

Sox2 inhibits Wnt- β -catenin signaling and metastatic potency of cisplatin-resistant lung adenocarcinoma cells

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Abstract. Lung cancer remains one of the most common cancer-associated mortalities worldwide, and platinum-based doublet chemotherapies are recommended as the first-line treatment for advanced non-small cell lung cancer (NSCLC). However, the frequent development of multidrug resistance, to cisplatin regimens in particular, is a major cause of chemotherapy failure in patients with aggressive NSCLC. Wnt/ β -catenin signaling and sex-determining region Y box 2 (Sox2) have been implicated in the development and progression and resistance to epidermal growth factor receptor-targeting therapy in lung cancer. The present study aimed to explore the effects of Wnt/ β -catenin and Sox2 signaling on the chemoresistance of cisplatin-resistant lung cancer cells by assessing the effects of Sox2 on Wnt/ β -catenin signaling activity, cell migration, invasion and clonogenicity, and susceptibility to cisplatin in lung adenocarcinoma A549 cells and cisplatin-resistant A549/DDP cells. The results demonstrated that an enforced expression of Sox2 led to inhibition of Wnt/ β -catenin signaling activity, potentially by upregulating glycogen synthase kinase

3 β in A549 and A549/DDP cells. An overexpression of Sox2 promoted cell migration and invasion, in addition to enhancing the clonogenic capacity in A549 cells. Notably, knockdown Sox2 using short hairpin RNA led to an enhanced susceptibility of A549 and A549/DDP cells to cisplatin, along with increased cell apoptosis. The present study thus suggests that Sox2 may be an important regulator in development of chemoresistance of lung cancer cells and may be a novel therapeutic target for treatment chemoresistant lung cancer.

Introduction

Lung cancer is a respiratory system malignancy with high mortality and its incidence has increased in recent years (1). Despite the number of novel agents specifically targeting oncogenic pathways that have been developed for lung cancer treatment, and a combination of histomorphological, immunohistochemical and genetic analysis currently employed in routine lung cancer diagnosis to stratify patients into clinically relevant subgroups for tailored treatment algorithms (2), metastatic lung cancer and the development of drug resistance to target therapy and chemotherapy mean that lung cancer remains incurable, and has poor patient outcomes with a 5-year survival rate of <20% (3,4).

The platinum-based doublet chemotherapy has been recommended as the first-line therapy for advanced non-small cell lung cancer (NSCLC) and has a 20% response rate in patients with NSCLC (5). However, the prognosis of this treatment in patients with aggressive NSCLC remains poor, mainly owing to the development of multidrug resistance, in particular against cisplatin regimens (6,7). Mechanistically, the cisplatin therapy induces DNA interstrand and intrastrand crosslinks in tumor cells, sequentially inhibiting cell replication and transcription (8). Therefore, understanding the mechanisms underlying the development of cisplatin therapy resistance may lead to the development of efficient therapeutic strategies for NSCLC.

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The Wnt/ β -catenin signaling pathway has been recognized as an oncogenic pathway with pivotal roles in numerous types of cancer, and aberrant activation of Wnt signaling was detected in 50% of human NSCLC cell lines and resected lung cancer samples (9), where it was associated with the increased proliferation and metastatic properties of lung cancer cells, in addition to resistance to conventional chemo-radiotherapies and targeted therapies, and a poor prognosis in patients with lung cancer (10-12). In this regard, mounting evidence has demonstrated that the Wnt/ β -catenin signaling pathway is an important factor in cancer stem cell (CSC) fate determination. A previous study (13) revealed an association between CSCs and the resistance to chemotherapeutic and/or targeted therapeutic agents. Indeed, previous studies in cancer cell lines (14,15) have demonstrated that cancer cells with elevated expression of Wnt1 were resistant to therapy-induced apoptosis. In addition, platinum-based chemotherapy was reported to induce stem cell-like properties and therapeutic resistance via the β -catenin signaling pathway in NSCLC cells (16).

The sex-determining region Y box-containing (Sox) family of transcriptional factors have emerged as potent modulators in embryonic development, stem cell maintenance, tissue homeostasis and carcinogenesis in numerous processes. A previous study (17) demonstrated that members of Sox family were important in the development and maintenance of the lung, and in the tumorigenesis of lung cancer. The Sox genes share the non-canonical 79 amino acid DNA-binding, high mobility group (HMG) domain, termed the HMG box domain. To date, 20 different Sox genes have been identified in mammals (18). Among them Sox2 is the most extensively studied, due to its crucial roles in embryonic development, stem cell maintenance and involvement in carcinogenesis, including in lung cancers (19-21). Sox2 is intricately involved in numerous cancer-associated processes including cell proliferation, evading cellular apoptosis and metastasis, via interactions with other oncogenic/developmental pathways and processes, and its expression has been demonstrated to be heavily associated with patient survival rates and prognosis in clinical settings (21). In addition, increasing evidence has revealed that Sox2 is involved in chemoresistance to conventional lung cancer therapies (16,22-24).

A previous study (25) demonstrated that Sox2 gene amplification was associated with a favorable prognosis in several types of lung cancers, including NSCLC. Other studies (25,26) have demonstrated that Sox2 may also exert its roles in carcinogenesis via the Wnt/ β -catenin signaling pathway in breast, colorectal and prostate cancers and in osteosarcomas. In lung cancer, the functions of Sox2 in pathogenesis and chemoresistance have been involved in the mitogen-activated protein kinase kinase kinase 4/Survivin, epidermal growth factor receptor (EGFR), SRC proto-oncogene non-receptor tyrosine kinase/Akt serine/threonine kinase 1 and bone morphogenetic protein 4 signaling pathways (21). However, the role of Sox2 in the chemoresistance of lung cancer and Wnt/ β -catenin signaling activity in lung cancer has not yet been identified.

