5544–55. ©2013 AACR.

Introduction
Pluripotency-associated stem cell factors such as OCT4 and SOX2 regulate cellular identity in embryonic stem cells and facilitate the reprogramming of terminally differentiated somatic cells back to a pluripotent stem cell state (1). SOX proteins are also important regulators of early development in different tissues, such as the foregut and lung, where for example SOX2 expression controls bronchogenesis by inhibiting airway branching (2, 3). In adult mice, SOX2 is expressed in different epithelial compartments marking cells with self-renewal properties (4), and targeted ablation lethally disrupts epithelial tissue homeostasis (4). SOX2 expression is also found in neural stem cells, where it promotes stemness by preventing default differentiation into neurons (5).

More recently, SOX2 expression has been shown in several tumor types such as lung (6–10), breast (11–14), skin (15, 16), prostate (17), ovarian (18, 19), sinonasal (20) as well as different types of squamous carcinomas (21). However, the SOX2 expression pattern and the correlation with histopathologic status and clinical outcome are highly variable among tumors, suggesting distinct roles of SOX2 in individual tumors. In breast carcinoma, SOX2 expression is mostly detected in a minor subset of tumor cells and seems to be an early event in tumor development (13), indicating potential roles in cancer stem cells (CSC) biology and involvement in reprogramming processes generating CSCs. In support of this notion, induction of SOX2 expression in breast carcinoma cell lines was shown to enhance CSC properties such as tumor sphere potential and in vivo tumorigenicity (12). Moreover, SOX2 expression was associated with positive lymph-nodal status in early-stage breast carcinoma (13). In contrast, in human squamous cell lung cancer, SOX2 protein overexpression was associated with smaller tumor size, lower probability of metastasis, and improved clinical outcome. Other than breast carcinoma, squamous cell lung cancer samples displayed homogeneous SOX2 expression, arguing against specific roles of SOX2 in lung CSCs.

Ovarian carcinoma has the seventh highest morbidity rate of cancer in women (22). Because of the lack of early specific symptoms, ovarian carcinoma is mostly diagnosed at advanced metastatic stages that cannot be cured by surgical
resection alone. Despite initially good response rates to Platinum-based chemotherapies, relapse is a common event during the clinical course of the disease (22). An explanation for ovarian carcinoma relapse is provided by the tumor stem cells hypothesis, proposing that conventional chemotherapeutic approaches target the fast proliferating bulk of the ovarian cancer cells, while sparing the tumor-initiating CSCs (23). The isolation and molecular characterization of ovarian CSCs are thus subjects of intense research. Previous studies have suggested ALDH1, CD133, CD44, and CD117 as ovarian CSC markers, but plasticity and transition between stem and non–stem cell states complicates their efficient isolation (24–32).

In this study, we hypothesized that SOX2 expression associates with stem cell state in ovarian carcinoma. SOX2 protein expression can be detected in 15% to 60.5% of ovarian carcinomas (18, 19), depending on the staining methodology. Supporting our hypothesis, we observed that the majority of SOX2-positive (SOX2+) samples displayed SOX2 protein expression in less than 10% of tumor cells. Moreover, SOX2 expression was enhanced by culture conditions enriching for tumor stem cells. Detailed analyses conducted on SOX2-modified human serous ovarian carcinoma (SOC) cell lines and primary cells show that indeed SOX2 expression induces CSC properties, such as expression of stemness markers, tumor sphere formation, in vivo tumor-initiating capacity as well as apoptosis resistance, thereby strongly promoting in vivo tumorigenicity and enabling selective resistance to conventional anticancer therapies.

Materials and Methods

Cell culture, tumor spheres, and cell growth assays

Ovarian cancer cell lines (Caov-3, OVCAR-3, OVCAR-5; DSMZ; last authentication on January 31, 2013 at DSMZ) and primary cells obtained through dissociation of tissue samples derived from 4 patients with SOC were cultured under standard conditions. For the tumor sphere culture assay, cells were grown in ultralow attachment plates (Corning) with sphere medium and daily-added growth factors [20 ng/mL fibroblast growth factor (FGF), 20 ng/mL EGF; Sigma-Aldrich] as described previously (33). Spheres were counted between day 5 and 9. To investigate serial sphere formation, spheres were washed with PBS and dissociated to single cells by trypsinization. To assess cell growth, 50,000 cells were plated under adherent conditions and counted on day 3, 6, and 9. For sphere cultures conducted to enrich for CSC activity, OVCAR-3 and Caov-3 cells were maintained under sphere culture conditions for 21 days and primary SOC cells for 10 days before undergoing assessment.

