

Original Article

SOX2 transactivates NRF2 to promote carboplatin resistance in lung squamous cell carcinoma

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Abstract

Lung squamous cell carcinoma (LUSC) remains a major therapeutic challenge because of its pronounced resistance to chemotherapy, particularly carboplatin. In this study, we investigate the role of SOX2, a lineage-survival oncogene, in mediating carboplatin resistance in LUSC. We demonstrate that SOX2 is highly expressed in LUSC and is significantly associated with poor prognosis. Our results show that SOX2 directly transactivates the expression of NRF2, a master regulator of cellular redox homeostasis, thereby increasing glutathione (GSH) synthesis and protecting cells from carboplatin-induced oxidative stress. Pharmacological or genetic inhibition of NRF2 effectively abrogates SOX2-mediated carboplatin resistance both *in vitro* and *in vivo*, resensitizing LUSC cells to chemotherapy. These findings highlight SOX2 as a critical redox regulator that modulates NRF2 signaling to promote carboplatin resistance in LUSC. The identification of the SOX2-NRF2 axis as a potential therapeutic target suggests that NRF2 inhibition may represent a promising strategy to overcome chemoresistance in LUSC.

Key words LUSC, SOX2, carboplatin resistance, NRF2, ROS

Introduction

Lung squamous cell carcinoma (LUSC) accounts for approximately 20%–30% of non-small cell lung cancer (NSCLC) cases and collectively comprises approximately 80% of all lung cancers [1]. Since the 1990s, the incidence of LUSC has declined, largely due to the success of smoking cessation programs [3], in contrast to lung adenocarcinoma (LUAD), another predominant NSCLC subtype that has benefited from targeted therapies against specific genetic alterations [4]. Despite significant advances in cancer therapeutics, effective therapies specifically tailored to LUSC are lacking [5]. Most LUSC patients are diagnosed at an advanced stage, characterized by limited treatment options and high disease-related mortality [6]. Treatment typically involves platinum-based chemotherapy regimens, such as carboplatin-containing combinations. Recent therapeutic approaches include the use of EGFR monoclonal antibodies (e.g., necitumumab) [7] and immune checkpoint inhibitors (e.g., pembrolizumab, pislelizumab, sintilimab) [8–10]. Platinum-immunotherapy combinations have now become the standard first-line

treatment for metastatic LUSC [6,11]. However, despite these advancements, durable responses and long-term survival benefits remain rare [12]. Therefore, understanding the mechanisms underlying chemoresistance in LUSC is critical for developing novel therapeutic strategies to overcome resistance and improve patient outcomes.

Lineage survival oncogenes are activated by somatic DNA alterations in cancers arising from the cell lineages where these genes contribute to normal development [13]. The transcription factor SOX2 is frequently subjected to genomic amplification on chromosome 3q26.33 in squamous cell carcinomas (SCCs) and is now recognized as a key driver oncogene that promotes tumorigenesis, proliferation, and invasion/metastasis [14,15]. Notably, SOX2 plays a crucial role in determining squamous cell fate across various cellular origins [16]. Additionally, SOX2, in cooperation with PKC δ or Δ Np63 α , drives tumorigenesis in LUSC [17,18]. Cancer stem cells (CSCs), a subset of tumor cells with self-renewal and differentiation capabilities, are widely acknowledged for their critical role in

therapeutic resistance [19]. SOX2 is a key transcription factor that not only maintains stem cell properties but also confers drug resistance [19]. Elevated SOX2 expression has been shown to be correlated with both drug resistance and poor patient survival [20]. Consequently, targeting SOX2 represents an attractive therapeutic strategy for cancer treatment [21]. However, progress in the development of SOX2 inhibitors has been limited, largely because of the “undruggable” nature of SOX2 as a transcription factor. Despite its well-established role in cancer therapy resistance, the contribution of SOX2 to carboplatin resistance in lung squamous cell carcinoma (LUSC) remains poorly understood.

In this study, we demonstrated that SOX2 is highly expressed in LUSC and associated with poor prognosis. Furthermore, SOX2 directly transactivates NRF2, a master regulator of redox homeostasis, to scavenge carboplatin-induced reactive oxygen species (ROS) and consequently promote carboplatin resistance. Pharmacological or genetic inhibition of NRF2 effectively reverses SOX2-mediated carboplatin resistance both in vitro and in vivo. Taken together, our findings identify the SOX2-NRF2 axis as a potential therapeutic target and highlight NRF2 inhibition as a promising strategy to overcome SOX2-mediated carboplatin resistance in LUSC.

Materials and Methods

Cell culture and drug treatment

Human lung cancer cell lines (NCI-H292, NCI-H520, and HCC95) and Human embryonic kidney cells (HEK-293T) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). All the cell lines were authenticated via short tandem repeat (STR) profiling, and mycoplasma contamination was routinely tested and confirmed to be negative. HEK-293T cells were cultured in DMEM (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; 16000-044; Gibco). NCI-H292, NCI-H520, and HCC95 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS. All the cells were maintained at 37°C in a humidified incubator with 5% CO₂. Carboplatin (S1215), ML385 (S8790), and N-acetylcysteine (NAC; S1623) were purchased from Selleck Chemicals (Shanghai, China).

Plasmid construction

Short hairpin RNAs (shRNAs) targeting human SOX2 were generated by inserting specific oligonucleotides into the pLKO.1-puromycin lentiviral vector (10878; Addgene, Cambridge, USA). The pLKO.1-scramble vector (1864; Addgene) was used as a negative control and contained a scrambled shRNA (shC). The oligonucleotide sequences used in this study are: shSOX2-#1, 5'-CAGCTCGCAGACCTACATGAA-3'; shSOX2-#2, 5'-TGGACAGTTACGCGCACATGA-3'; and shC, 5'-CCTAAGGTAAAGTCGCCCTCG-3'. The construct encoding human SOX2 was generated via PCR amplification with specific primers and subsequently cloned and inserted into the pLVX-puro vector (632164; Clontech, Mountain View, USA). The construct encoding human NRF2 was described in a previous study [22]. For the promoter assay, a fragment (−3000 bp to +1000 bp) of the human *NFE2L2* gene containing putative SOX2 binding sites was inserted into the Gluc-On promoter reporter vector (pEZX-PG04; GeneCopoeia, Guangzhou, China) and designated NFE2L2-Gluc. The mutants of SOX2^{R74P}, NRF2^{ΔNeh1}, and NFE2L2^{ΔP3}-Gluc were generated via the KOD-Plus-Mutagenesis kit (SMK-101; Toyobo, Shanghai, China). All plasmids were confirmed by DNA sequencing.

Lentiviral infection

Lentivirus was generated as described previously [23]. Briefly, HEK-293T cells were transfected with the pMD2.G and psPAX2 packaging plasmids, along with the appropriate backbone plasmid, using Lipofectamine 3000 (L3000008; Invitrogen, Shanghai, China) when the cells reached 80%–90% confluence. Viral supernatants were collected 72 h post-transfection, filtered, and used to infect cells at 50% confluence in the presence of 10 µg/mL polybrene. To establish stable cell lines, the cells were treated with 2 µg/mL puromycin (A1113803; Invitrogen) 48 h post-infection.

