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Original Article

The cancer stemness inhibitor napabucasin suppresses small cell lung cancer growth through SOX2 expression

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Abstract: Small cell lung cancer (SCLC) is a high-grade malignancy of neuroendocrine origin characterized by aggressive cell growth and a poor survival rate of patients. Currently, the treatment options for SCLC remain limited despite platinum-based chemotherapy. Systemic chemotherapy is effective for SCLC, but most patients eventually acquire drug resistance, which leads to treatment failure. Stemness-high cancer cells show characteristics of advanced tumorigenesis and metastasis and have high potential in promoting treatment resistance and disease relapse. Napabucasin (BBI608), a novel small-molecule drug targeting on signal transducer and activator of transcription 3 (STAT3), was shown to suppress the progression and metastasis of stemness-high cancer stem cells in various cancers. Here, we demonstrated that napabucasin significantly decreased viability and colony formation and induced the arrest of S-phase cell cycle and apoptosis in cisplatin-resistant SCLC cells. Findings from mechanistic studies further indicated that napabucasin directly downregulated the expression of SOX2 in cisplatin-resistant SCLC cells; however, dysfunctional SOX2 expression in SCLC cells was associated with interference in the napabucasin-mediated reduction of cell viability. In contrast, napabucasin-induced viability reduction was restored in these cells when SOX2 expression was upregulated. Furthermore, napabucasin significantly inhibited cisplatin-resistant SCLC cell xenograft growth *in vivo* by downregulating SOX2 and inducing apoptosis. These data demonstrate that napabucasin may be a novel drug for the clinical treatment of cisplatin-resistant SCLC.

Keywords: Small cell lung cancer, napabucasin, stemness, SOX2, cisplatin, resistant

Introduction

Small cell lung cancer (SCLC) is an extremely aggressive malignancy of neuroendocrine origin [1], accounting for approximately 10-20% of all lung cancer in the histological classification [2]. SCLC is characterized by low differentiation, rapid disease progression, and a poor prognosis. SCLC development involves several gene aberrations, such as the inactivation of *TP53* and *Rb1* mutations and mutations activating *EGFR* and *KRAS* [3, 4]. In a previous study, we observed the amplification and overexpression of sex determining region Y-box 2 (SOX2) in 80 human SCLCs [5]. Although patients with SCLC exhibit significant sensitization to standard treatment, including chemotherapy with cisplatin and etoposide, immunotherapy, and radiotherapy, in the beginning, drug resistance remains an important challenge in clinical settings [6, 7].

Cancer stem cells (CSCs) constitute a heterogeneous population of cells that possess the ability of self-renewal and differentiation [8]. A recent study showed that CSCs in SCLC are characterized by a side-population (SP) fraction (< 1% of the bulk cell population) [9]. These cells exhibit stem cell-like characteristics with increased stemness-associated marker expressions. When present within the neoplasm, they mediate tumor initiation, progression, and resistance to conventional chemotherapy and radiotherapy [10, 11]. Moreover, different types

of therapies can induce stemness-related gene expression for the transformation of stemness-low to stemness-high cancer cells [12]. The biological activities of CSCs have been reported to be upregulated by several pluripotent transcription factors, including SOX2, c-Myc, octamer-binding transcription factor 4, and Nanog, eventually conferring the cells the ability to form tumorspheres [13].

SOX2 and c-Myc, transcription factors that maintain the unique characteristics of embryonic stem cells, have been investigated extensively [14, 15]. A recent study revealed that SOX2 plays an important role in lung carcinogenesis [16]. In 2012, Rudin *et al.* reported *SOX2* amplification in 27% of SCLC samples (15 of 56 samples) [5]. Moreover, *SOX2* amplification is also higher in SCLC than in non-small cell lung cancer (NSCLC) [17], and SOX2 overexpression in SCLC and esophageal squamous cell carcinoma has been associated with more aggressive tumors [18, 19]. Chen *et al.* showed that shRNA-mediated silencing of *SOX2* induced NSCLC cell apoptosis through the MAP4K4-survivin signaling pathway [20]. Clinically, the mRNA and protein expression levels of SOX2 and survivin are significantly higher in stages III-IV in salivary adenoid cystic carcinoma and are positively correlated [21].

c-Myc, an important oncogene, frequently undergoes dysregulation, which is associated with unfavorable survival in patients with lung cancer [22]. Cosgrave *et al.* demonstrated that c-Myc overexpression induces survivin upregulation and promotes hematopoietic stem cell differentiation [23] and breast cancer tumorigenesis [24]. The silencing of c-Myc expression directly decreases the protein and mRNA levels of survivin in breast cancer cell lines [25]. Survivin is one of the members in apoptosis inhibitor family of proteins acting as a suppressor of apoptosis and plays a crucial role in promoting tumor initiation, cancer progression, and drug resistance [26]. Yano *et al.* reported a positive relationship between the clinical disease stage of SCLC and nuclear survivin expression [27]. In our previous study, we showed that the silencing of survivin expression enhances the radiosensitivity of lung cancer cells [28].

Napabucasin (BBI608) is a novel small-molecule inhibitor of stemness/signal transducer and activator of transcription 3 (STAT3) that blocks the stemness markers expression and induces apoptosis in prostate cancer and NSCLC [29, 30]. MacDonagh *et al.* showed that napabucasin can be administered in combination with numerous chemotherapeutic agents in several prospective clinical trials [30]. Furthermore, napabucasin was shown to suppress the phosphorylation of STAT3 to downregulate the expression of downstream target genes, including the c-Myc and survivin genes, in diffuse large B-cell lymphoma [31]. Chemotherapy and radiotherapy have been reported to induce cancer cells stemness in previous studies [32, 33], and napabucasin has been shown to effectively inhibit stemness marker expression and block relapse and metastasis in xenografted human cancers [34]. However, the mechanisms of action and therapeutic potential of napabucasin in SCLC *in vitro* and *in vivo* have not yet been investigated.

In this study, we used commercial SCLC tissue arrays obtained from 79 patients to evaluate the significance of SOX2 expression in clinical settings. We also found two cisplatin-resistant SCLC cell lines and further showed that napabucasin did inhibit cell proliferation and colony formation from these cells *in vitro*. Napabucasin-mediated SOX2 and c-Myc downregulation primarily induced cell cycle arrest and apoptosis. Furthermore, our results show that SOX2 expression dysfunction in SCLC cells led to napabucasin resistance. Thus, napabucasin may serve as a novel drug for suppressing progression and improving prognosis in SCLC.