Analysis of a tissue microarray of primary human ovarian carcinomas

SOX2 protein expression and Ki67 positivity were investigated by immunohistochemistry using polyclonal goat anti-human SOX2 (AF2018; R&D Systems) and monoclonal mouse anti-human Ki67 (clone MiB-1, M7240; DakoCytonom) on a tissue microarray (TMA) including 215 human primary ovarian carcinomas from patients treated at the University of Tuebingen (Tuebingen, Germany). Detailed information about the TMA construction and analysis are provided in the Supplementary Data. The study was approved by the Ethics Committee of the University of Tuebingen.

Lentiviral transduction

Lentiviruses carrying SOX2 short hairpin RNA (shRNA), SOX2 overexpression, corresponding empty GFP-, and SOX2-enhancer reporter constructs (34) were designed, produced, and used for transduction as previously reported (35–37). Details on lentiviral constructs and protocols are provided in the Supplementary Data.

Flow cytometry analysis of stem cell markers, cell cycle, and BrdU assays

To detect aldehyde dehydrogenase (ALDH) activity, the ALDEFLUOR assay was used according to the manufacturer's guidelines (STEMCELL Technologies). Cells were incubated in ALDEFLUOR assay buffer for 30 minutes. Cells from each sample additionally treated with the ALDH inhibitor diethylaminobenzaldehyde (DEAB) served as negative controls. For flow-cytometric analyses, anti-CD133 (Miltenyi Biotec) and anti-TRAIL receptor 1 and 2 antibodies (BioLegend) were used. Dead cells were detected by 4',6-diamidino-2-phenylindole (DAPI) staining (100 ng/mL). Cell-cycle analysis using propidium iodide (PI; Sigma) staining and bromodeoxyuridine (BrdU, Roche) incorporation assays using mouse anti-BrdU V450 antibody (BD Biosciences) were conducted as previously described (36). Flow-cytometric analyses were conducted using a FACS Canto II and data analysed using the FACS Diva software (BD Biosciences).

Apoptosis assays

Cells seeded at 50,000 cells/cm² were incubated overnight and then treated for 24 hours with staurosporine (2.5 μmol/L; Sigma-Aldrich) or Superkiller TRAIL (25 ng/mL; Enzo Life Sciences), or for 96 hours with cisplatin (5 μmol/L; Medac), carboplatin (100 μmol/L; Medac), or paclitaxel (5 nmol/L; Bristol-Myers Squibb). Cells were harvested by trypsinization, fixed in 70% ice-cold ethanol, and incubated in PBS containing 50 μg/mL PI and 100 μg/mL RNase A. Cells with subdiploid DNA content (sub-G₁) were assessed by flow cytometry. Caspase-3/7 activity was assessed by the Caspase-Glo 3/7 assay (Promega) and normalized to protein content following treatment with staurosporine (4 hours) or TRAIL (6 hours).

Immunoblot and immunocytochemistry analyses

Immunoblot and immunocytochemistry analyses were conducted using mouse anti-actin (LI-COR Biosciences), rabbit anti-caspase-3 (Cell Signaling Technology), rabbit anti-cleaved caspase-3 (Asp175; Cell Signaling Technology), and rabbit anti-SOX2 (D6D9) XP (Cell Signaling Technology) antibodies. Detection was carried out using IRDye 800CW-conjugated goat anti-rabbit immunoglobulin G (IgG) or IRDye 680 anti-mouse IgG antibodies (LI-COR Biosciences) and an Odyssey Imager (LI-COR Biosciences). For detection of the SOX2 knockdown horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (Cell Signaling Technology) and ECL Prime Western Blotting Detection Reagent (GE Healthcare) were used.
Gene expression analyses
RNA isolation, cDNA preparation, and real-time gene expression analyses were conducted as described previously (13, 38) using a LightCycler 480 instrument and LightCycler probes master mix (for SOX2, ALDH1, LIN28, NANOG, OCT4, and GAPDH; Roche) or SYBR Green assay (for BCC3, PMAIP1, and BCL2; Eurogentec). Primers and probes are listed in the Supplementary Data. Relative expression levels were calculated after normalization to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Probe or PBGD (SYBR Green) by using the ΔΔCt method.