Cell viability analysis

Cell viability was assessed via CCK8 assay. The cells were seeded in 96-well plates at a density of 2×10^4 cells per well and treated with carboplatin. After 2 h of incubation with CCK8 solution (C0039; Beyotime Biotechnology, Shanghai, China), the absorbance was measured at 450 nm to assess cell viability via a microplate reader (Synergy H1; BioTek, Winooski, USA).

Clonogenic survival assay

Chemosensitivity was evaluated via clonogenic survival assay. The cells were seeded in triplicate into 6-well plates at a density of 1×10^3 cells per well and treated with carboplatin. After 8–10 days, colonies containing more than 50 cells were stained with 0.5% crystal violet. The number of colonies in the entire well was counted for every replicate. The survival fraction was calculated via GraphPad Prism 8.

ROS detection

Reactive oxygen species (ROS) levels were measured as previously described [22]. Briefly, after treatment, the cells were incubated with the redox-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA; S0033S-1; Beyotime) at 37°C. The fluorescence intensity of oxidized DCF was measured at an excitation wavelength of 488 nm and an emission wavelength of 535 nm via a FACS Scan flow cytometer (FACS Calibur; BD Biosciences, Franklin Lakes, USA). The geometric mean fluorescence intensity (MFI) of the DCF channel for the entire cell population was analyzed via FlowJo software and used for comparison.

Apoptosis assay

Apoptosis was assessed via an Annexin V-FITC/PI Apoptosis Detection Kit (C1383M; Beyotime) according to the manufacturer's protocol. The cells were stained with Annexin V-FITC and propidium iodide (PI) and then analyzed via the FACS Scan flow cytometer. Data were displayed as Annexin V/PI dot plots and were analyzed via FlowJo software for comparison.

Measurement of GSH and GSSG

The cells were seeded in 6-well plates at a density of 1×10^6 cells/well. After treatment, the cells were collected and lysed in cell lysis buffer. Following three freeze-thaw cycles, the supernatants were collected via centrifugation at 12,000 g for 10 min at 4°C. The concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured via an assay kit (S0053; Beyotime) according to the manufacturer's instructions.

Quantitative PCR analysis

Quantitative RT-PCR (qPCR) was used to assess mRNA levels as described previously [24]. Total RNA was extracted via the

NucleoSpin® RNA Plus Kit (740984; MACHEREY-NAGE, Dueren, Germany), followed by reverse transcription via ReverTra Ace qPCR RT Master Mix (FSQ-201; Toyobo). The sequences of primers used for qPCR are listed in Table 1.

Immunoblot analysis

Cells were lysed in EBC250 lysis buffer (250 mM NaCl, 25 mM Tris, 50 mM NaF, 0.5% Nonidet P-40) supplemented with 0.5 mM Na₃VO₄, 0.2 mM PMSF, 20 µg/mL aprotinin, and 10 µg/mL leupeptin, pH 8.0. Equal amounts of total protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (FPF28; Beyotime). Membranes were blocked in 5% nonfat milk in TBST for 1 h and then incubated with primary antibodies overnight at 4°C. After washing, HRP-conjugated secondary antibodies (A0181 and A0208; Beyotime) were applied, followed by detection via enhanced chemiluminescence reagent (P0018S; Beyotime). Antibody against SOX2 (AF2018; 1:500) was purchased from R&D Systems (Shanghai, China). Antibody against actin (AB0035; 1:5000) was purchased from Abways Technology (Shanghai, China). Antibody against cleaved caspase 3 (9664; 1:1000) was purchased from Cell Signaling Technology (Beverly, USA). Antibodies against NRF2 (ab62352; 1:1000) and NQO1 (ab34173; 1:1000) were purchased from Abcam (Shanghai, China).

Immunofluorescence analysis

For immunofluorescence staining, cells grown on coverslips were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 4% bovine serum albumin in PBS. Cells were then incubated overnight at 4°C with goat anti-SOX2 (AF2018, 1:100; R&D Systems) and rabbit anti-NRF2 (ab62352, 1:100; Abcam) antibodies. After incubation, the cells were treated with rhodamine (TRITC)-conjugated donkey anti-goat IgG (705-025-003, 1:1000; Jackson ImmunoResearch, West Grove, USA) or fluorescein (FITC)-conjugated donkey anti-rabbit IgG (711-095-152, 1:1000; Jackson ImmunoResearch) for 1 h at room temperature in the dark. The nuclei were stained with DAPI for 5 min at room temperature. Coverslips were mounted with Antifade Mounting Medium (P0126; Beyotime). Images were acquired via a laser confocal microscope (TCS SP5 II system; Leica Microsystems, Wetzlar, Germany).

Dual-luciferase reporter assay

Luciferase reporter assay was performed via the Secrete-Pair™ Dual Luminescence Assay Kit (LF031; GeneCopoeia, Rockville, USA) according to the manufacturer's instructions. For gene promoter analysis, cells were cotransfected with the indicated reporter plasmids (NFE2L2-Gluc or NFE2L2^{AP3}-Gluc) and SOX2 expression plasmids (SOX2^{WT} or SOX2^{R74P}) or an empty vector (EV). After 48 h, the cell culture media were collected, and the activities of Gluc and SEAP were measured. Gluc activity was normalized to SEAP activity.

Chromatin immunoprecipitation (ChIP) assay

Potential transcription factor-binding sites in enhancer or promoter regions were predicted via the JASPAR database (<http://jaspar.genereg.net/>). On the basis of these predictions, ChIP assay was conducted to confirm the binding of SOX2 to gene enhancers or promoters in HCC95 cells via a ChIP-IT Kit (53009; Active Motif, Shanghai, China). Antibodies specific for SOX2 (AF2018, 1:50; R&D Systems) or normal goat serum (5425, 1:50; Cell Signaling

Technology) were used as controls, as described previously [25]. Immunoprecipitated DNA was subjected to PCR amplification of gene enhancer or promoter regions. The primers used for ChIP-PCR are listed in Table 1.

CDX tumors and drug sensitivity assay

Animal care and experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Chengdu Medical College, and the procedures were carried out according to the guidelines established by the China Council on Animal Care. Athymic nude mice (8 weeks old, male) were purchased from GemPharmatech (Nanjing, China) and maintained on a standard chow diet. Stable NCI-H292 cells (5 × 10⁵ cells per flank) were subcutaneously injected into the mice. Tumor size was measured with a digital caliper at 8-day intervals. Tumor volumes were calculated via the following formula: length × width² × 0.5. Once