Materials and methods

Cell lines

Cells from the SCLC cell lines H146, H1417, H1688, H446, H720, H209, and BEAS-2B were purchased from the American Type Culture Collection. H146, H1417, H1688, H446, and H209 cells were cultured in the RPMI1640 medium (Invitrogen, Carlsbad, CA, USA). H720 cell was cultured in DMEM:F12 medium supplemented with 0.005 mg/mL insulin, 0.01 mg/mL transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, and 10 nM beta-estradiol (Invitrogen). BEAS-2B cell was cultured in LHC-9 medium. All media were supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics (100 U/mL penicillin and 100 mg/

mL streptomycin), in accordance with the manufacturer's instructions.

Viability assay

Cell viability was assayed by using the CCK-8 cell counting kit (Roche Applied Science, Penzberg, Germany) [35]. Briefly, SCLC cells (1×10^4/well) were seeded in a 96-well plate. After overnight incubation, the cells were treated with cisplatin and napabucasin at various concentrations for 24, 48, and 72 hours (h).

Cell cycle

For analyzing cell cycle progression, propidium iodide (PI) (BD Biosciences Inc., San Jose, CA, USA) was used to confirm the effects of napabucasin on SCLC cells. H146 and H446 cells were starved in serum-free medium for 24 h to synchronize their cell cycles. After starvation, H146 and H446 cells were treated with napabucasin at various concentrations for 24 h, collected, fixed for PI staining, and finally analyzed by using flow cytometry. The percentage of cells in the G1, S, and G2/M phases was determined by using ModFit.

Isolation of SCLC cells with dysfunctional SOX2 expression using non-SP fraction cell sorting

The approach in Non-SP fraction cell sorting was cell-labelled with Hoechst 33342 (Sigma, St Louis, MO, USA) [9]. 1×10^6 H146 and H446 cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and stained with Hoechst 33342 for 90 min. Control cells was incubated with Verapamil for 15 min before Hoechst 33342 addition. After treatment, the cells were then washed in PBS and resuspended in PBS/1% FBS for sorting cells with dysfunctional SOX2 expression in the non-SP fraction using the BD FACS Aria II instrument.

Antibodies and reagents

Antibodies against cyclin A (1:1000), cyclin E, Cdk2 (1:1000), cdc2 (1:1000), and cdc25c purchased from Cell Signaling Technology (Danvers, MA, USA) were used to evaluate cell cycle progression. Antibodies against SOX2 (1:1000), ABCG2 (1:1000), Nanog (1:1000), OCT-4 (1:1000), c-Myc (1:1000), cleaved PARP (1:1000), and Mcl-1 (1:1000) purchased from Cell Signaling Technology were used to evaluate stemness marker expression and cellular apoptosis. Antibodies against survivin (1:1000) were purchased from Abcam (Cambridge, UK). An anti-β-actin antibody (1:5000) (Santa Cruz Biotechnology, Dallas, TX, USA) was used as a loading control.

Apoptosis analysis

For the evaluation of apoptosis, fluorescein isothiocyanate (FITC)-labeled annexin-V and PI (BD Biosciences Inc.) were used as double stains to assess early and late apoptosis in live and necrotic cells. H146 and H446 cells were seeded in 6-well plates and treated with napabucasin at various concentrations in the culture medium for 48 h. Following treatment, the cells were washed and stained with annexin-V-FITC and PI and finally analyzed by flow cytometry.

Evaluation of ectopic SOX2 expression in SCLC cells

To construct the lentivirus-based SOX2-expressing plasmid *psox2*, a 1000 bp fragment of human *SOX2* was generated by PCR using the pMSCV-Flag-hSOX2 plasmid (Addgene, #200-73, Watertown, MA, USA) as the template. The oligonucleotide primers used were as follows: forward primer, 5'-GGG ATA TCA TGT ACA ACA TGA TGG AGA C-3' and reverse primer, 5-GGG AAT TCT CAC ATG TGT GAG AGG GGC A-3'. To produce viral particles, *psox2* was transfected into HEK293T cells, following which the SOX2-expressing lentiviral supernatant was collected [36] and used to infect SCLC cells with dysfunctional SOX2 expression. Green fluorescent protein-positive SCLC cells were sorted and maintained at 37°C in an incubator for other assays.

In vivo tumorigenicity assay

All the animal experiments had been approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Chiayi, Taiwan). To evaluate the effect of napabucasin on the growth of SCLC xenograft tumors *in vivo*, 6-week-old nude mice (nu/nu) (BioLASCO, Taipei, Taiwan) were used for the establishment of xenograft mouse model. For the experiment, 5×10^6 H146 or H446 cells were suspended in 50 μL of PBS, mixed with an equal volume of Matrigel® (Corning Inc., Oneonta, NY, USA), and implanted subcutane-

ously into the flank of mice. After the tumors had grown to approximately 100 mm^3, the mice were treated with PBS or napabucasin (20 mg/kg/mouse) injected intraperitoneally once a day. Tumor volume was measured every 3 days, and the tumor growth by a volume greater than 2000 mm^3 was not allowed according to the policy of Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (Chiayi, Taiwan). After 21 days, the tumors were excised, photographed, fixed, and frozen for further analysis.

Immunofluorescent terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining analyses

An immunofluorescent TUNEL staining kit (No. 11767291910; Sigma-Aldrich Corp., St Louis, MO, USA) was used to detect apoptosis in the H146- and H446-xenograft tumors according to the manufacturer's instructions (Roche Applied Science). 4,6-diamidino-2-phenylindole nuclear staining was used for counterstaining control.

SCLC tissue microarrays

An SCLC tissue microarray (LC818t) containing cells from stage I (n = 39), stage II (n = 27), and stage III (n = 13) was purchased from US Biomax Inc. (Rockville, MD, USA). Briefly, the tissue microarray slides were stained with primary rabbit anti-human SOX2 antibody (Cell Signaling Technology, Inc.) to determine the clinical relevance of SOX2 expression in patients with SCLC. Pathological diagnosis was performed using the manufacturer's data sheet, and a pathologist scored SOX2 expression.