Xenotransplantation model
NOD.Cg-Prkd6ermIL2rgtmWj/+Sz (also termed NOD/SCID/IL2rgtmWj; abbreviated as NSG) mice (39) were purchased from The Jackson Laboratory and maintained under pathogen-free conditions. Control and SOX2-overexpressing Caov-3 cells mixed with Matrigel (1:2; BD Biosciences) were implanted subcutaneously in individual flanks of the same mouse. Tumor growth was monitored by palpation of the injection site and positron emission tomography (PET)/magnetic resonance imaging (MRI) analysis conducted using intravenously administered 11 to 15 MBq of [18F]fluoro-2-deoxy-D-glucose (FDG) as described previously (40, 41) and summarized in the Supplementary Data. Mice were euthanized 7 to 15 weeks after implantation. For histologic analysis, mouse tissues were fixed in 4% formaldehyde, paraffin-embedded, cut in 3 to 5 µm sections, and stained with hematoxylin and eosin (H&E). Immunohistochemical analysis was conduct as described previously (42) on an automated immunostainer (Ventana Medical Systems) according to the company’s protocol for open procedures with slight modifications. The antibody panel used included SOX2 (SP76; Cell Marque), cleaved caspase-3 (Asp 175; Cell Signaling Technology), Ki67 (SP6; DCS Innovative Diagnostik Systeme), and EpCAM (BerEp4; Dako).

Statistical analyses
For all experiments, mean values are presented and error bars represent the SE if not otherwise indicated. P values are derived via the application of a two-tailed, unpaired Student t test.

Results
SOX2 expression is enhanced in CSC-enriched SOC cell cultures
SOX2 mRNA expression was investigated by real-time PCR in SOC patient samples and cell lines including the lines Caov-3 and OVCAR-3 harboring amplifications on the chromosome 3q (Fig. 1A). Heterogeneous expression of SOX2 was noted (Fig. 1A and Supplementary Fig. S1) mirroring the results documented in TMA of human SOC samples (18, 19). Previous data on ovarian and breast cancer cells reported that sphere cultures increase CSC frequency as compared with two-dimensional (2D) adherent cultures (27, 33). Indeed, ovarian cancer cell lines grown as spheres for 21 days showed a higher frequency of ALDHhighCD133+ putative CSCs (OVCAR-3 cells; Fig. 1B) and enhanced expression of putative stem cell markers in comparison with 2D cultures (Caov-3 cells; Fig. 1C). Consistent with a role of SOX2 as a stem cell marker in SOC, SOX2 expression was also enhanced in sphere cultures of Caov-3 as well as primary ovarian carcinoma cells (Fig. 1C).

SOX2 modulates CSC properties in human SOC cells
To explore the functional role of SOX2 in ovarian carcinoma, we stably suppressed SOX2 expression in OVCAR-3, the SOC line with the highest basal SOX2 expression, using two different lentiviruses containing SOX2-inhibitory shRNAs (Fig. 2A and Supplementary Fig. S2A). Furthermore, Caov-3 cells displaying low-basal SOX2 expression as well as primary SOC cells derived from patients were treated with SOX2 lentiviruses to study the effects of SOX2 overexpression (Fig. 2B and Supplementary Fig. S3A). Cells transduced with empty GFP-lentiviruses were used as controls.

Induction of SOX2 expression in both Caov-3 and primary SOC cells was able to enhance the expression of other putative stem cell markers (LIN28, NANOG, OCT4, and ALDH1; Fig. 3A and B), suggesting that activation of SOX2 expression is sufficient to facilitate the transition to a stem cell–like state. Consistent with this notion, enhanced tumor spheres formation was observed in SOX2-overexpressing cells, whereas SOX2 knockdown induced the opposite effect (Fig. 4A). Notably, the effect on tumor sphere formation was also documented in primary SOC cells and was even more pronounced upon serial replating (Fig. 4B). Single cell tumor sphere assays further confirmed the higher frequency of sphere-initiating cells in SOX2-expressing versus control cells (Fig. 4C).