Table 1. Sequence of primers used for q-PCR or ChIP

Primer	Sequence (5'→3')
SOX2-PCR-F	GATTACGCTGGAGGAGAATTCATGTACAACATGATGGAGAC
SOX2-PCR-R	TTATCTAGAGTCGCGGGATCCTCACATGTGTGAGAGGGGCA
GAPDH-QPCR-F	AAGGTGAAGGTCGGAGTCAA
GAPDH-QPCR-R	AATGAAGGGGTCATTGATGG
GCLC-QPCR-F	AGAGAAGGGGAAAGGACAAAC
GCLC-QPCR-R	AAGTTATTGTGCAAAGAGCCTGAT
GCLM-QPCR-F	TGTCTTGGAATGCACTGTATCTC
GCLM-QPCR-R	CCCAGTAAGGCTGTAAATGCTC
GSR-QPCR-F	CACGAGTGATCCCAAGCCC
GSR-QPCR-R	CAATGTAACCTGCACCAACAATG
XCT-QPCR-F	TCTCCAAAGGAGGTTACCTGC
XCT-QPCR-R	AGACTCCCCTCAGTAAAGTGAC
GPX1-QPCR-F	TGCAACCAGTTTGGGCATCA
GPX1-QPCR-R	ACCGTTCACCTCGCACTTC
GPX4-QPCR-F	CAGTGAGGCAAGACCGAAGT
GPX4-QPCR-R	CTGCTTCCCGAAGTGTTAC
GSTM1-QPCR-F	TCTGCCCTACTTGATTGATGGG
GSTM1-QPCR-R	TCCACACGAATCTTCTCCTCT
GSTP1-QPCR-F	CCCTACACCGTGGTCTATTTC
GSTP1-QPCR-R	CAGGAGGCTTTGAGTGAGC
NQO1-QPCR-F	GAAGAGCACTGATCGTACTGGC
NQO1-QPCR-R	GGATACTGAAAGTTCGCAGGG
NFE2L2-QPCR-F	TCAGCGACGGAAAGAGTATGA
NFE2L2-QPCR-R	CCACTGGTTTCTGACTGGATGT
P1-ChIP-F	CTGCTAAGACTCCAGCAGACTT
P1-ChIP-R	CCGTGGATAAATGGATAAACCG
P2-ChIP-F	CTCCTCGGAGAAAAATAGAACC
P2-ChIP-R	GTCATATGTGAGGGTAACAGG
P3-ChIP-F	GATAGCACGTGAAAAGTGCTCC
P3-ChIP-R	GTGTTCTTCAGATTCTCTGC
NC-ChIP-F	CTGTGGTTCCTTCTTCTCTATC
NC-ChIP-R	CACATCAAAAGCTTCCACAGAGG

the tumors reached approximately 50 mm³, the tumor-bearing mice were treated with carboplatin (5 mg/kg, i.p. injection, 5 times/week, for 3 weeks) and/or ML385 (30 mg/kg, i.p. injection, 5 times/week, for 3 weeks). At the end of the treatment period, the mice were euthanized, and the tumors were excised, weighed, fixed, embedded, and sectioned. Tumor sections were subsequently subjected to immunohistochemistry (IHC) staining. Since this was an exploratory animal study, no statistical tests were used to determine the sample size, and no mice were excluded from the analysis.

Immunohistochemistry (IHC)

Paraffin-embedded slides were deparaffinized and rehydrated via xylene and a graded ethanol series (100%, 95%, 80%, and 75%), followed by three washes with PBS (5 min each). Antigen retrieval was performed by incubating the slides in citrate antigen retrieval solution in a microwave for 15 min, followed by natural cooling and washing with PBS three times for 5 min each. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ solution at room temperature. Nonspecific binding was blocked by incubation with 3% BSA for 1 h, followed by overnight incubation with primary antibodies at 4°C. After being washed with PBS, the slides were incubated with secondary antibodies for 1 h at room temperature. Following PBS washes, the slides were incubated with DAB solution for staining, counterstained with hematoxylin, dehydrated with a graded alcohol series, and mounted with neutral balsam. The primary antibodies used were anti-Ki67 (27309-1-AP, 1:100; Proteintech) and anti-cleaved caspase 3 (#9664, 1:100; Cell Signaling Technology). Positively stained cells were scanned via a NanoZoomer (Hamamatsu, Japan). The percentage of positively stained cells was independently evaluated by four authors (H.G., C.L., J.S., and L.D.). The average optical density (AOD) score was calculated as described previously [24] to compare protein expression levels in different samples.

Human data from publicly available databases

The mRNA expression of *SOX2* in LUAD and LUSC samples was analyzed via the GEPIA2 database (<http://gepia2.cancer-pku.cn>). The correlation of mRNAs between *SOX2* and *NFE2L2* in LUSC, HNSCC, and ESCA was obtained from the TCGA Firehose Legacy datasets on the cBioPortal platform (cBioPortal, www.cbioportal.org). Kaplan-Meier survival curves for patients with lung cancer were generated via data from the KM Plotter database (www.kmplotter.com).

Statistical analysis

Cell culture data were derived from three independent experiments and are presented as the mean ± SD. All the statistical analyses were performed via GraphPad Prism 8 software. Unless otherwise specified, differences between two groups were assessed via two-tailed unpaired Student's *t*-test, whereas comparisons involving more than two groups were assessed via one-way ANOVA. The homogeneity of variances in the clinical data was assessed via Levene's test in SPSS 16 software. Statistical significance was set at *P* < 0.05.

Results

SOX2 overexpression leads to poor prognosis and carboplatin resistance in LUSC

SOX2 is an essential regulator of stem cells and functions as an

oncogene in various tumor types. In human lung cancer patients, elevated SOX2 expression is associated with poor overall survival (OS) (Figure 1A). Oncogenes are often activated by somatic DNA alterations in cancers arising from the cell lineages where these genes play a critical role in normal development. To explore this, we analyzed DNA alterations in LUAD and LUSC using TCGA datasets. We found that the frequency of SOX2 gene amplification is significantly greater in LUSC (48%) than in LUAD (4%) (Figure 1B). Similarly, the proportion of tumors with high SOX2 mRNA expression is greater in LUSC than in LUAD (Figure 1C). We further examined SOX2 expression in LUSC cell lines, finding one with low SOX2 expression (NCI-H292) and two with high SOX2 expression (NCI-H520 and HCC95) (Figure 1D).

Previous studies have reported that high SOX2 expression is linked to drug resistance [26]. To investigate the role of SOX2 in LUSC therapy resistance, we first assessed the effect of carboplatin, a first-line chemotherapeutic agent for LUSC treatment, on the survival of these cell lines. The results revealed that the half-maximal inhibitory concentration (IC₅₀) of carboplatin is greater in SOX2-high-expressing cells than in control cells (Supplementary Figure S1A). The inhibition of cell viability by carboplatin increased with both drug concentration and treatment duration, accompanied by an increase in the protein level of the apoptosis marker cleaved caspase-3 (Supplementary Figure S1B,C). However, carboplatin treatment did not significantly alter SOX2 protein expression in HCC95 cells, suggesting that SOX2 may play a critical role in carboplatin resistance. Next, we knocked down SOX2 in HCC95 cells and treated them with carboplatin. As shown in Figure 1E–G, knockdown of SOX2 significantly increased the sensitivity of HCC95 cells to carboplatin. To further confirm the role of SOX2 in LUSC therapy resistance, we ectopically expressed wild-type SOX2 (SOX2^{WT}) or mutant SOX2 (SOX2^{R74P}), a loss-of-function DNA-binding domain mutant identified in an individual with congenital tracheoesophageal fistula [13,27]. As expected, ectopic expression of SOX2^{WT}, but not its transcriptional defect mutant SOX2^{R74P}, significantly reduced the sensitivity of NCI-H292 cells to carboplatin (Figure 1H–J). Collectively, these data suggest that SOX2 mediates carboplatin resistance in LUSC cells in a transcription-dependent manner.