Statistical analyses

GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. Data are expressed as means (± standard deviation (SD)). The Bonferroni post-hoc test was used to compare the cell viability curves of the cisplatin- and napabucasin-treated groups and either of the single groups *in vivo*. Statistical significance was defined by $P < 0.05$, 0.01, and 0.001.

Results

SOX2 expression is associated with high-grade tumor development in SCLC clinical specimens

A previous report showed that high SOX2 expression is associated with the clinical stage, drug resistance, and metastasis in cancers [37]. SOX2 expression was scored according to the intensity of nuclear staining (no staining = 0, weak staining = 1+, moderate staining = 2+, and strong staining = 3+) (**Figure 1A**). **Figure 1B** shows the expression patterns of SOX2 observed at various stages of SCLC. The proportion of moderate and strong SOX2 expression (2+ and 3+) was considered to represent significant SOX2 expression. SOX2 expression was observed in 28.2% (11/39) of stage-1, 37.0% (10/27) of stage-2, and 53.8% (7/13) of stage-3 specimens. Previously, high SOX2 expression has been reported in lung cancer [17]. Our findings are consistent with these reports and further support that *SOX2* is a genuine driver gene in an advanced stage of the disease in patients with SCLC.

SOX2 high-expression results in the resistance of SCLCs to cisplatin treatment

Platinum agents including cisplatin and carboplatin, are widely used to treat patients with SCLC; however, some patients eventually develop resistance to this drug and continue to exhibit metastasis post treatment [38]. Cisplatin treatment for 24 h significantly suppressed the viability of H720, H209, and BEAS-2B cells (IC_{50} 52.7 μM, 43.7 μM, 7.1 μM, respectively), mildly repressed the viability of H1688 and H446 cells (IC_{50}, 106.1 μM, 137.6 μM, respectively), and marginally altered the viability of H146 and H1417 cells (IC_{50} 857.8 μM, 772.9 μM, respectively) in a dose-dependent manner (**Figure 2A**). After continuous administration for 48 and 72 h, cisplatin significantly reduced the viability of H1688, H720, H209, and BEAS-2B cells (IC_{50} 56.4 and 35.9 μM, 17.1 and 8.1 μM, 0.5 and 0.5 μM, and 0.1 and 0.1 μM, respectively) and mildly repressed the viability of H146, H1417, and H446 cells (IC_{50} μM, 462.7 and 52.1 μM, 268.8 and 139.7 μM, 10.7 and 39.0 μM, respectively) (**Figure 2B**, **2C**).

Because some SCLC cells are resistant to cisplatin, the expression of stemness-related markers in various SCLC cell lines was verified using western blotting. The expression of stemness-related markers in cisplatin-resistant SCLC cells was considerably higher than that in cisplatin-sensitive SCLC and normal cells (**Figure 2D**). Both SOX2 and c-Myc expression levels were higher in H146 and H446 cells than in H720, H1417, H1688, H209, and BEAS-2B cells. These results (**Figure 2**) suggested that

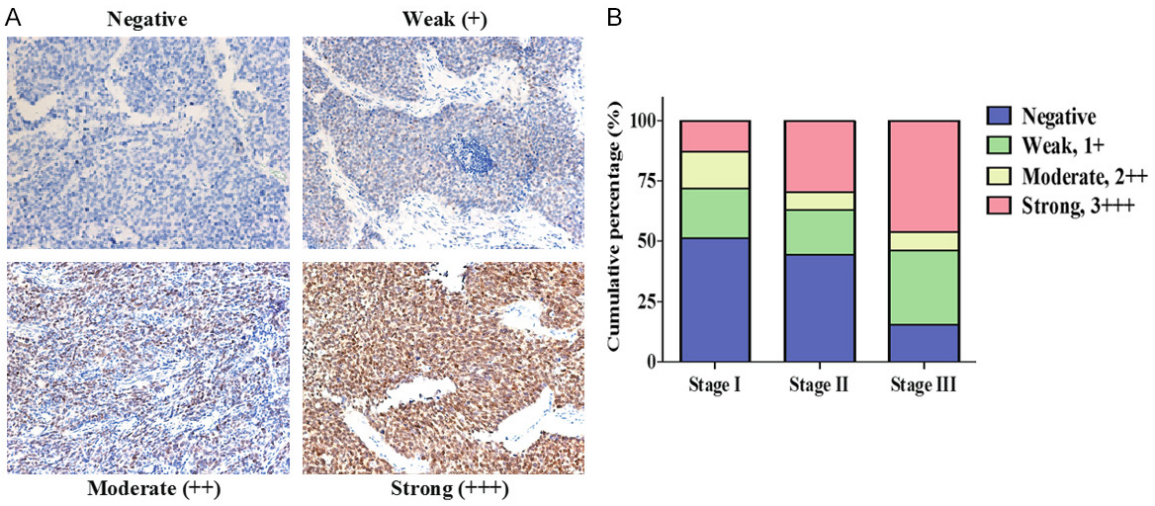

Figure 1. Assessment of protein expression and clinical significance of SOX2 in SCLC. A. Representative immunohistochemistry images of SCLC tissues. Scoring with respect to negative, weak (+), moderate (++), and strong (+++) positive expression. B. Comparison of SOX2 expression level in 79 patients with SCLC. SOX2 expression was low in stage I tissues but was elevated in stages II and III tissues. SOX2, sex determining region Y-box 2; SCLC, small cell lung cancer.