To further explore the role of SOX2 as a CSC marker in human SOC, we treated OVCAR-3 cells with a lentiviral SOX2-reporter construct, previously described to recognize cells with high SOX2 promoter activity (SOX2+) in breast carcinoma and neural stem cells (Supplementary Fig. S4A; refs. 12, 43). Puromycin selection was applied to select for efficiently transduced cells and SOX2+ cells were visualized by fluorescence. Supporting our previous data, SOX2+ cells were enriched in OVCAR-3 cells cultured as spheres, as compared with 2D cultures (Supplementary Fig. S4B). In primary sphere assays, nearly every SOX2+ cell isolated by fluorescence-activated cell sorting (FACS) gave rise to an individual tumor sphere (SOX2+/sphere), and SOX2+ spheres were larger than those derived from reporter-negative cells [SOX2-negative (SOX2−) spheres; Fig. 4D; Supplementary Fig. S4C]. Importantly, flow cytometry of SOX2+ primary spheres revealed a mixture of SOX2+ and SOX2− cells, suggesting that SOX2+ cells undergo both self-renewal and differentiation processes giving rise to both populations of cells. In contrast, primary SOX2− spheres remained SOX2− (Supplementary Fig. S4C). Consistent with these data, cells derived from SOX2− spheres exhausted their sphere generation potential upon serial replating, whereas cells derived from SOX2+ spheres maintained sphere formation (Fig. 4D).

Together, these data indicate that cells with self-renewal capacity segregate to the SOX2+ compartment and suggest that SOX2 induction can activate CSC molecular pathways and functional properties in human SOC cells.
Induction of SOX2 strongly enhances in vivo tumorigenicity in a NSG mouse model

To explore the relevance of SOX2 expression in SOC cells in vivo, we carried out xenotransplantation experiments of SOX2-overexpressing and control Caov-3 cells in immunoposphine NSG mice (39). The same numbers of SOX2-overexpressing and control cells were implanted subcutaneously in the flanks of 8-week-old female mice as indicated, and tumor induction was monitored every second week by palpation of the injection sites. To avoid bias through different animal hosts, SOX2-overexpressing and control cells were injected in the right and left flank of the same mouse. When 500,000 cells were injected per flank, control Caov-3 cells generated tumors at 4 weeks postinjection in 1 of 4 animals (Fig. 5A). Lowering the number of transplanted cells to 100,000 or 50,000 cells per animal delayed tumor formation from control Caov-3 cells (Fig. 5A). In contrast, SOX2-overexpressing cells robustly induced tumors in all transplanted animals and accelerated the appearance of palpable tumor masses (Fig. 5A).

To further consolidate these observations, we conducted in vivo PET/MRI analyses and ex vivo immunohistologic analyses at the end of the experiment. At week 15 postinoculation of 100,000 cells, tumors were detected on both sites in all mice by the sensitive PET/MRI method (Fig. 5D). However, quantitative image analysis of tumor volumes revealed that SOX2-overexpressing cells induced much larger tumors than control cells (118.2 ± 19.0 vs. 40.4 mm³ at 8 weeks following injection of 500,000 cells, and 25.2 ± 4.3 vs. 7.2 mm³ at 8 weeks following injection of 100,000 cells).
respectively 618.6 ± 244.6 vs. 50.98 ± 21.8 mm³ at 15 weeks when 100,000 cells were injected (Fig. 5B), which was also confirmed by immunohistologic analysis (Fig. 5C). Interestingly, PET-quantification of FDG uptake (40) revealed similar metabolic activity in SOX2-overexpressing and control tumors at both measured time points (8 and 15 weeks postinjection, postinoculation; Fig. 5B), indicating that the inductive effects of SOX2 on tumor formation were not mediated by modulation of metabolic activity. However, due to their larger mass, tumors derived from SOX2-overexpressing cells partially displayed necrotic areas revealing a heterogeneous uptake of FDG at the measured time points.

Interestingly, the pronounced difference of in vivo tumorigenicity between SOX2-overexpressing and control cells was

Figure 2. Modulation of SOX2 expression. Modulation of SOX2 mRNA and protein expression in ovarian cell lines after lentiviral SOX2 knockdown (A) or SOX2 overexpression (B) in comparison with control lentiviruses. Top, relative gene expression levels normalized to GAPDH, as measured by real-time PCR analysis of 3 representative independent biologic experiments, each carried out in triplicates. Bottom, immunoblot analyses representative for 3 independent biologic replicates.

Figure 3. SOX2 expression enhances expression of putative stem cell genes. Caov-3 (A) and primary SOC patient-derived cells (B) were analyzed by real-time PCR and normalized for GAPDH. Shown are the fold changes in relative gene expression in cells cultured in spheres (gray box) versus cells cultured in 2D (dashed line). Shown are data from one representative out of 3 independent biologic experiments carried out in triplicates for Caov-3 cells and from technical triplicates in primary cells (\( P < 0.05; \quad \ast \ast \ast \ast \ast \ast \ast \ast \ast , \quad P < 0.01; \quad \ast \ast \ast \ast \ast \ast \ast \ast \ast , \quad P < 0.005; \quad \text{n.s.}, \quad \text{not significant}).
SOX2 overexpression
Caov-3