The present study therefore attempted to investigate the potential role of Sox2 in Wnt/ β -catenin signaling and the chemoresistance of NSCLC cells to cisplatin. The results

suggest that Sox2 may be involved in the chemoresistance of NSCLC to platinum-based doublet chemotherapy.

Materials and methods

Cell lines and reagents. A549 lung cancer cell line (cat. no. CCL-185) was purchased from American Type Culture Collection (Manassas, VA, USA). The cisplatin-resistant A549/DDP cell line was purchased from the Bank of Cancer Cell Lines of the Chinese Academy of Medical Science (Beijing, China) and its drug resistance phenotype was maintained in a medium containing 10 nM cisplatin (Cayman Chemical Company, Ann Arbor, MI, USA). The cells were cultured and maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air in Dulbecco's modified Eagle medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.).

Construction and transfection of plasmids. To generate a plasmid capable of overexpressing Sox2 in mammalian cells, human Sox2 cDNA (NM_003106) was cloned into the pcDNA3.1 backbone plasmid downstream of a cytomegalovirus (CMV) promoter (Invitrogen; Thermo Fisher Scientific, Inc.), which was referred to as pcDNA-Sox2. To construct a plasmid able to inhibit Sox2 expression, a small hairpin RNA (shRNA) construct was generated by annealing the sense oligonucleotide, 5'-CCGGCGCTCATGAAGAAGGATAAC TCGAGTTATCCTTCTTCATGAGCGTTTTTG-3', and the anti-sense oligonucleotide 5'-AATTCAAAAACGCTCATG AAGAAGGATAACTCGAGTTATCCTTCTTCATG ACG-3'. The resulting double stranded shRNA was cloned into a GV248 vector (Shanghai GenePharma Co., Ltd., Shanghai, China). The canonical Wnt reporter plasmid carrying a tandem of 7 T cell factor (TCF) binding sites upstream of a minimal c-fos promoter driving the firefly luciferase gene (BATflash) and its control plasmid (BOTflash, containing mutated TCF binding sites) were produced by EMD Millipore (Billerica, MA, USA). The transfection of control plasmid expressing Renilla luciferase (RL) from (Promega Corporation, Madison, WI, USA) was used for assessing the transfection efficiency. The plasmid DNA transfection was performed using Lipofectamine LTX reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Cells transfected with plasmid pcDNA3.1 served as the untreated control. To investigate the effect of Sox2 on the chemoresistance of lung cancer cells to cisplatin, the transfected A549 or A549/DDP cells were then exposed to culture medium containing cisplatin at a final concentration of 10 μ M for 24 h prior to being harvested for analysis. Control cells were untreated with cisplatin. The pcDNA3.1 plasmid (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was always included as a control.

3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cell proliferation was determined by using an MTT cell proliferation kit (Beijing Solarbio Science & Technology Co.,

Ltd., Beijing, China). A549 or A549/DDP cells were cultured in a 6-well plate and transfected with pcDNA3.1 control plasmid or plasmids expressing Sox2 or Sox2 shRNA (shSox2) for 12 h, then the cells were divided and seeded into a 96-well plate at a density of 2×10^4 per well and allowed to adhere overnight. The cells were then used for MTT assay at indicated time points following the manufacturer's protocol (Beijing Solarbio Science & Technology Co., Ltd.).

Dual-luciferase reporter assay. Wnt/ β -catenin signaling was assessed using a dual luciferase reporter assay, which was determined using a Dual-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA) on a 20/20n Luminometer (Turner Designs, Sunnyvale, CA, USA) according to the manufacturer's protocols. The A549 or A549/DDP cells were cultured in a 24-well plate and transfected with plasmid BATflash or BOTflash for 24 h. The activity of Wnt/ β -catenin was assessed by determining the relative activity of firefly luciferase. The transfection efficiency was assessed by luciferase activity of the co-transfected RL plasmid, pCMV-RL (Promega Corporation).

Cell scratch assay. The A549 or A549/DDP cells transfected with a plasmid expressing Sox2 or shSox2 were seeded at 80% confluence and exposed to cisplatin for 24 h (cells were cultured to confluence) in 6-well culture plates. The cells were then scratched with a 200 μ l pipette tip. The resultant unattached cells were removed by washing with pre-warmed PBS three times and the wounded monolayers were cultured for an additional 24 h prior to staining with 0.1% crystal violet solution. The closure of the wounded areas was observed under a light microscope at x40 magnification and images were captured. The distance of closure and unrecovered area were quantified with the NIH Image J image processing program version 1.46 (National Institutes of Health, Bethesda, MD, USA). The experiments were performed in triplicate. Each condition was tested in duplicate and each experiment was repeated at least three times.

Clonogenic assay. A clonogenic assay was used for assessing the degree of stemness of the A549 and A549/DDP cells. For clonogenicity, $1-2 \times 10^3$ of cells treated under the different conditions were seeded onto separated 35 mm dishes. Cells were continuously cultured for 10 days with a refreshment of an appropriate medium (e.g., containing 10 nM cisplatin for cisplatin-resistant cells or regular medium for cisplatin-sensitive cells) at intervals of 3 days. For colony counting, the medium was removed and the cells were rinsed with PBS prior to being fixed with 4% paraformaldehyde at room temperature for 5 min. Following the removal of the fixative, the cells were then stained with 0.5% crystal violet solution and incubated at room temperature for 30 min. The staining solution was carefully removed, and the cells were rinsed with H₂O to remove residual staining solution, prior to air-drying the sample at room temperature for up to a day. The number of colonies were counted and calculated under a light microscope. Each condition was tested in duplicate and each experiment was repeated three times.