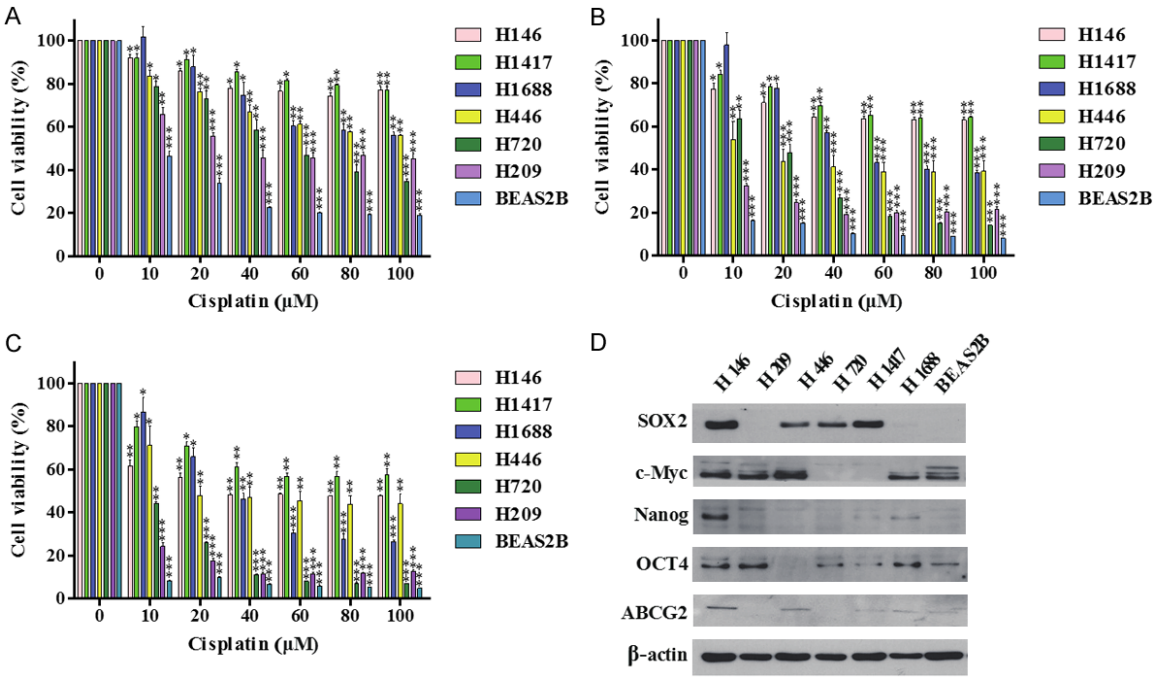

Figure 2. SOX2 overexpression enhanced the viability of cisplatin-resistant SCLC cell lines after treatment. A-C. Various SCLC cells were treated with cisplatin at different concentrations for 24, 48, and 72 h, and their viability was assayed using CCK8 reagent. Values are expressed in terms of cell viability [%]. D. Relative expression of stemness molecular markers was determined by western blotting analysis in various SCLC cells. Each value represents the mean ± SD obtained from quadruplicate assays. Similar results were obtained from more than three independent experiments. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with the control. SOX2, sex determining region Y-box 2; SCLC, small cell lung cancer.

SOX2 and c-Myc overexpression may play a critical role in SCLC cells, as they are associated with the induction of resistance to platinum-based drugs.

Napabucasin inhibits the viability of SCLC cells by suppressing SOX2 and c-Myc expression

A previous report showed that CSCs in SCLC contribute to the generation of drug-resistant, recurrent, and metastatic lung tumors by inducing the overexpression of stemness-related markers [39]; however, the effectiveness of chemotherapy in tumor growth inhibition in these patients in clinical settings remains poor. Napabucasin treatment for 24 h significantly suppressed the viability of H146, H209, and BEAS-2B cells (IC_{50} 1.3 μM, 0.5 μM, 0.9 μM, respectively) and mildly repressed the viability of H1417, H1688, H446, and H720 cells (IC_{50} 3.4 μM, 1.7 μM, 4.4 μM, 11.6, respectively) in a dose-dependent manner (**Figure 3A**). When administered continuously for 48 and 72 h, napabucasin significantly reduced the viability of H146, H1417, H1688, H446, H209, and BEAS-2B cells (IC_{50} 1.2 and 0.8 μM, 18.3 and 0.6 μM, 1.2 and 0.9 μM, 1.7 and 1.0 μM, 0.6 and 0.6 μM, 0.5 and 0.4 μM, respectively) and mildly repressed the viability of H720 cells (IC_{50} 5.4 and 3.2 μM, respectively) (**Figure 3B**, **3C**). Since napabucasin could significantly inhibit the viability of cisplatin-resistant SCLC cells, the expression of SOX2 and c-Myc in H146 and H446 cell lines was confirmed after napabucasin treatment for 48 h. SOX2 and c-Myc expression in H146 and H446 cells reduced significantly in a dose-dependent manner after napabucasin treatment (**Figure 3D**). Moreover, compared to that in the control group, the percentage of colony-forming H146 and H446 cells reduced significantly in the treatment groups (**Figure 3E**, **3F**). These data highlight the potential of napabucasin in suppressing SCLC cell survival and inhibiting cisplatin resistance.

Napabucasin induces the arrest of S-phase cell cycle and apoptosis in SCLC

Since napabucasin inhibited the viability of H146 and H446 cells by inhibiting SOX2 and c-Myc expression, the mechanism underlying the effect of napabucasin on cell cycle progression was investigated. Napabucasin induced S-phase cell cycle arrest in H146 and H446 cells after 24 h of administration (**Figure 4A**, **4B**). The functional mechanism underlying napabucasin-induced cell cycle arrest was further investigated. Among S-phase cell cycle-related proteins, cyclin E, cyclin A, CDK2, cdc2, and cdc25c were significantly downregulated in H146 and H446 cells after 24 h of napabucasin treatment (**Figure 4C**, **4D**). H146 and H446 cells had an increase in apoptosis significantly after treatment with napabucasin for 48 h, as revealed by staining with annexin-V and PI (**Figure 4E**, **4F**). Moreover, H146 cells treated with napabucasin were more sensitive than H446 cells. To evaluate the molecular mechanism underlying apoptosis, H146 and H446 cells were treated with napabucasin in a dose-dependent manner for 48 h. PARP cleavage increased and the expression of anti-apoptotic proteins, including Mcl-1 and survivin, decreased after H146 and H446 cells were treated with napabucasin at various doses for 48 h (**Figure 4G**, **4H**). These results indicate that SOX2 and c-Myc are involved in S-phase cell cycle arrest and induce apoptosis following the suppression of cisplatin-resistant SCLC cells. However, the mechanism underlying the interaction between stemness markers and cisplatin resistance in SCLC cells remains unclear.