Number of spheres per 100 cells

Primary spheres
Secondary spheres
Tertiary spheres
Quaternary spheres

SOX2 overexpression
OVCAR-3

Number of spheres per 100 cells

Primary spheres
Secondary spheres
Tertiary spheres
Quaternary spheres

SOX2 overexpression - single spheres
Caov-3

Number of spheres from one dissociated single sphere

Primary spheres
Secondary spheres
Tertiary spheres
Quaternary spheres

SOX2 reporter cells
OVCAR-3

Number of spheres per 100 cells

Primary spheres
Secondary spheres
Tertiary spheres
Quaternary spheres

Figure 4. SOX2 increases the sphere-forming potential of ovarian cell lines and primary tumor cells. A, SOX2-modified and control Caov-3 and OVCAR-3 cells (plated 1,250 cells/well) were scored for primary sphere formation after 9 and 5 days, respectively. Shown are data from 3 independent biologic experiments carried out in triplicates. B, primary SOC cells were transduced with lentiviruses for GFP-tagged SOX2 or a GFP control. Cells were then directly plated at a density of 625 cells per well without prior FACS. After 5 days, sphere formation of transduced (GFP+) and nontransduced (GFP−) cells was microscopically scored. Primary spheres were subsequently dissociated to single cells and used for the replating experiments to investigate secondary sphere formation. Shown are data from technical triplicates. C, single SOX2-overexpressing and control Caov-3 cells were assessed for their sphere-forming potential in 96-well plates. Shown are data from 3 independent biologic experiments. Secondary spheres were generated by replating of cells dissociated from one individual primary sphere in each well. For tertiary and quaternary spheres, pooled spheres dissociated to single cells were replated at a density of 20 cells per well. D, SOX2− and SOX2+ OVCAR-3 cells isolated by FACS were plated in sphere conditions 100 cells per well and spheres counted after 7 days. For all replating assays, spheres were pooled, dissociated to single cells, and replated as indicated or at a density of 100 cells per well. Primary and secondary sphere formation was analyzed in 3 or more, tertiary and quaternary sphere formation in 2 independent biologic experiments carried out in triplicates (C and D).

not due to enhanced cellular proliferation, as revealed by the similar results of the Ki67 staining conducted on explanted tumors (Fig. 5C). SOX2-overexpressing tumors displayed more necrotic areas and a higher apoptotic activity, as shown by the active caspase-3 staining (Fig. 5C). These findings are in line with the in vivo PET/MRI results where necrotic areas were detected in all SOX2-overexpressing tumors (Fig. 5B).

Overall, these data strongly suggest that SOX2 mediates tumorigenicity in SOC cells by facilitating transition to a CSC state with enhanced tumor-initiating properties. To further explore this hypothesis, we conducted a limiting dilution
**in vivo** transplantation assay: 10,000, 1,000, 100, and 10 SOX2-overexpressing and control Caov-3 cells were transplanted as described earlier in the contralateral flanks of n = 5 mice per group. In contrast to the results observed with higher numbers of cells (Fig. 5A), no palpable tumors were documented at 7 weeks posttransplantation. However, immunohistologic analysis of the injection sites revealed microscopic human tumor cell clusters in animals injected with 10,000 or 1,000 cells, but not 100 or 10 cells (H&E staining; Fig. 5D and E). Staining with antibodies against human EpCAM (Supplementary Fig. S5) and CA125 (not shown) confirmed correct detection of human SOC cells. Notably, microscopic tumors were detected more frequently from SOX2-overexpressing as compared with control cells, which was especially evident with the lowest number of injected cells (Fig. 5D and E).

**SOX2 expression does not affect cell proliferation but enhances the apoptosis resistance of SOC cells**

Modulation of SOX2 expression did not alter cell-cycle progression, BrdU incorporation (Fig. 6A), or cell growth of **in vitro** 2D-cultured OVCAR-3 and Caov-3 cells (Fig. 6B). To further explore whether SOX2 regulates SOC cell proliferation, we analyzed SOX2 protein expression and Ki67 staining on a TMA of 215 human primary ovarian carcinomas comprising...
143 high-grade SOC (see Supplementary Data for details on TMA construction). In 136 of 143 high-grade SOC samples, both SOX2 and Ki67 stainings were available. Although SOX2 expression was found in 64.6% of high-grade SOC samples (Fig. 6C; Supplementary Fig. S1; data not shown), Ki67 positivity was not dependent on the SOX2 expression level, which is in contrast to findings in other tumor entities (11, 17).