Transwell assay. In order to assess the effect of Sox2 on cisplatin-resistant lung cancer cells, the invasive capacity of

A549 or A549/DDP cells transfected with the plasmid either expressing Sox2 or shSox2 was ascertained by a Transwell assay using Transwell migration chambers (BD Biosciences, Franklin Lakes, NJ, USA). The 8- μ m filters were coated with 100 μ l Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), which was diluted to 1:2 of concentration using serum-free 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), and incubated at 37°C in a 5% CO₂ atmosphere for 30 min for gelling. A total of 10^4 cells resuspended in 100 μ l DMEM basal medium were seeded in the upper chamber, and 700 μ l of DMEM medium supplemented with 10% FBS was added in the lower chamber. The culture was then incubated at 37°C in a 5% CO₂ atmosphere for 12 h. The medium was then removed and the cells were washed twice with cool PBS. Cells were then fixed with 4% paraformaldehyde for 20 min, prior to being stained with 1% crystal violet for 20 min. The crystal violet was removed from the top of the membrane with a pipette tip or cotton tipped applicator, then the rinsed the Transwell membrane with distilled water to remove the excess crystal violet and allowed to dry for a day. The number of cells in 10 different fields of view was counted under a Upright light microscope (Leica DM4B, equipped with DFC450 camera, Leica, Shanghai, China) to obtain an average sum of cells that had migrated from the top of membrane toward the basolateral side of the membrane. The percentage of invasive cells was calculated as (the average sum of cells attached on basolateral membrane/the average sum of cells (attached on the top membrane + attached on basolateral membrane) x100%.

Apoptosis assay. Cell apoptosis was assessed by Annexin V analysis using flow cytometry. For flow cytometry, cells were detached and labeled using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (NeoBioscience Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol. Apoptotic and necrotic cells were quantified using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) and the CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). For each sample, $\geq 10,000$ cells were analyzed. Cells negative for Annexin V and PI were considered to be viable, and cells stained with Annexin V but not PI were considered to be apoptotic.

Immunoblotting analysis. Whole cell lysates were prepared in a lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), and cell nuclear proteins were extracted with the NucBuster Protein Extraction kit following the manufacturer's protocol (Novagen; EMD Millipore, Billerica, MA, USA). Whole cell extract or nuclear extract of cells (40 μ g) were resolved by a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by being transferred to a PVDF membrane (EMD Millipore). The membranes were blocked with blocking buffer (5% fat-free milk in PBS-0.1% Tween-20) at room temperature for 1 h prior to being probed with the primary antibody at 4°C overnight, followed by being incubated with the appropriate horseradish peroxidase-labeled secondary antibody (Donkey anti-mouse immunoglobulin G, cat. no. 109415; donkey anti-rabbit immunoglobulin G, cat. no. 108894; or donkey anti-goat immunoglobulin G, cat. no. 109291). All secondary antibodies were applied by 1:2,000 dilution in blocking

buffer. Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 2 h. The blots were developed using the enhanced chemiluminescence (ECL) reagent (Advansta, Menlo Park, CA, USA) after they were incubated with the appropriate peroxidase labeled secondary antibodies. Antibodies against β -actin (cat. no. sc-8432; 1:1,000 dilution), lymphoid enhancer-binding factor-1 (LEF-1; cat. no. sc-8592; 1:1,000 dilution) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); the antibody to phosphorylated (phos)-catenin (cat. no. ab75777; 1:1,000 dilution) was purchased from Abcam (Cambridge, MA, USA); antibodies against phos-glycogen synthase kinase (GSK)3 β (cat. no. 05-413; 1:1,000 dilution), acetyl-Histone H3 (cat. no. 06-942; 1:1,000 dilution) and active β -catenin (cat. no. 05-665; 1:10,000 dilution) were purchased from EMD Millipore; the antibody to GSK3 β (cat. no. 610202; 1:1,000 dilution) was purchased from BD Biosciences (San Jose, CA, USA); antibodies against Sox2 (cat. no. 66411; 1:500 dilution), B-cell lymphoma 2 (Bcl-2) (cat. no. 12789; 1:500 dilution), caspase-3 (cat. no. 10380; 1:1,000 dilution), Bcl-2-like protein 4 (Bax; cat. no. 15422; 1:1,000 dilution), myeloid cell leukemia sequence 1 protein (Mcl-1; cat. no. 66026; 1:1,000 dilution) and apoptosis inducing factor (AIF; cat. no. 17984; 1:1,000 dilution) were purchased from Proteintech (Wuhan Sanying Biotechnology, Wuhan, China). The expressions of proteins of interest were semi-quantified by optical densitometry using Image J software version 1.46 (National Institutes of Health, Bethesda, MD, USA). The ratio between the net intensity of each sample divided by the respective internal controls (β -actin or Histone H3) was calculated as densitometric arbitrary units, which served as an index of the relative expression of a protein of interest, the fold of induction for a specific protein under an indicated condition was calculated by comparing its relative expression over the control (27).

Statistical analysis. All data collected in this study were obtained from at least three independent experiments for each set of circumstances. SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and PRISM 5 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. Statistical evaluation of the data was performed by one-way analysis of variance when more than two groups were compared with a single control, and by t-test for comparison of differences between the two groups. $P < 0.05$ was considered to indicate a statistically significant difference. Data was presented as the mean \pm standard deviation.