Dysfunction in SOX2 expression attenuates napabucasin-inhibited cell viability, and restoration of SOX2 expression reverses the effect

Napabucasin significantly reduced SOX2 and c-Myc expression in H146 and H446 cells, based on which the molecular significance of SOX2 expression in SCLC cells altered by napabucasin treatment was further examined. SOX2 was overexpressed in H146 and H446 parental cells, but it was not expressed in H146 and H446 cells with dysfunctional SOX2 expression (**Figure 5A**, **5B**). To investigate the cytotoxic effect of napabucasin in SCLC cells with SOX2 dysfunction, we performed a dose-dependent analysis of cell viability. In contrast to parental cells, cells with dysfunctional SOX2 expression exhibited a reversal in viability after napabucasin treatment (**Figure 5C**, **5D**). We used a lentivirus-based SOX2-expressing vector (psox2) to restore SOX2 expression in cells with SOX2 expression dysfunction. SOX2 was ectopically expressed in these cells (**Figure 5E**, **5F**). The enforced SOX2 expression reversed the napabucasin-inhibited viability of cells with SOX2 dysfunction (**Figure 5G**, **5H**). Additionally, we also used lentivirus-expressing SOX2 shRNA to silence SOX2 expression in H146 and H446

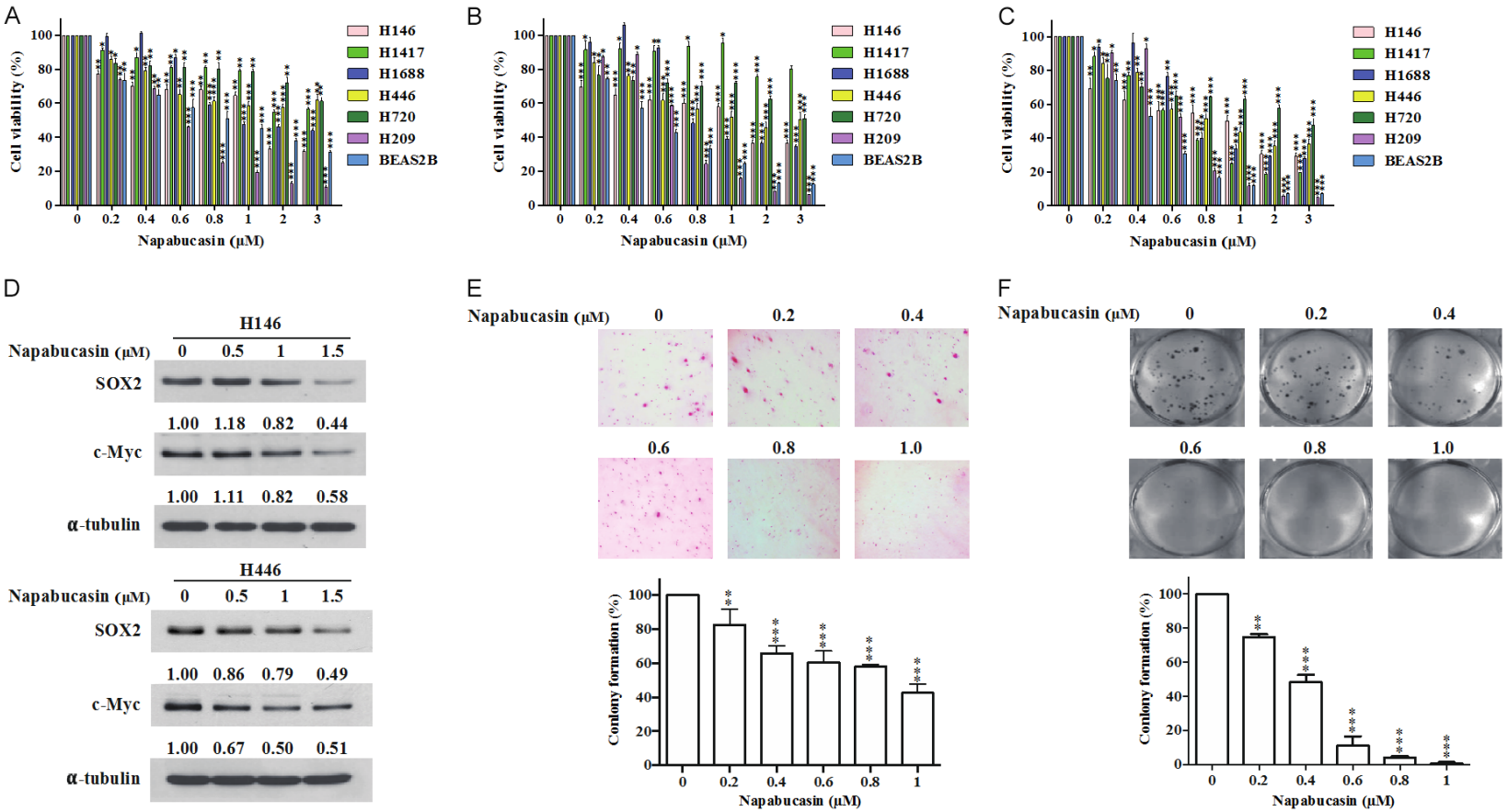

Figure 3. Napabucasin decreased the viability and clonogenicity of cisplatin-resistant SCLC cell lines. A-C. Various SCLC cells were treated with napabucasin at different concentrations for 24, 48, and 72 h, and their viability was assayed using CCK8 reagent. Values are expressed in terms of cell viability [%]. D. Napabucasin significantly suppressed SOX2 and c-Myc expression in H146 and H446 cells. E, F. A representative image of the results of the clonogenic cell survival assay. H146 and H446 cells were treated with napabucasin at various concentrations for 12 days (top panels). Napabucasin induced a significant reduction in the survival of H146 and H446 cells compared to that of untreated cells. Each value represents the mean ± SD obtained from quadruplicate assays. Similar results were obtained for more than three independent experiments. Statistical significance was defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with the control. SCLC, small cell lung cancer; SOX2, sex determining region Y-box 2.