As mentioned earlier, the histologic analysis of the murine tumors revealed higher levels of active caspase-3 in SOX2-overexpressing tumors (Fig. 5C). However, enhanced caspase-3 activation is most likely a secondary effect in response to restrictive in vivo environmental factors (e.g., insufficient blood supply due to disproportionate tumor growth overriding tumor’s capacity of vessel recruitment), as in vitro 2D culture experiments showed reduced levels of spontaneous apoptosis in SOX2-overexpressing Caov-3 as well as OVCAR-3 cells (Fig. 6D). To test whether SOX2 expression modulates apoptosis sensitivity, we incubated the cells with staurosporine and the death ligand TRAIL to activate the intrinsic and extrinsic pathways of apoptosis. Flow-cytometric quantitation of sub-G1 cell populations revealed enhanced apoptosis in response to staurosporine and TRAIL in the SOX2 knockdown cells, whereas SOX2 overexpression conferred enhanced resistance (Fig. 6A and B and Supplementary Figs. S2 and S6). We also assayed activity of caspase-3/7 in substrate cleavage assays (Fig. 7A and B) and processing of caspase-3 by immunoblot analysis for cleaved caspase-3 (Supplementary Fig. S7), confirming the resistance-mediating potential of SOX2 expression. Importantly, SOX2 expression in Caov-3 cells also mediated resistance to carboplatin, cisplatin, and paclitaxel (Fig. 7C and Supplementary Fig. S7), indicating SOX2 expression as a molecular driver of chemotherapy resistance in ovarian carcinoma. Notably, the
enhanced sensitivity of OVCAR-3 cells due to SOX2 knockdown was reverted by lentiviral reexpression of ectopic SOX2 (Fig. 7D). These data show that the observed phenotype specifically depends on SOX2 expression levels. Analog experiments carried out in a third cell line (OVCAR-5) and using an alternative SOX2 shRNA sequence furthermore confirmed these results (Supplementary Fig. S2; data not shown).

In an attempt to elucidate the molecular basis for SOX2-induced resistance to TRAIL-mediated apoptosis, we initially analyzed surface expression of the TRAIL receptors 1 and 2 by flow cytometry. However, no significant difference in the expression level of TRAIL-R1 or TRAIL-R2 was detected in OVCAR-3 and Caov-3 cells in response to SOX2 knockdown and overexpression, respectively (Fig. 5E). Therefore, SOX2 expression modulates apoptosis sensitivity downstream of these death receptors, as usually seen in so-called type II cells that depend on amplification of death receptor signaling via the intrinsic apoptotic pathway. As the intrinsic pathway is
controlled by the BCL2 protein family, we analyzed the expression of proapoptotic (PUMA/BBC3 and NOXA/PMAIP1) and antiapoptotic genes (BCL2) by real-time PCR. In line with the observed apoptosis resistance, overexpression of SOX2 induced enhanced expression of antiapoptotic BCL2, whereas reducing expression of the proapoptotic proteins PUMA/BBC3 and NOXA/PMAIP1 (Fig. 7F).

Discussion

SOX2 is a key regulator for maintaining the pluripotency and self-renewal of embryonic stem cells and contributes to the reprogramming of differentiated somatic cells back to a pluripotent stem cell state. More recently, enhanced SOX2 expression has been detected in several epithelial tumors (6, 7, 9–19) suggesting that SOX2 also regulates tumorigenesis. On the basis of its prominent role in pluripotent stem cell stemness, SOX2 expression has been proposed as a general feature of CSCs (12, 27, 29, 44). Emerging data, however, show divergent SOX2 expression patterns and functions across tumors, suggesting that SOX2 adopts specific roles in individual tumor types. In breast cancer cells, for instance, SOX2 was shown to promote CSC characteristics such as in vitro tumor sphere formation and in vivo tumorigenicity (12). When cultured under nonadherent sphere conditions that enrich for CSCs, breast cancer cells upregulated SOX2 expression, indicating a tight link between SOX2 expression and functional stem cell state. Furthermore, immunohistochemical analysis of primary breast carcinomas revealed a heterogeneous SOX2 protein expression in only a minority of tumor cells (13), consistent with the putative role of SOX2 as a breast CSC marker. In contrast, squamous cell lung cancers (9) mostly display homogenous distribution of SOX2 protein among tumor cells, suggesting that in this tumor entity SOX2 might also influence non-CSCs. The difference in upstream regulatory mechanisms reported for SOX2 in individual tumor types further support this hypothesis. In squamous cell lung cancers, SOX2 overexpression is mostly linked to SOX2 gene amplification on the chromosome 3q26 (9). This is in line with the observed homogenous SOX2 protein expression in all tumor cells, as genetic amplification events likely persist upon CSC differentiation. In contrast, in breast carcinomas elevated SOX2 expression has been largely detected in the absence of chromosomal amplifications and relies on yet unknown upstream regulatory mechanisms (13). Because epigenetic mechanisms essentially participate in stem cell reprogramming, it is possible that SOX2 expression in breast CSCs is triggered by epigenetic events, such as altered SOX2 promoter methylation as previously reported in glioblastoma (45).