SOX2 inhibits carboplatin-induced ROS to maintain cell survival by enhancing GSH synthesis in LUSC

In addition to inducing DNA double-strand breaks, carboplatin also generates reactive oxygen species (ROS) [28,29], which contribute to cell death. To investigate whether SOX2 is involved in the regulation of carboplatin-induced ROS, we knocked down SOX2 via two specific shRNAs in NCI-H520 and HCC95 cells (Figure 2A). We found that SOX2 depletion significantly increased cellular ROS levels (Figure 2B). Notably, the removal of ROS via N-acetylcysteine (NAC) rescued the SOX2 knockdown-induced sensitization of cells to carboplatin (Figure 2C,D). Glutathione (GSH) is the most abundant antioxidant in cells. We observed that SOX2 knockdown led to a reduction in the level of reduced glutathione (GSH) and an increase in oxidized glutathione (GSSG) (Figure 2E,F). Furthermore, supplementation with GSH rescued SOX2 knockdown-induced sensitivity to carboplatin (Figure 2G–H). Taken together, these data demonstrate that SOX2 inhibits carboplatin-induced ROS accumulation by promoting GSH synthesis, thereby increasing cell survival.

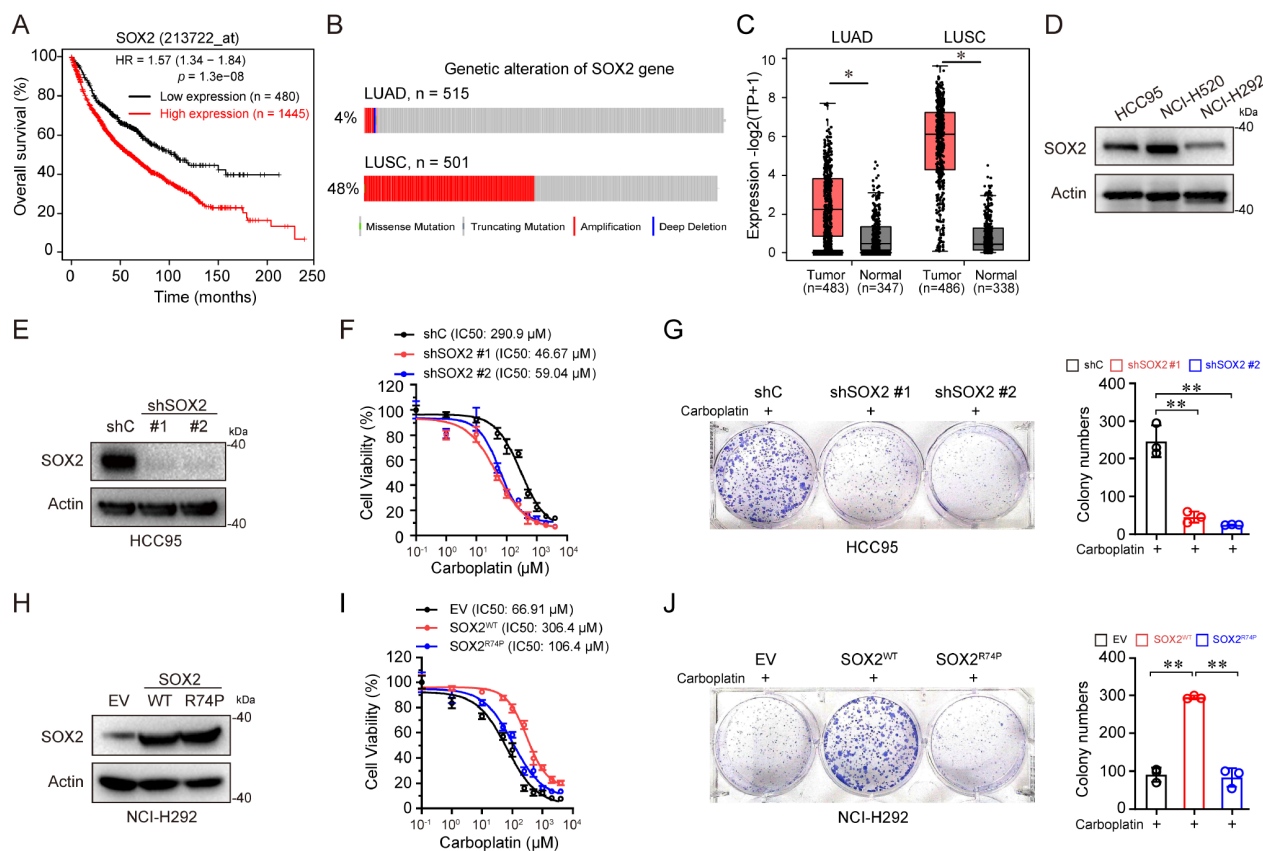


Figure 1. High expression of SOX2 is correlated with poor prognosis and carboplatin resistance in LUSC (A) Kaplan-Meier survival analysis of overall survival (OS) in human lung cancer patients stratified by *SOX2* mRNA expression levels. (B) TCGA database analysis of *SOX2* gene alterations in LUAD and LUSC samples. (C) GEPIA2 database analysis of *SOX2* expression levels in LUSC and LUAD samples. (D) Immunoblot analysis of whole-cell lysates from the HCC95, NCI-H520, and NCI-H292 cell lines. (E–G) HCC95 cells stably expressing shSOX2 or shC were analyzed as follows: (E) Immunoblot analysis of SOX2 expression; (F) Cells were treated with increasing concentrations of carboplatin (0, 5, 10, 20, 50, 100, 200, and 500 μM) for 24 h, followed by a CCK8 assay to assess cell viability, curve fitting, and calculation of IC_{50} values; (G) Clonogenic survival assay after carboplatin treatment. (H–J) NCI-H292 cells stably expressing SOX2 or empty vector (EV) were analyzed as follows: (H) Immunoblot analysis of SOX2 expression. (I) Cells were treated with increasing concentrations of carboplatin for 24 h, followed by a CCK8 assay to assess cell viability, curve fitting, and calculation of IC_{50} values. (J) Clonogenic survival assay after carboplatin treatment. The results are presented as the mean $\pm$ SD from three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

SOX2 promotes GSH synthesis through NRF2

To investigate how SOX2 affects the cellular GSH content, we analyzed the expression of genes involved in GSH metabolism following SOX2 depletion. As shown in Figure 3A, SOX2 knockdown significantly reduced the expression of genes involved in GSH regeneration and utilization. Notably, both the protein and mRNA levels of the transcription factor NRF2, the master regulator of GSH synthesis enzymes, were significantly decreased after SOX2 depletion (Figure 3B–D). Consistently, ectopic expression of wild-type SOX2, but not the R74P mutant, upregulated NRF2 expression (Figure 3E). To investigate whether NRF2 mediates the effect of SOX2 on GSH synthesis, we restored NRF2 expression in SOX2-depleted cells (Figure 3F). Restoration of wild-type NRF2, but not its transcriptionally defective mutant ΔNeh1 , completely rescued the decreased GSH content caused by SOX2 depletion (Figure 3G). As expected, NRF2 restoration fully reversed the increase in cellular ROS levels induced by SOX2 knockdown (Figure 3H). Consequently, cell viability was significantly restored following NRF2 re-expression under SOX2 depletion conditions (Figure 3I). Taken together, these data demonstrate that SOX2 promotes GSH synthesis

to confer carboplatin resistance in LUSC cells through the activation of NRF2 signaling.