Results

Sox2 suppresses the Wnt/ β -catenin signaling activity in lung cancer cells. In order to examine the potential role of Sox2 in canonical Wnt signaling, lung cancer A549 and cisplatin-resistant A549/DDP cells, enforced expression of Sox2 or shSox2, and the Wnt/ β -catenin signaling activity was ascertained in terms of a dual luciferase Wnt reporter assay, in addition to the expression of key components in the Wnt/ β -catenin signaling cascade. The results demonstrated decreased Wnt activity in cells transfected with Sox2 compared with control cells ($P < 0.01$), and marginally increased activity of Wnt signaling in A549 and A549/DDP cells which overexpressed

shSox2 in comparison with cells transfected with TOPflash and pcDNA3.1 plasmids (Fig. 1A). However, no change of luciferase activity was detected between BOTflash-transfected cells co-transfected with pcDNA3.1, Sox2 or shSox2 plasmids (data not shown). Molecular analysis by immunoblotting assay furthermore revealed that the quantities of active β -catenin, transcription factor LEF-1 and the Wnt signaling target gene, cyclin D1, were decreased, whereas the quantities of GSK3 β and phos- β -catenin protein were increased in cells transfected with Sox2 (Fig. 1B), suggesting that Sox2 may inhibit Wnt/ β -catenin signaling activity in lung cancer cells.

Sox2 reduces the cisplatin-induced apoptosis of lung cancer cells. The present study also investigated the effect of Sox2 on cisplatin-mediated cell apoptosis in lung cancer cells. The results from the MTT assay revealed that a transient expression of Sox2 or shSox2 had no effect on cell proliferation, but overexpression of Sox2 may increase the survival rate of A549 cells in the presence of cisplatin, although it had no effect on cisplatin-resistant A59/DDP cells. Notably, a suppression of Sox2 expression by transfection of shRNA led to an increase in the cisplatin-induced cell death in A549/DDP cells ($P < 0.05$; Fig. 2A). The cytometric analysis also demonstrated that an inhibition of Sox2 by shSox2 significantly enhanced cisplatin-induced apoptosis in A549 and A549/DDP cells ($P < 0.05$; Fig. 2B), despite the evidence that the overall fraction of apoptotic cells was small. In the absence of cisplatin, molecular analysis further indicated that an overexpression of Sox2 tended to increase the expression of pro-apoptotic proteins (caspase-3 and Bax), but decreased expression of AIF and anti-apoptotic protein Bcl-2 and Mcl-1 in A549 lung cancer cells; however, decreased caspase-3, AIF, Mcl-1 protein expression and increased BAX and Bcl-2 protein expression were observed in A549/DDP cells overexpressing Sox2. Similarly in the absence of cisplatin, an introduction of shSox2 led a reduced abundance of caspase-3 and Mcl-1 proteins in both A549 and A549/DDP cells. Decreased AIF and Bcl-1 protein expression was also detected in A549 cells, but expression levels increased in cisplatin-resistant A459/DDP cells. In contrast, in the presence of cisplatin, a reduced expression of caspase-3, Bcl-2 and Mcl-1 was observed, but increased BAX and AIF expression was observed in A549 cells transfected with either Sox2 expressed plasmid or shSox2. Caspase-3 expression was increased in A549 cells ectopically expressing shSox2; in A549/DDP cells, however, an overexpression of Sox2 induced increased caspase-3 protein expression but decreased expressions of BAX, AIF, Bcl-1 and Mcl-1, and an introduction of shSox2 resulted in upregulated expression of caspase-3, AIF and Bcl-2 but downregulated BAX and Mcl-1 protein expression (Fig. 3). Of note, certain controversial results were also observed between the A549 cells and cisplatin-resistant A549/DDP cells, as well as between cells with a gain and loss of Sox2 function in the present study, which requires further elucidation. Nonetheless, the data from the functional studies implied that Sox2 may be a target for sensitizing cisplatin-resistant lung cancer cells to chemopreventive agents, including cisplatin.

Effect of Sox2 on the migration and invasion of lung cancer cells *in vitro*. In order to investigate whether Sox2 has an effect on the metastatic properties of lung cancer cells, the capability