In SOC, high SOX2 protein expression is associated with histopathologically and clinically aggressive disease (18, 19, 46). Similar as in breast carcinoma (13), we found that SOX2+ expressing ovarian carcinomas display a heterogeneous expression pattern with mostly less than 10% of tumor cells expressing SOX2 protein, indicating that SOX2 might preferentially regulate the ovarian CSC compartment. Indeed, SOC sphere cultures enriched for putative ovarian CSCs induced increased SOX2 enhancer activity and SOX2 expression as compared with 2D cultures. Consistently, SOX2+ cells enriched by detection of the SOX2 enhancer reporter generated tumor spheres from nearly every cell and showed self-renewal and differentiation properties in serial replating assays. SOX2+ cells, in contrast, gave rise to significantly less primary spheres, and most importantly, could not preserve sphere initiation properties beyond tertiary spheres.

To explore whether SOX2 expression is sufficient to mediate stemness in ovarian carcinoma cells, we modulated SOX2 expression in human SOC cell lines and primary cells by lentiviral SOX2 knockdown and overexpression. Ectopic SOX2 expression enhanced the in vitro tumor sphere potential and expression of stemness genes such as OCT4, LIN28, NANOG, and ALDH1, whereas the SOX2 knockdown showed opposite effects. However, although the frequency of sphere-initiating cells was greatly enhanced by SOX2 overexpression in Caov3 and patient-derived SOC cells, primary tumor spheres were initiated by only a fraction of SOX2-overexpressing cells. In contrast, SOX2+ cells isolated via positivity for the SOX2 enhancer reporter generated spheres from nearly every cell. A possible explanation for this finding is that, even though SOX2 can facilitate transition to a stem cell state, this transition occurs only in a subset of tumor cells. Alternatively, particularly high SOX2 expression levels, as detected by the SOX2 enhancer reporter, are needed to accomplish the transition to a CSC state, which might not be uniformly induced in all cells by lentiviral SOX2 expression.

Upon xenotransplantation in NSG mice, SOX2-overexpressing cells induced tumors earlier and more frequent than control SOC cells. In vivo PET/MRI analyses as well as histologic analyses of xenotransplanted mice confirmed larger tumor volumes from SOX2-overexpressing than control SOC cells. Because data in prostate as well as breast cancer suggested that SOX2 promotes tumorigenesis by inducing cell proliferation (11, 17), we tested whether cell growth and proliferation were affected by SOX2. Surprisingly, mouse tumors derived from SOX2-overexpressing and control cells showed similar Ki67 staining. In addition, no differences in cell-cycle distribution, BrdU incorporation, or cell growth were observed in 2D cultures of SOX2-modified and control cells. Tumors generated from SOX2-expressing and control cells showed also similar metabolic activity in the PET assay. These results were further corroborated by TMA analyses of primary ovarian carcinomas, which revealed no correlation between the SOX2 protein expression level and Ki67 positivity. Furthermore, limiting dilution experiments suggest that SOX2 overexpression enhanced the frequency of tumor-initiating cells, as increased tumor cell clusters could microscopically be detected in animals transplanted with low numbers of SOX2-overexpressing as compared with control cells. Thus, our data suggest that the enhanced tumorigenicity of SOX2-overexpressing ovarian carcinoma cells does not rely on enhanced cell proliferation, but is rather due to the induction of a CSC state.