SOX2 directly transactivates NRF2 expression

To elucidate the molecular mechanism by which SOX2 regulates NRF2 expression, we analyzed the correlation between SOX2 and *NFE2L2* (encoding NRF2) mRNA expression via the TCGA database. As shown in Figure 4A and Supplementary Figure S2A, B, SOX2 mRNA levels were positively correlated with *NFE2L2* mRNA levels in LUSC, head and neck squamous cell carcinoma (HNSCC), and esophageal carcinoma (ESCA). Given that SOX2 affects both the protein and mRNA levels of NRF2, we hypothesized that SOX2 might directly regulate NRF2 transcription. To investigate whether SOX2 binds to the regulatory regions of the *NFE2L2* gene, we identified SOX2-enriched regions near the *NFE2L2* genomic locus via the UCSC genome browser (Figure 4B). Three conserved putative SOX2-binding sites, designated P1, P2, and P3, were found within the human *NFE2L2* genomic sequence (Figure 4C). We then cloned the *NFE2L2* gene promoter, including these three putative SOX2-binding sites, into a luciferase reporter construct (Figure 4D).

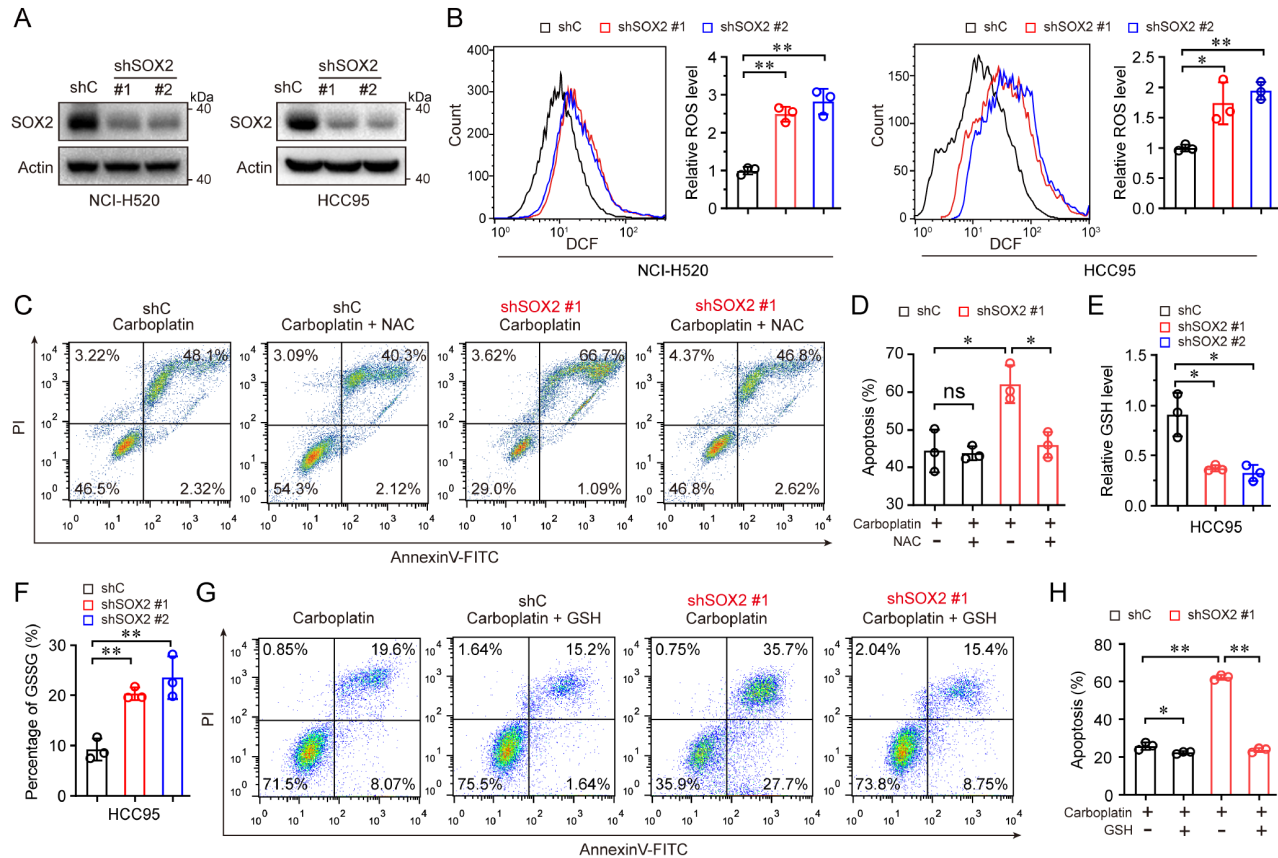


Figure 2. SOX2 inhibits carboplatin-induced ROS production by increasing GSH synthesis in LUSC (A,B) NCI-H520 and NCI-HCC95 cells stably expressing shSOX2 (#1 or #2) or shC were analyzed as follows: (A) Immunoblot analysis of SOX2 expression; (B) DCFH-DA staining to measure intracellular reactive oxygen species (ROS) levels. (C,D) HCC95 cells stably expressing shSOX2 (#1) or shC were treated with 100 μM carboplatin with or without 5 mM NAC for 24 h, followed by FACS analysis to assess apoptosis (C) and quantification (D). (E,F) HCC95 cells stably expressing shSOX2 (#1 or #2) or shC were analyzed for (D) GSH and (E) GSSG content. (G,H) HCC95 cells stably expressing shSOX2 (#1 or #2) or shC were treated with 100 μM carboplatin with or without 5 mM GSH for 24 h, followed by FACS analysis to assess apoptosis (G) and quantification (H). The results are presented as the mean ± SD from three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

As shown in Figure 4E, SOX2, but not the R74P mutant, significantly enhanced *NFE2L2* promoter activity. Furthermore, *NFE2L2* promoter activity was dependent on SOX2 protein level (Figure 4F). ChIP assay demonstrated that SOX2 directly binds to the P3 region of the *NRF2* promoter (Figure 4G). Moreover, deletion of the P3 site partially impaired the ability of SOX2 to bind to the *NRF2* promoter (Figure 4H), suggesting that P3 is one of several regulatory sites through which SOX2 activates *NRF2* transcription. Taken together, these data demonstrate that SOX2 directly binds to the *NFE2L2* gene and activates its transcription.