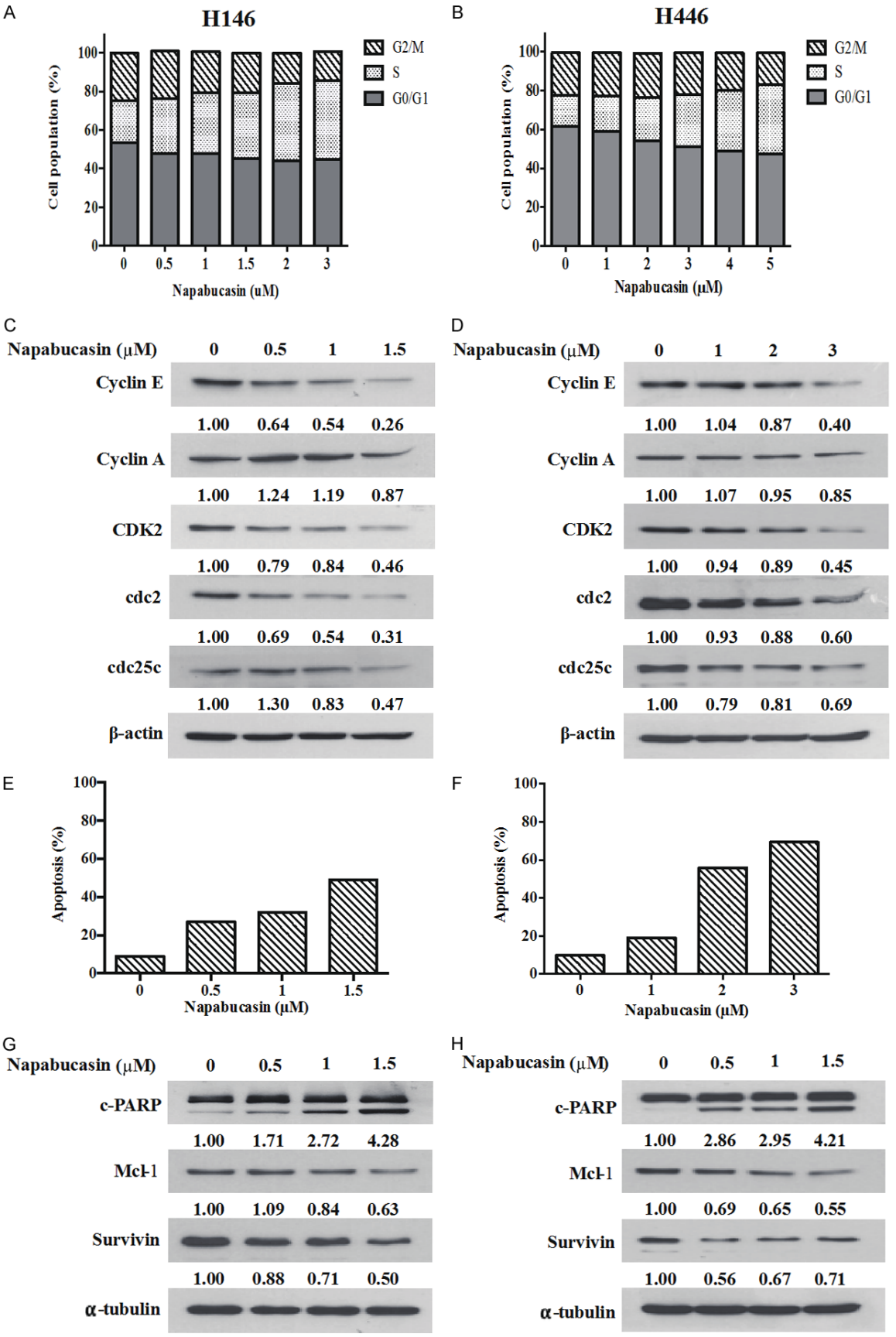
A
H146
Cell population (%)
G2/M
S
G0/G1
Napabucasin (uM)
B
H446
Napabucasin (μM)
C
Napabucasin (μM) 0 0.5 1 1.5
Cyclin E
1.00 0.64 0.54 0.26
Cyclin A
1.00 1.24 1.19 0.87
CDK2
1.00 0.79 0.84 0.46
cdc2
1.00 0.69 0.54 0.31
cdc25c
1.00 1.30 0.83 0.47
β-actin
D
Napabucasin (μM) 0 1 2 3
Cyclin E
1.00 1.04 0.87 0.40
Cyclin A
1.00 1.07 0.95 0.85
CDK2
1.00 0.94 0.89 0.45
cdc2
1.00 0.93 0.88 0.60
cdc25c
1.00 0.79 0.81 0.69
β-actin
E
Apoptosis (%)
Napabucasin (μM)
F
G
Napabucasin (μM) 0 0.5 1 1.5
c-PARP
1.00 1.71 2.72 4.28
Mcl-1
1.00 1.09 0.84 0.63
Survivin
1.00 0.88 0.71 0.50
α-tubulin
H
Napabucasin (μM) 0 0.5 1 1.5
c-PARP
1.00 2.86 2.95 4.21
Mcl-1
1.00 0.69 0.65 0.55
Survivin
1.00 0.56 0.67 0.71
α-tubulin

Figure 4. Napabucasin induced S-phase cell cycle arrest and apoptosis in cisplatin-resistant SCLC cells. A, B. Cell cycle distribution of H146 and H446 cells was assayed and quantified after napabucasin treatment for 24 h. Values are expressed in terms of cell population [%]. C, D. Napabucasin downregulated cyclin A, cyclin E, CDK2, cdc2, and cdc25c protein expression after 48 h of treatment. E, F. Napabucasin-induced apoptosis in H146 and H446 cells was assayed and quantified after 48 h of treatment. G, H. Napabucasin promoted the cleavage of PARP and downregulated the expression of the pro-survival protein Mcl-1 and survivin. SCLC, small cell lung cancer; SOX2, sex determining region Y-box 2.