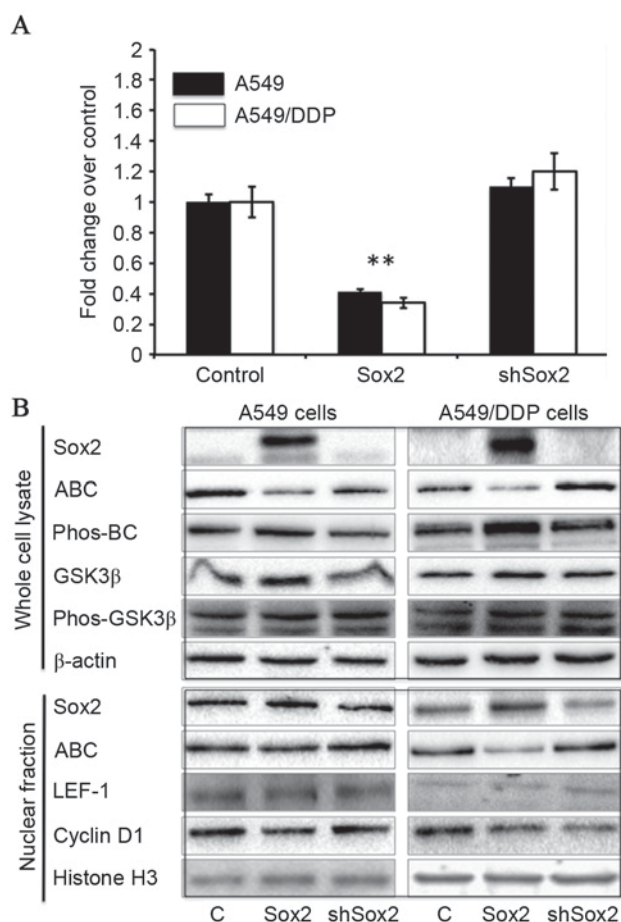


Figure 1. Sox2 suppresses the Wnt/ β -catenin signaling activity in A549 and A549/DDP cells. A549 and A549/DDP were transfected with canonical Wnt signaling reporter BATflash and a plasmid expressing Renilla luciferase, along with a plasmid expressing Sox2 or shSox2, or a pcDNA3.1 plasmid for 24 h. The cells were then harvested for analysis of luciferase activity and the expression of key components of Wnt/ β -catenin signaling cascade. (A) Wnt/ β -catenin signaling luciferase reporter demonstrates that Sox2 may inhibit Wnt signaling activity in A549 and A549/DDP cells ($P < 0.01$), whereas the cells transfected with shSox2 exhibited a moderately enhanced luciferase activity, as compared with cells transfected with BATflash and pcDNA3.1 plasmids ($n = 9$). (B) Molecular analysis by immunoblotting demonstrated a decreased expression of indicated Wnt signaling activators including ABC, LEF-1 and cyclin D1, and an increased expression of Wnt signaling inhibitor, GSK3 β , and phos-BC in Sox2-transfected cells. All data are presented as the mean \pm standard deviation of at least three independent triplicated experiments. ** $P < 0.01$ vs. control. Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA; ABC, active β -catenin; phos-, phosphorylated; BC, β -catenin; GSK3 β , glycogen synthase kinase 3 β ; LEF-1, lymphoid enhancer-binding factor-1.

of migration and invasion in A549 and A549/DDP cells introduced with a plasmid expressing Sox2 or shSox2 was evaluated by scratch assay (Fig. 4) and Transwell analysis (Fig. 5), respectively. The results demonstrated that A549 cells overexpressing Sox2 exhibited enhanced capacities of migration (Fig. 4A) and invasion compared with the control cells (Fig. 5A; $P < 0.05$), although a similar effect was not observed in A549/DDP cells (Figs. 4B and 5B). Notably, a reduced expression of Sox2 by shSox2 demonstrated the potential to promote cell migration (Fig. 4B) and invasion (Fig. 5B) in A549/DDP cells ($P < 0.05$), but not in A549 cells (Figs. 4A and 5A). These results suggest that Sox2 may play a regulatory role in the migration and invasion of lung cancer cells in a cell-context-dependent manner.

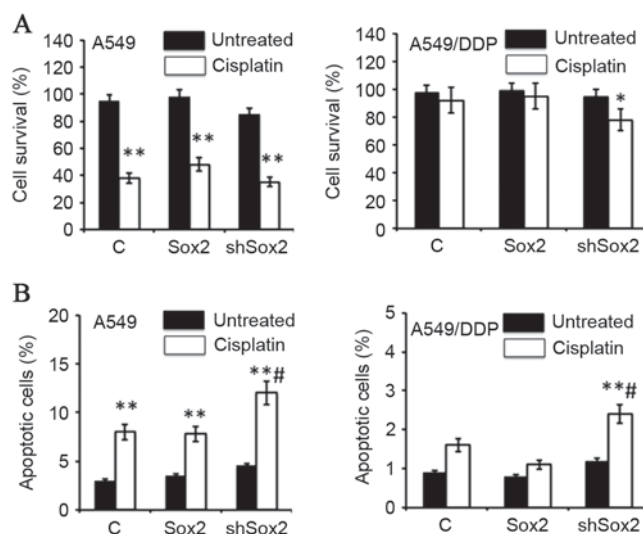


Figure 2. Sox2 inhibits cisplatin-induced apoptosis in lung cancer cells. A549 and A549/DDP cells were transfected with a plasmid expressing Sox2 or shSox2, or a pcDNA3.1 plasmid for 12 h, and then cultured in medium containing 10 μ M cisplatin for an additional 24 h prior to being harvested for analysis. (A) MTT assay determined the proliferation of cells in the presence of cisplatin. The transient transduction of Sox2 or shSox2 had no effect on cell proliferation. Overexpression of Sox2 increased the survival rate of A549 cells in the presence of cisplatin, but had no effect on cisplatin-resistant A549/DDP cells. Notably, inhibition of Sox2 expression by short hairpin RNA increased the cisplatin-induced cell death in A549/DDP cells. (B) Cell apoptosis analyzed by a cytometric assay. An inhibition of Sox2 by shSox2 significantly enhanced cisplatin-induced apoptosis in A549 and A549/DDP cells ($P < 0.05$). All data are presented as the mean \pm standard deviation of three independent triplicated experiments ($n = 9$). * $P < 0.05$, ** $P < 0.01$ vs. the corresponding non-cisplatin-treated group, # $P < 0.05$ vs. the cisplatin-treated pcDNA3.1-transfected cells. Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

Sox2 enhances the stemness of lung cancer cells. Since Sox2 is a well-characterized marker of pluripotency of stem cells and CSCs (24,28,29), the capacity for clone formation of lung cancer cells altered by an overexpression of Sox2 was using a clonogenic assay (Fig. 6). Notably, cells overexpressing Sox2 demonstrated an enhanced clonogenicity in A549 and A549/DDP cells compared with control cells ($P < 0.05$), although a decreased expression of Sox2 by shSox2 only marginally reduced the clonogenicity in A549 and A549/DDP cells, regardless of their resistance to cisplatin (Fig. 6). Notably, the clonogenicity of A549/DDP cells was markedly increased compared with A549 cells (Fig. 6A and B), indicating that a more abundant lung CSC population may exist in the cisplatin-resistant A549/DDP cells compared with their parent cells.