Targeting NRF2 overcomes SOX2-induced carboplatin resistance

To investigate whether targeting NRF2 can reverse SOX2-mediated carboplatin resistance, we treated SOX2-overexpressing lung cancer cells with carboplatin in combination with ML385, a specific NRF2 inhibitor [30]. Our results showed that inhibition of NRF2 significantly enhanced cell sensitivity to carboplatin (Figure 5A, B). To further validate the ability of NRF2 inhibition to reverse SOX2-mediated carboplatin resistance *in vivo*, we generated xenograft models by subcutaneously injecting transfected LUSC cells into nude mice, followed by treatment with carboplatin and ML385 (Figure 5C). As shown in Figure 5D,E, ectopic expression of

SOX2 promoted tumor growth and increased tumor weight. While carboplatin significantly reduced tumor growth and weight, ectopic expression of SOX2 maintained tumor growth despite carboplatin treatment. Notably, the combination of carboplatin and NRF2 inhibition significantly suppressed SOX2-driven tumor growth, indicating that NRF2 inhibition can counteract SOX2-mediated carboplatin resistance. Furthermore, IHC assay revealed that, compared with empty vector (EV) treatment, SOX2 increased the number of Ki67-positive cells and decreased the number of cleaved caspase 3 (CC3)-positive apoptotic cells. However, NRF2 inhibition combined with carboplatin treatment significantly reduced the number of Ki67-positive cells and increased the number of CC3-positive apoptotic cells (Figure 5F,G). Taken together, these results demonstrate that NRF2 inhibition effectively overcomes SOX2-mediated carboplatin resistance in NSCLC.

Discussion

LUSC has not benefited from targeted therapies [31]. Although immunotherapy has improved outcomes for many cancers, its efficacy in LUSC is often limited by low response rates and adverse events [6]. Therefore, a deeper understanding of the mechanisms underlying LUSC pathogenesis is crucial. In this study, we demonstrated that SOX2, a lineage survival oncogene in LUSC, is

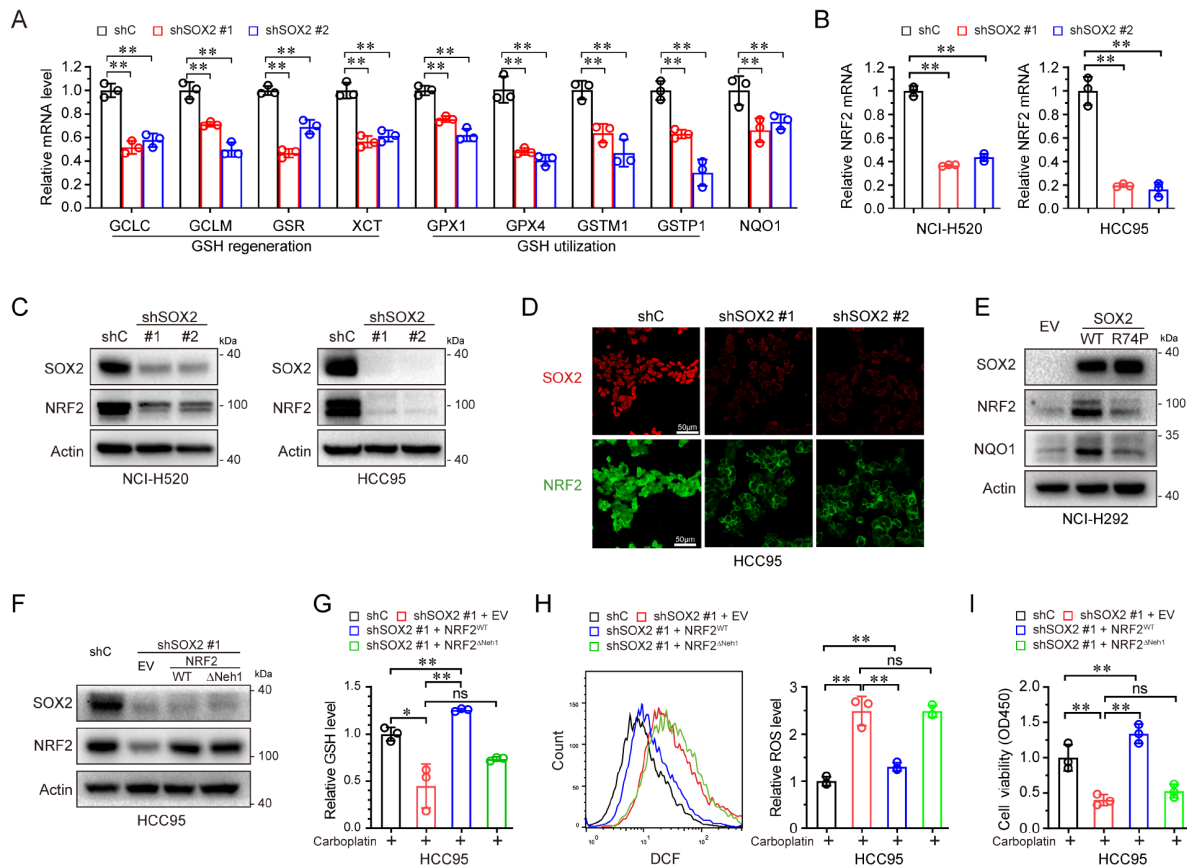


Figure 3. SOX2 promotes GSH synthesis through NRF2 (A) NCI-H520 cells stably expressing shSOX2 (#1 or #2) or shC were analyzed via qPCR for the expression of genes involved in GSH metabolism. (B-D) NCI-H520 or HCC95 cells stably expressing shSOX2 (#1 or #2) or shC were subjected to the following assays: (B) qPCR analysis of NRF2, (C) immunoblotting analysis to assess protein expression, and (D) immunofluorescence staining for protein localization. (E) NCI-H292 cells stably expressing SOX2^{WT}, SOX2^{R74P}, or the empty vector (EV) were analyzed via immunoblot analysis. (F-H) HCC95 cells stably expressing shSOX2 (#1) were infected with lentivirus encoding either NRF2^{WT} or NRF2^{ΔNeh1} and subsequently analyzed by (F) immunoblot analysis for NRF2 protein expression; (G) GSH content analysis; (H) ROS level measurement; and (I) cell viability assays following treatment with 100 μ M carboplatin. The results are presented as the mean $\pm$ SD from three independent experiments performed in triplicate. * P < 0.05, ** P < 0.01. ns: not significant.

overexpressed in lung cancer and is associated with poor prognosis. We show that SOX2 promotes carboplatin resistance by enhancing the scavenging of reactive oxygen species (ROS). Mechanistically, SOX2 directly transactivates NRF2, thereby increasing glutathione (GSH) biosynthesis. Notably, inhibition of NRF2 can reverse SOX2-mediated carboplatin resistance *in vivo*. This study identified the SOX2-NRF2 axis as a potential therapeutic target for overcoming carboplatin resistance in LUSC.