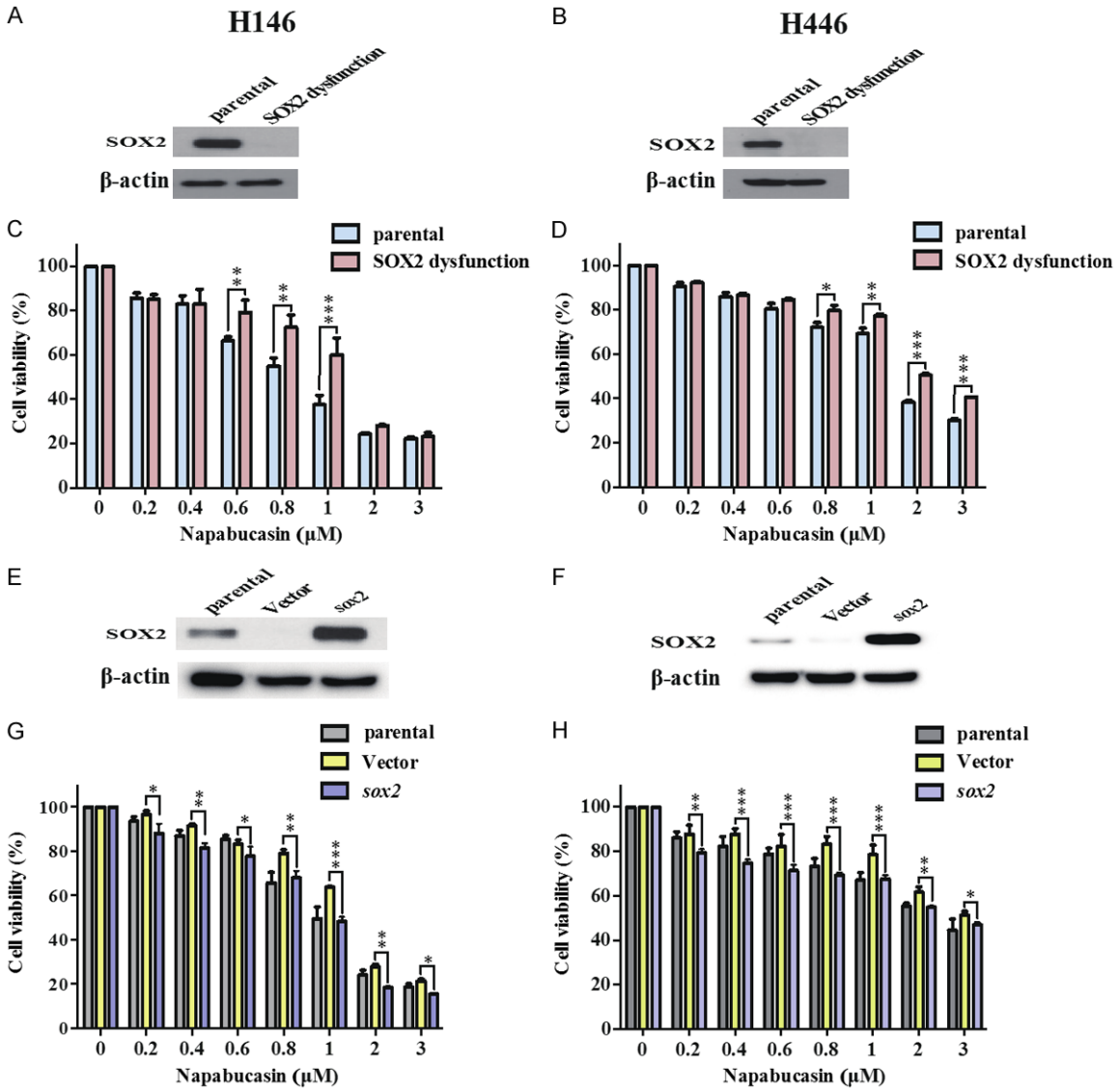

Figure 5. Napabucasin reduced the viability of cisplatin-resistant SCLC cells in a SOX2-expression-dependent manner. A, B. SOX2 protein was depleted in SCLC cells with SOX2 dysfunction. C, D. SOX2 depletion inhibited the reversal of napabucasin-mediated viability reduction in cells with SOX2 dysfunction. E, F. SOX2 protein expression was strongly upregulated upon the introduction of *sox2* into SCLC cells with SOX2 dysfunction. G, H. Enhancement of SOX2 expression helped reverse the napabucasin-mediated viability reduction in SCLC cells with SOX2 dysfunction. SOX2, sex determining region Y-box 2; SCLC, small cell lung cancer.

cells. Our results showed that *sox2* gene knockdown by shRNA significantly decreased the viability in SCLC cells for 72 h (Supplementary Figure 1), and the results were compatible with napabucasin treatment. The findings showed that SOX2 expression is related to the napabu-