Discussion

Chemotherapy is a common treatment for lung cancer and regimens containing cisplatin remain the main treatment in clinical settings (30). However, the development of resistance to chemotherapeutic agents eventually leads the failure of lung cancer chemotherapy (5,31). Therefore, an improved understanding of mechanisms underpinning the chemoresistance of lung cancer may aid the identification of novel targets for reversing drug-resistance in cancer therapy. The present study investigated the potential roles of the Sox2 gene in

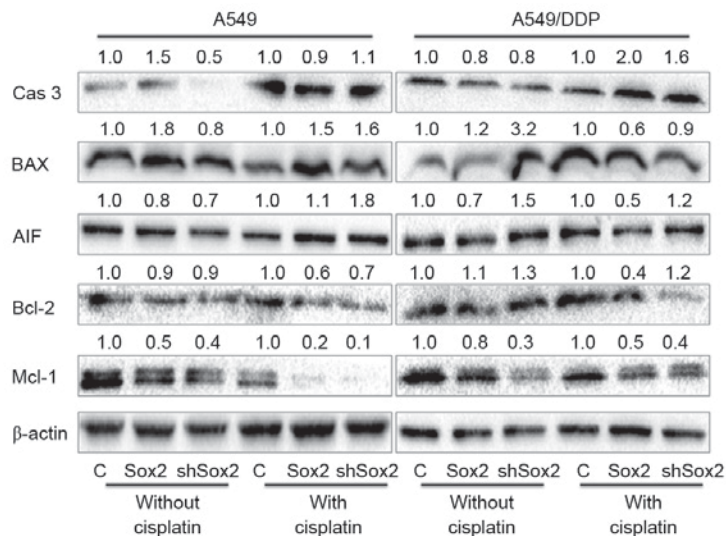


Figure 3. Apoptosis associated proteins determined by an immunoblotting analysis. A549 and A549/DDP cells were transfected with plasmid expressing Sox2 or shSox2, or control pcDNA3.1 plasmid for 12 h, and then cultured in medium containing 10 μ M cisplatin for additional 24 h prior to being harvested for immunoblotting analysis for indicated proteins. The values labeled on the top of each bands represented the relative expression levels of proteins over their respective pcDNA3.1 control as determined by a densitometric assay. Overexpression of Sox2 demonstrated a trend to reduce the expression of pro-apoptotic proteins (caspase-3, Bax), but increased the expression of anti-apoptotic proteins Bcl-2 in lung cancer cells. Cas 3: caspase-3; Bax, Bcl-2-like protein 4; AIF, apoptosis inducing factor; Bcl-2, B-cell lymphoma 2; Mcl-1, myeloid cell leukemia sequence 1 protein; C, control; Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA.

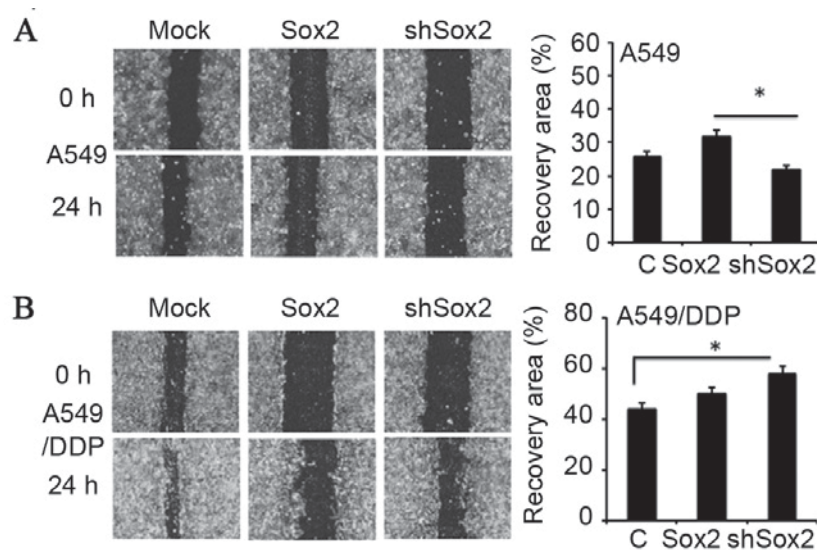


Figure 4. Effect of Sox2 on the migration of lung cancer cells *in vitro*. A549 and A549/DDP cells were transfected with a plasmid expressing Sox2 or shSox2, or a pcDNA3.1 plasmid for 12 h and the capability of cell migration was assessed using a scratch assay. (A) Representative images of scratch assays for A549 cells (left panel) and its relevant quantification of the results of cell migration index (right panel). (B) Representative images of scratch assays for A549/DDP cells (left panel) and its relevant quantification of the results of cell migration index (right panel). Overexpression of Sox2 enhanced cell migration in A549 cells, but had no effect on A549/DDP cells. By contrast, inhibition of Sox2 by shSox2 promoted cell migration in A549/DDP cells. * $P < 0.05$ vs. pcDNA3.1 group. Data are presented as the mean \pm standard deviation from three independent triplicated experiments ($n=9$). Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA; C, control.

cisplatin-resistant lung cancer cells. The results demonstrated that Sox2 may inhibit Wnt/ β -catenin signaling activity and effect the proliferation, metastasis and chemoresistance of lung cancer cells. In this context, Sox2 repressed Wnt/ β -catenin signaling, promoted cell proliferation and clonogenicity, and inhibited cisplatin-induced cell apoptosis in lung cancer cells. Notably, cell context-dependent Sox2-promoted cell migration and invasion were also observed, that is, Sox2 may enhance the migratory and invasive capacity of A549 cells; by

contrast, a reduced expression of Sox2 by shRNA increased the migration and invasion of A549/DDP cells. More importantly, targeting Sox2 using shRNA demonstrated a potential to sensitize A549/DDP cells to cisplatin, suggesting that Sox2 may be a novel target for chemotherapy in lung cancer.