SOX2 is closely associated with the initiation and progression of LUSC [13,14]. SOX2 confers resistance to various tumor therapies, including chemoresistance, tamoxifen resistance, ferroptosis resistance and radioresistance [26,32–34]. Therefore, targeting SOX2 represents a potential strategy for overcoming therapy resistance [35]. Inducing SOX2 degradation has been proposed as a viable approach [19], and the ubiquitin-proteasome system has been implicated in regulating SOX2 stability. Consequently, targeting the expression or enzymatic activity of E3 ubiquitin ligases or deubiquitinases represents a novel therapeutic strategy for SOX2-driven cancers [36–38]. Additionally, LSD1-specific inhibitors have been shown to selectively impair the growth of SOX2-expressing LUSC cells without affecting SOX2-negative cells [39]. Unfortunately, little progress has been made in the development of SOX2 inhibitors,

largely due to the “undruggable” nature of SOX2 as a transcription factor [35]. In this study, we demonstrated that SOX2 promotes carboplatin resistance in preclinical models of LUSC by transactivating the expression of NRF2. Inhibition of NRF2 effectively reverses SOX2-mediated carboplatin resistance in LUSC. Therefore, targeting downstream effectors of SOX2 may represent a promising strategy for treating LUSC and overcoming therapy resistance.

SOX2 plays a central role in cancer stem cell (CSC)-related signaling [20]. CSCs are considered key drivers of tumorigenesis, therapy failure, and resistance to treatment, highlighting the critical importance of eliminating CSCs to reverse resistance [19]. While CSCs represent a small fraction of tumor cells in solid tumors [40], it is unlikely that all SOX2-positive LUSC cells are CSCs. Interestingly, a study has shown that breast and liver cancer stem cells typically exhibit low ROS levels due to the expression of ROS-scavenging systems [41], suggesting that SOX2 may play a role in regulating ROS [42]. However, the detailed mechanism by which SOX2 regulates ROS remains unclear. In this study, we demonstrated that SOX2 scavenges ROS by directly transactivating NRF2 to increase GSH synthesis, thereby expanding the functional repertoire of SOX2.

NRF2, the master regulator of cellular antioxidant responses and a key cytoprotective protein, is tightly regulated by a complex

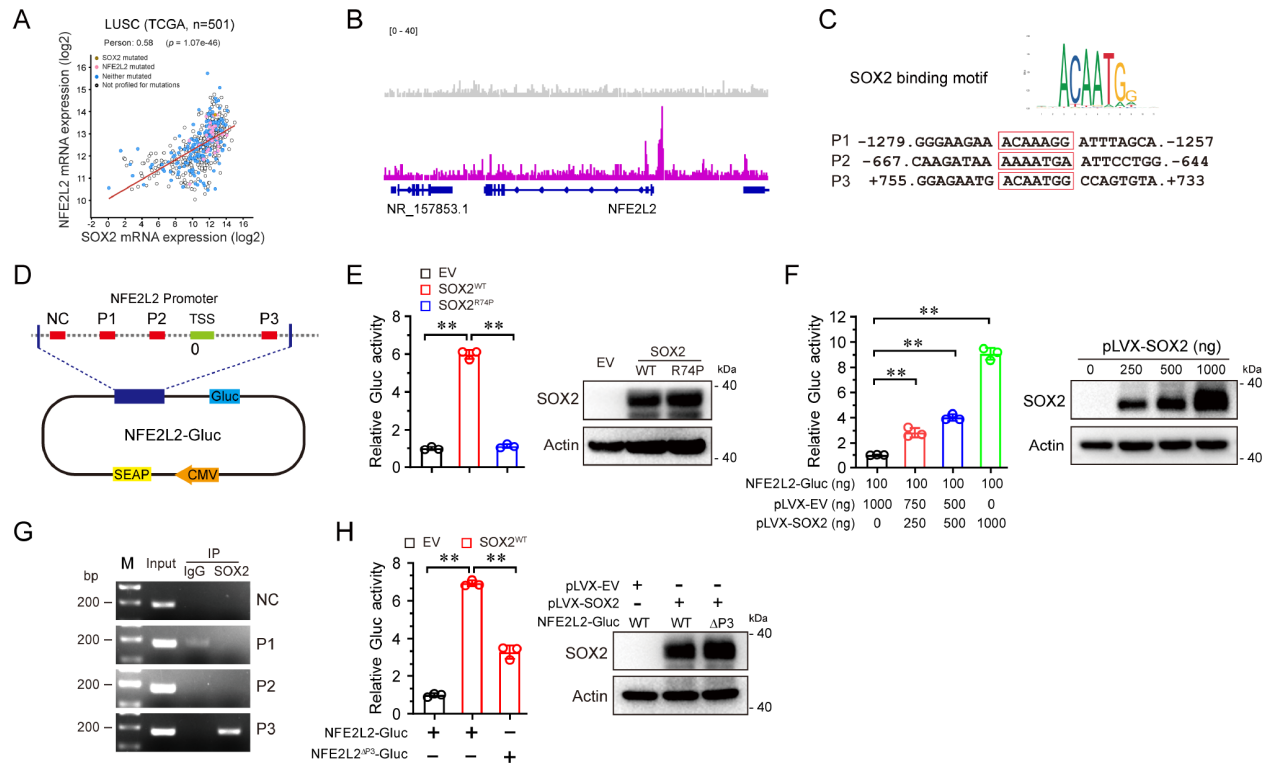


Figure 4. SOX2 directly transactivates NRF2 expression (A) The Pearson correlation coefficient (R value) and two-tailed probability test (P value) between SOX2 and NFE2L2 were analyzed via data from the TCGA database for LUSC. (B) Chromatin immunoprecipitation (ChIP) sequencing data from HCC95 cells (GEO accession number GSM1139038) identified putative SOX2-binding sites within the *NFE2L2* promoter. (C) JASPAR database analysis of potential SOX2-binding sites on the *NFE2L2* gene promoter. (D) Diagram of the *NFE2L2* gene promoter reporter vector construction. (E) HEK-293T cells were transfected with the pLVX-SOX2^{WT} or pLVX-SOX2^{R74P} plasmid and the pEZX-NRF2 promoter plasmid. The medium was harvested for dual luciferase reporter assays, and the cell lysates were subjected to immunoblot analysis to confirm the transfection efficiency. (F) HEK293T cells were transfected with 0 ng, 250 ng, 500 ng, or 1000 ng of the pLVX-SOX2 plasmid or the pEZX-NRF2 promoter plasmid. The medium was harvested for dual luciferase reporter assays, and the cell lysates were subjected to immunoblot analysis to confirm the transfection efficiency. (G) HCC95 cell lysates were subjected to ChIP to assess the binding capacity of SOX2 to the *NRF2* promoter region at P1, P2, and P3. NC served as a negative control. (H) HEK-293T cells were transfected with the pLVX-SOX2 plasmid and either the pEZX-NRF2^{WT} promoter or the pEZX-NRF2^{ΔP3} promoter plasmid. The medium was harvested for dual luciferase reporter assays, and the cell lysates were subjected to immunoblot analysis to confirm the transfection efficiency. The results are presented as the mean $\pm$ SD from three independent experiments performed in triplicate. ** $P < 0.01$.