Another feature regulating tumor formation is the apoptosis sensitivity of tumor cells. CSCs are assumed to possess enhanced apoptosis resistance, facilitating tumor generation and escape from conventional chemotherapies. Intriguingly, SOX2-expressing tumors from transplanted Caov-3 cancer cells showed enhanced caspase-3 activity...
as compared with tumors from control cells. Nevertheless, analyses of SOX2-modified Caov-3, OVCAR-3, and OVCAR-5 cells consistently showed that higher SOX2 levels prevented apoptosis in response to both intrinsic (e.g., staurosporine, chemotherapies, etc.) and extrinsic (e.g., TRAIL, etc.) stimuli, indicating that SOX2 confers apoptosis resistance, a property classically attributed to CSCs. The obviously discrepant results between caspase-3 activation observed in vitro and in vivo might be explained by several reasons, including a potentially increased hypoxia of the larger SOX2-expressing tumors, which might result in secondary necrosis.

In ovarian cancer cells, SOX2 seems to regulate stemness, tumor-initiating capacity, and apoptosis resistance, which are main features characterizing CSCs, while not modulating proliferation. The molecular mechanisms of SOX2-mediated stemness remain largely unexplored. In this study, we observed robust induction of OCT4, LIN28, NANOG, and partly ALDH1 upon SOX2 activation. This could be a direct SOX2-induced transcriptional effect or mediated by the fact that SOX2 activation induces a stem cell state characterized by expression of these markers. In embryonic stem cells, SOX2 interacts with the pluripotency proteins OCT4, NANOG, and LIN28. In line, suppression of OCT4 and LIN28 by RNA interference was recently shown to inhibit ovarian cancer cell growth and survival (29). To elucidate the pathways underlying SOX2-mediated apoptosis resistance, we first studied the surface expression of TRAIL receptors, which was not affected by SOX2 expression and, hence, indicated an involvement of receptor downstream events. Indeed, expression analysis of apoptosis-regulatory genes revealed that SOX2 modulated the expression of certain BCL2 members. In SOX2-overexpressing Caov-3 cells, expression of the antiapoptotic gene BCL2 was enhanced, whereas the expression of the proapoptotic genes PUMA/BBC3 and NOXA/PMAIP1 was reduced. These data indicate that SOX2 modulates the balance of central apoptosis regulators, thereby changing apoptosis sensitivity. New therapeutic approaches that target BCL2 proteins to enhance apoptosis may therefore be a valuable tool for targeting SOX2+ putative ovarian CSCs. Further studies are needed to explore in detail the mechanisms of apoptosis protection governed by SOX2 and to investigate whether BCL2, PUMA/BBC3, and NOXA/PMAIP1 or related genes are direct transcriptional targets of SOX2 in ovarian carcinoma. In support of this assumption is the recent identification of SOX2-binding regions in the BCL2 and NOXA/PMAIP1 genes using a ChiP-Seq approach of glioblastoma cells (47).

In summary, our data in ovarian carcinoma cell lines and patient-derived tumor samples suggest that in this tumor entity SOX2 expression is a CSC marker and can induce CSC properties such as stemness, tumor-initiating capacity, and apoptosis resistance. SOX2 expression in putative ovarian CSCs enables their selective survival to conventional chemotherapies and promotes their in vivo tumorigenicity. We propose that SOX2-expressing CSCs contribute to therapy resistance and disease relapse in patients with ovarian carcinoma and that targeting SOX2 will improve clinical treatment of ovarian carcinoma by enhancing apoptotic responses to conventional chemotherapies and exhausting the CSC fraction.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P.M. Bareiss, K. Schulze-Osthoff, C. Lengerke
Development of methodology: P.M. Bareiss, A. Paczulla, H. Wang, R. Schairer, S. Wiehr, L. Quintanilla-Martinez, F. Essmann
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.M. Bareiss, A. Paczulla, S. Schaller, S. Wiehr, U. Kohlhof, A. Fischer, A. Staebler, D. Wallwiener, F. Fend, T. Fehm, L. Quintanilla-Martinez, F. Essmann
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.M. Bareiss, A. Paczulla, R. Schairer, S. Wiehr, U. Kohlhof, O.C. Rothfuss, A. Staebler, F. Fend, B. Pichler, L. Quintanilla-Martinez, F. Essmann, C. Lengerke
Writing, review, and/or revision of the manuscript: P.M. Bareiss, S. Schaller, U.C. Rothfuss, S. Perner, A. Staebler, D. Wallwiener, F. Fend, T. Fehm, K. Schulze-Osthoff, F. Essmann, C. Lengerke
Administrative, technical, or material support (e.g., reporting or organizing data, constructing databases): P.M. Bareiss, B. Pichler, L. Kanz, F. Essmann, C. Lengerke
Study supervision: C. Lengerke

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References


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Petra M. Bareiss, Anna Paczulla, Hui Wang, et al.


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