Wnt signaling has been recognized as serving multiple functions in cell proliferation and migration, organogenesis and tissue homeostasis (32). Increasing evidence suggests an interaction between the Sox2 and Wnt signaling

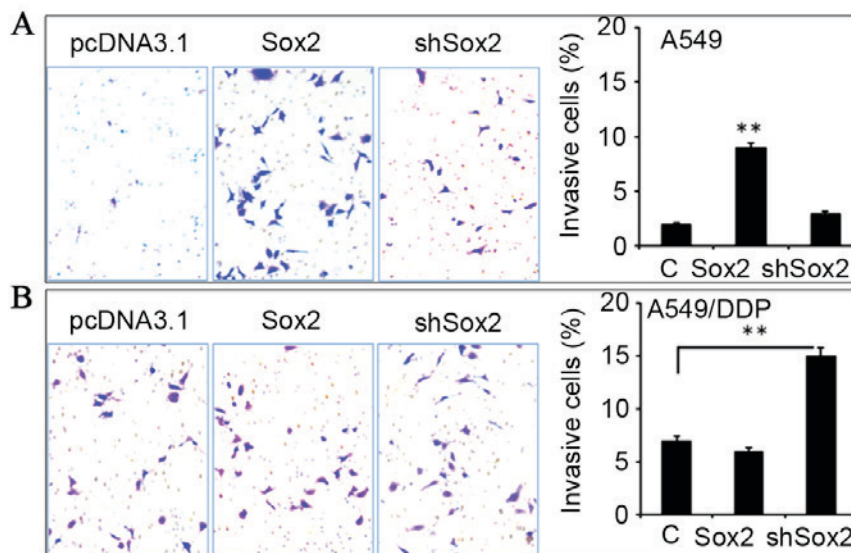


Figure 5. Effect of Sox2 on the invasion of lung cancer cells *in vitro*. A549 and A549/DDP cells were transfected with a plasmid expressing Sox2 or shSox2, or a pcDNA3.1 plasmid for 12 h, the capability of cell invasion was accessed by a Transwell analysis, and cell proliferative ability was ascertained by an MTT assay. (A) Representative images of Transwell assay for A549 cells (left) and its relevant quantification of the numbers of invasive cells (right). (B) Representative images of Transwell assay for A549/DDP cells (left) and its relevant quantification of the numbers of invasive cells (right). Overexpression of Sox2 enhanced cell invasion in A549 cells, but had no effect on A549/DDP cells. By contrast, an inhibition of Sox2 by shSox2 promoted cell invasion in A549/DDP cells. Data are presented as the mean \pm standard deviation from three independent triplicated experiments (n=9). **P<0.01 vs. pcDNA3.1 group. Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA; C, control.

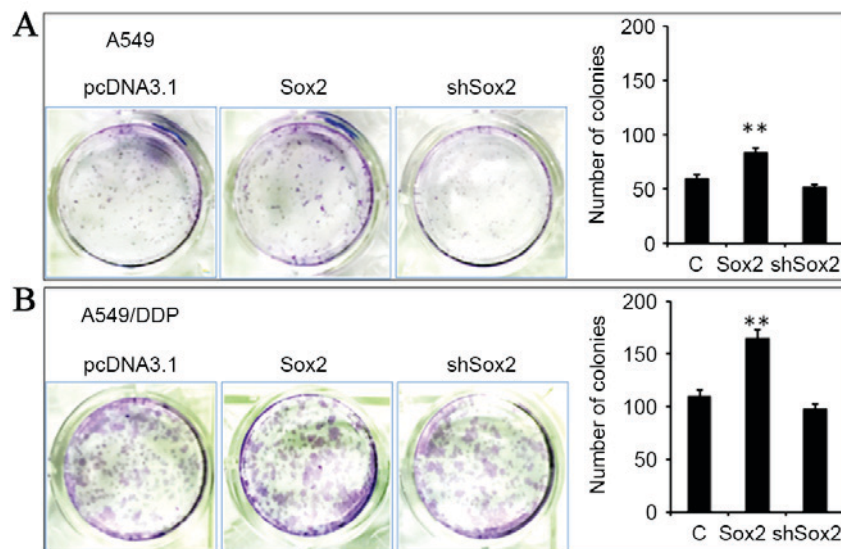


Figure 6. Sox2 enhances the stemness of lung cancer cells determined by a clonogenic assay. A549 and A549/DDP cells were transfected with a plasmid expressing Sox2 or shSox2, or a pcDNA3.1 plasmid and their capacity for clone formation was analyzed using a clonogenic assay in 35 mm dishes. (A) Representative images of clonogenic assay for A549 cells (left) and its relevant quantification of the number of colonies (right). (B) Representative images of clonogenic assay for A549/DDP cells (left) and its relevant quantification of the number of colonies (right). An overexpression of Sox2 demonstrated an ability to enhance the clone formation in A549 and A549/DDP cells, and a shRNA-mediated knockdown of Sox2 marginally reduced the clone formation. Data represented the mean \pm standard deviation from three independent triplicated experiments (n=9). **P<0.01 vs. pcDNA3.1 group. Sox2, sex-determining region Y box 2; shSox2, Sox2 short hairpin RNA; C, control.

pathways (33-35). For example, Sox2 has been identified as being able to bind to β -catenin and inhibit the differentiation of stem cells into osteoblast lineage by attenuating Wnt signaling through post-transcriptional and transcriptional mechanisms. Sox2 regulates the expression of Wnt signaling inhibitors dickkopf-1, adenomatous polyposis coli and GSK3 β , enhances the stemness of cancer stem cells and increases the tumorigenic capacity of osteosarcoma (26,36).