control system [43]. In the absence of oxidative stress, the KEAP1 homodimer, which acts as an adapter protein for the E3-ubiquitin ligase complex, binds to NRF2 and promotes its ubiquitination and degradation [44]. Therefore, NRF2 levels can be influenced by either disruption of the KEAP1-NRF2 complex or a reduction in KEAP1 expression. Loss or mutation of KEAP1 activates NRF2, promoting lung cancer progression [36,37]. Notably, mutations and copy number alterations in *KEAP1* and *NFE2L2*, or deletions or mutations in *CUL3*, occur in 34% of LUSC patients [38], resulting in reduced cellular ROS levels and increased resistance to chemo-, radio-, or immunotherapy [37]. Our previous work demonstrated that Δ Np63 α , another lineage survival oncogene in LUSC, stabilizes the NRF2 protein to promote radioresistance by transactivating PLECs, which facilitates the release of NRF2 from KEAP1 [22]. Interestingly, SOX2 can directly activate the transcription of Δ Np63 α [39]. These findings suggest that SOX2 may transactivate *NRF2* mRNA expression and stabilize NRF2 protein levels through the Δ Np63 α -PLEC-KEAP1 axis. Therefore, targeting NRF2 represents a critical strategy to overcome SOX2- or Δ Np63 α -mediated therapeutic resistance in LUSC. Several NRF2 inhibitors, including ML385, MSU38225, and brusatol, have been described over the past few decades [30,45]; however, none of these inhibitors have been

explored in clinical trials or incorporated into the drug development pipeline [46], highlighting the inherent challenges in targeting NRF2 with small molecules.

NRF2 activation in nontransformed cells protects against DNA damage, whereas constitutive activation of NRF2 in cancer cells confers resistance to chemo- and radiotherapies, rendering them refractory to treatment [44]. NRF2 also provides protection to cancer cells against toxic insults from a broad spectrum of chemicals through the antioxidant response system, particularly via the Xc catalytic subunit xCT (SLC7A11) [47]. Notably, SOX2 has been shown to transactivate SLC7A11, promoting ferroptosis resistance in LUAD [26]. In this study, we revealed that SOX2 transactivates the expression of NRF2, a key transcriptional regulator of SLC7A11, to promote carboplatin resistance in LUSC. These findings suggest that SOX2 may directly activate SLC7A11 expression or indirectly regulate it through NRF2. Therefore, targeting SLC7A11 may provide a promising approach to overcome the limitations of the use of SOX2 and NRF2 as drug targets.

In summary, we identified SOX2 as a critical regulator of NRF2 expression, driving carboplatin resistance in LUSC. By transactivating NRF2, SOX2 enhances the cellular antioxidant response, thereby

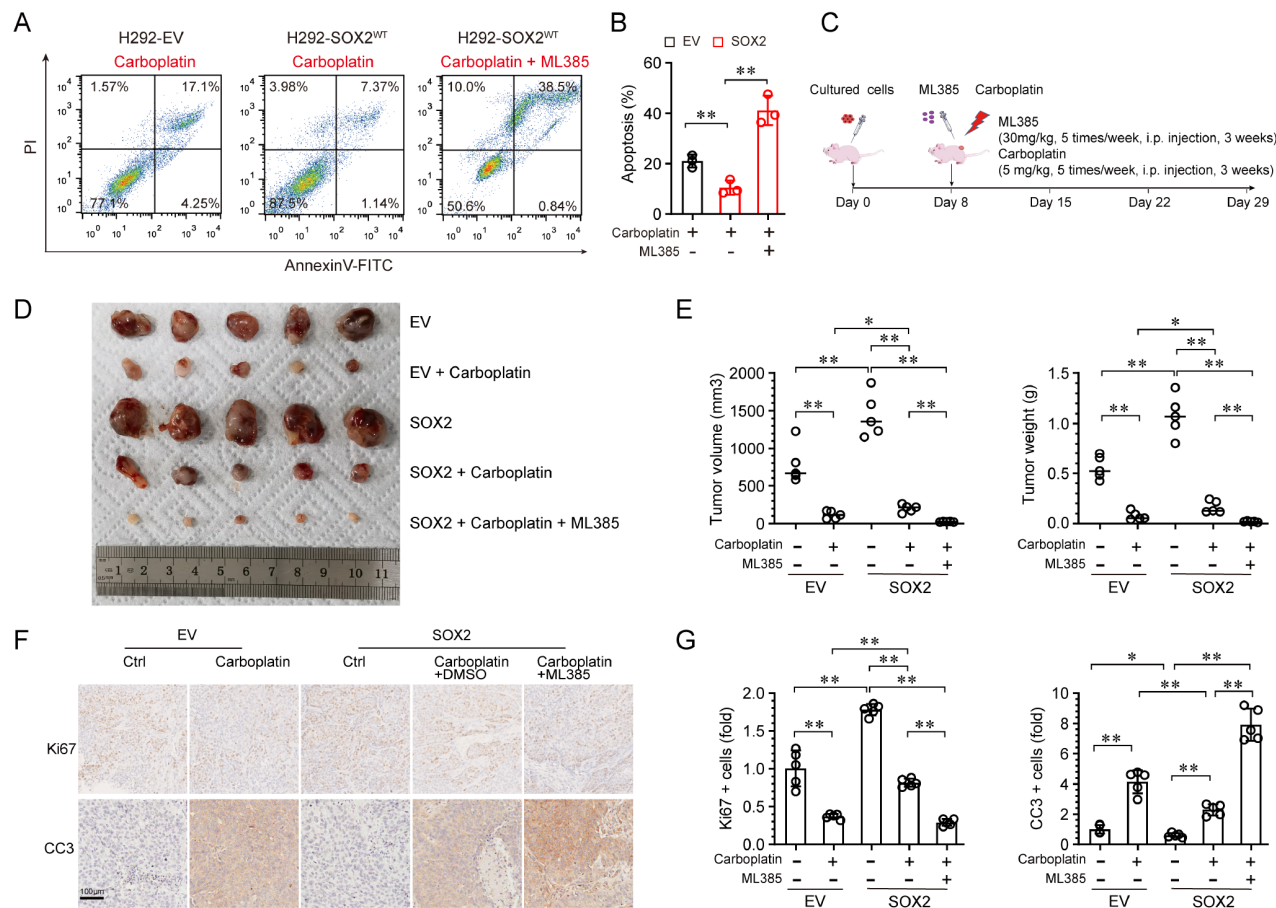


Figure 5. Targeting NRF2 overrides SOX2-induced carboplatin resistance (A,B) NCI-H292 cells stably expressing SOX2^{WT} or empty vector (EV) were treated with 100 μ M carboplatin and/or 10 μ M ML385 for 24 h, followed by FACS analysis to assess apoptosis (A) and quantification (B). (C) Schematic overview of the animal experiment design. (D) Representative images of xenograft tumors dissected from the indicated groups at the end of the experiment. (E) Tumor weights and volumes of the subcutaneous xenografts in the indicated groups. (F) Representative IHC images showing Ki67 and cleaved caspase 3 (CC3) staining in tumor sections from the indicated groups. (G) Quantification of Ki67 and CC3 protein levels via average optical density (AOD) scores. The results are presented as the mean $\pm$ SD from five mice per group. * P < 0.05, ** P < 0.01. ns: not significant.

protecting cells from carboplatin-induced oxidative stress. Targeting NRF2 effectively reverses SOX2-mediated resistance, positioning NRF2 as a promising therapeutic target in LUSC.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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