In agreement with this finding, an inhibitory role of Sox2 in Wnt/ β -catenin signaling was also identified in A549 cells and A549/DDP cells, partially by regulating the expression of GSK3 β . Conversely, Sox2 may synergistically act with β -catenin to transcriptionally regulate cyclin D1 gene expression and promote cell proliferation and tumorigenesis by facilitating the G1/S transition in breast cancer cells (37). Results from these studies and the present study may imply a

cell context-dependent bifunctional role of Sox2 in regulation of Wnt/ β -catenin signaling activity.

As an important pluripotent marker of stem cells, Sox2 has been recognized as serving a crucial role in maintaining the properties of cancer stem cells that contribute to resistance to therapeutic agents (20,24,28,38,39). Therefore, inhibition of Sox2 may result in decreased metastatic characteristics of cancer cells and an increased sensitivity of these cells to chemotherapeutic and/or targeted therapeutic agents (21). Recently, the role of Sox2 and its mechanisms in CSC maintenance and regulation have prompted an increased interest (13,15,34,40,41). In this way, the regulatory role of Sox2 in CSC self-renewal and maintenance has been investigated in numerous types of cancer, including breast, prostate, gastric, ovarian, pancreatic and lung cancers (24,42-44). Notably, a knockdown of Sox2 gene expression by small interfering RNA (siRNA) or shRNA demonstrated abilities to reduce CSC properties in several types of cancer. For example, siRNA-mediated Sox2 knockdown in gastric cancer cells led to a reduced spheroid colony formation and increased apoptosis within sphere cells (24). In human glioma cells, siRNA of Sox2 demonstrated an ability to attenuate S-phase entry and induce a RhoA-dependent switch to protease-independent amoeboid migration (45). Another example is the enhanced self-renewal capacity of prostate CSCs induced by EGFR-mediated Sox2 expression (46). With respect to lung cancer, a siRNA-mediated Sox2 knockdown in NSCLC cells also exhibited a significant reduction of sphere formation (47). In the current study, an increased expression of Sox2 demonstrated the potential to enhance clonogenicity in A549 lung cancer cells, although the shRNA-mediated Sox2 knockdown only moderately reduced the clone formation. It was hypothesized that the inefficient inhibition of shSox2 in A549 clone formation may be due to the expression of shRNA being transiently introduced rather than persistently expressed, in addition to the effect of transfection efficacy. More pronounced clonogenic capacity was observed in A549/DDP cells compared with the parent A549 cells, partially due to that cisplatin-resistant A549 cell populations may be selected fractions of cells with CSC potentials. These studies thus emphasize the importance of the Sox2 gene in the maintenance of the stemness of CSCs.

Increasing evidence has suggested that Sox2 expression is associated with the cancer hallmarks of sustained proliferative signaling, activation of invasion and metastasis, and evasion of cell death (48). In this respect, Sox2 has been reported to promote cellular proliferation in breast, prostate, pancreatic and cervical cancers (39), evade apoptosis in prostate and gastric cancer, and NSCLC (39,42), and promote invasion, migration and metastasis in melanoma, colorectal, glioma, gastric and ovarian cancers, and in hepatocellular carcinoma (49). Notably, the involvement of Sox2 in cancer cell physiology was demonstrated to vary between different types of cancer cell (21). In the present study, we also identified that Sox2 may promote migration and invasion in A549 cells but not in A549/DDP cells. By contrast, a knockdown of Sox2 increased the migration and invasion in A549/DDP cells but not in A549 cells, although the underlying mechanism remains to be elucidated. Together with the present study and others, these data suggest that the Sox2 gene serves a cell context-dependent role in maintaining the physiological phenotype of cancer cells.

Aside from its role in cancer cell migration and invasion, Sox2 also serves an important role in evading apoptotic signals. In this context, an overexpression of Sox2 may induce the increased apoptotic resistance in prostate cancer cells and xenograft models (39). Equally noteworthy, a knockdown of Sox2 may induce apoptosis in NSCLC cell lines (42). For example, the shRNA-mediated knockdown of Sox2 in EGFR mutated lung cancer HCC827 cells exhibited a decreased proliferation and an increased sensitivity of cells to erlotinib (50). In agreement with these findings, the present study also identified that a knockdown of Sox2 expression demonstrated the potential to enhance the sensitivity of A549/DDP cells to cisplatin. Therefore, targeting Sox2 gene in lung cancer may be therapeutically beneficial.

In summary, the present study demonstrated that an overexpression of the Sox2 gene led to the decreased activity of Wnt/ β -catenin signaling in lung adenocarcinoma A549 cells and the cisplatin-resistant A549/DDP cells through an upregulation of the Wnt/ β -catenin signaling negative regulator GSK3 β . Notably, the increased expression of the Sox2 gene was able to promote cell migration and invasion, in addition to enhancing clonogenic capacity in A549 cells. Conversely, a knockdown of Sox2 expression by shRNA led to an enhanced susceptibility of A549 and A549/DDP cells to cisplatin, along with an increased cisplatin-induced apoptosis of cancer cells. The present study therefore suggests that the Sox2 gene may be a novel target for the treatment of chemoresistant lung cancers.

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