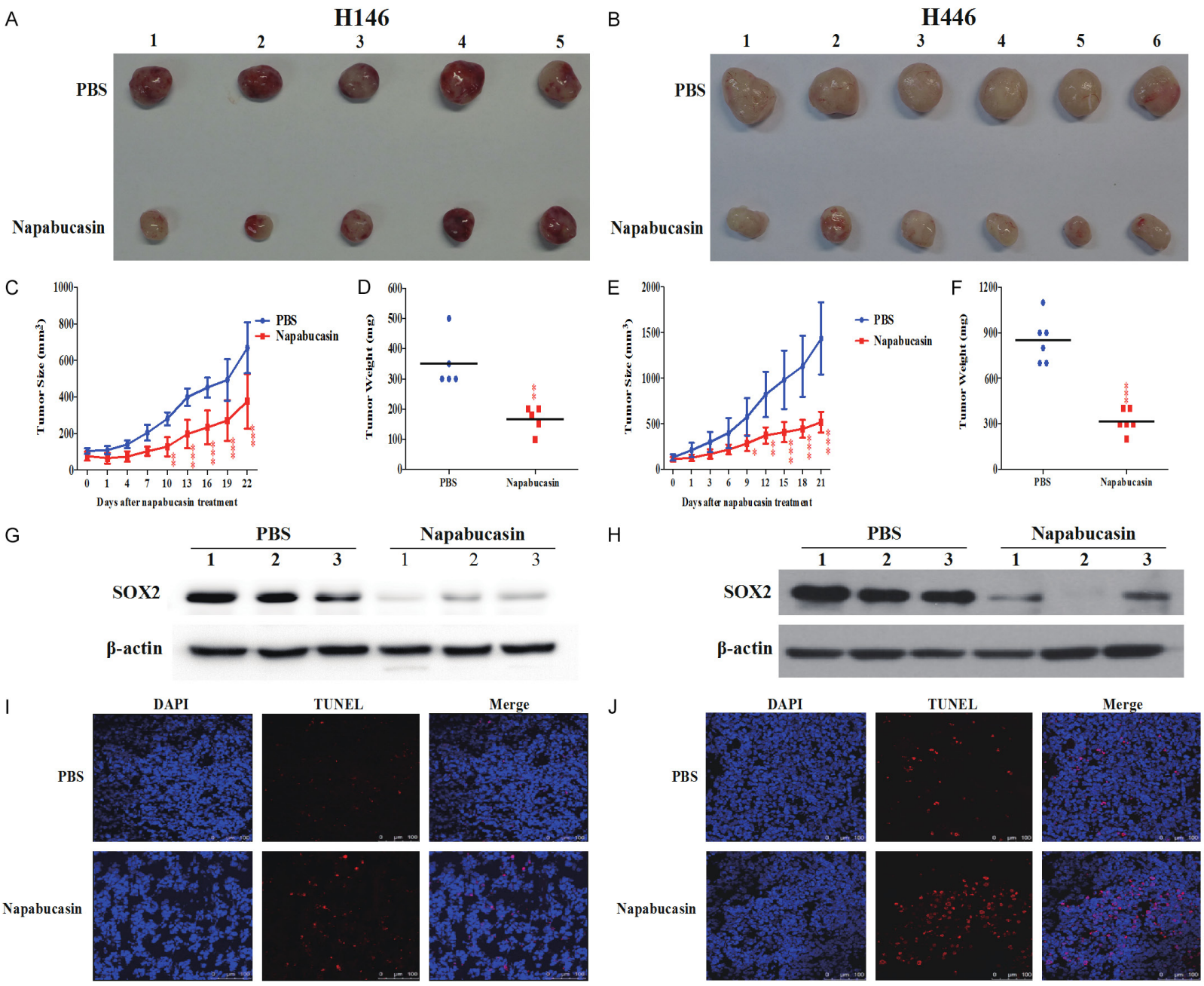
A
H146
1
2
3
4
5
PBS
Napabucasin
B
H446
1
2
3
4
5
6
PBS
Napabucasin
C
Tumor Size (mm³)
1000
800
600
400
200
0
0 1 4 7 10 13 16 19 22
Days after napabucasin treatment
PBS
Napabucasin
D
Tumor Weight (mg)
600
500
400
300
200
100
0
PBS
Napabucasin
E
Tumor Size (mm³)
2000
1500
1000
500
0
0 1 3 6 9 12 15 18 21
Days after napabucasin treatment
PBS
Napabucasin
F
Tumor Weight (mg)
1200
900
600
300
0
PBS
Napabucasin
G
PBS
Napabucasin
1 2 3 1 2 3
SOX2
β-actin
H
PBS
Napabucasin
1 2 3 1 2 3
SOX2
β-actin
I
DAPI
TUNEL
Merge
PBS
Napabucasin
J
DAPI
TUNEL
Merge
PBS
Napabucasin

Figure 6. Napabucasin significantly inhibited tumor growth in H146 and H446 SCLC xenograft models by decreasing SOX2 expression. H146- and H446-xenograft-bearing mice were treated either with PBS or napabucasin (20 mg/kg/day). A, B. Representative images of excised tumors from each group. C, D. Tumor volumes were measured to determine tumor growth. E, F. Tumor weights were compared on the last day of treatment. ***$P < 0.001$ compared with PBS-treated group. G, H. SOX2 expression levels in H146 and H446 tumors were evaluated by western blotting. I, J. Apoptosis in H146 and H446 tumors was measured by TUNEL staining. SCLC, small cell lung cancer; SOX2, sex determining region Y-box 2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

casin-induced inhibition of SCLC cell survival, implying that SOX2 gene expression in SCLC cells is important for chemotherapy (**Figure 5**).

Napabucasin significantly suppresses H146 and H446 xenograft tumor growth by inhibiting SOX2 in vivo

Since napabucasin significantly suppressed the viability and induced apoptosis in SCLC cells *in vitro*, the therapeutic efficacy of napabucasin in H146- and H446-xenograft tumor growth *in vivo* was further investigated. **Figure 6A** and **6B** depict H146- and H446-xenograft tumors, respectively. Napabucasin inhibited the growth of H146- and H446-xenograft tumors substantially, as indicated by the volume and weight of the tumors after 3 weeks of treatment (**Figure 6C-F**). To further evaluate the inhibition of SOX2 expression by napabucasin *in vivo*, SOX2 expression in H146- and H446-xenograft tumors was assessed using western blotting. Consistent with the results obtained *in vitro*, SOX2 expression was suppressed in response to napabucasin treatment compared with that in PBS-treated control tumors (**Figure 6G**, **6H**). Furthermore, in contrast to PBS, napabucasin induced significant apoptosis in H146- and H446-xenograft tumors (**Figure 6I**, **6J**). The results indicate that napabucasin was effective at blocking tumor growth in SCLC by suppressing SOX2 expression *in vivo*.

Discussion

SCLC is the most aggressive lung cancer and the leading cause of cancer-related deaths worldwide [2]. The prognosis of SCLC patients is extremely poor (5% of 5-year survival rate overall) [40]. Currently, the most effective SCLC chemotherapy involves the use of cisplatin and etoposide; however, in clinical settings, the efficacy of the treatment is hampered owing to the development of drug resistance. A previous report indicated that SP cells from SCLC, similar to CSCs, showed higher expression levels of stemness markers and tumorigenesis than did non-SP cells [9]. Moreover, in SCLC, CSCs with high stemness have been shown to cause resistance and directly induce cancer recurrence after first-line chemotherapy. Among stemness markers, SOX2 and c-Myc are major regulators of embryogenesis and neurogenesis in CSCs [41]. In the present study, SOX2 expression was observed in 58.3% (7/13) of stage-III specimens collected from patients with SCLC, which is consistent with findings from a previous study [42]. However, the underlying mechanisms and protective effects of stemness in cisplatin-resistant SCLC cells remain unknown.

In this study, we aimed to evaluate the possibility of using a stemness inhibitor against cisplatin-resistant SCLC cells and explore whether SOX2 dysfunction in SCLC induced desensitization to napabucasin treatment. Napabucasin, a novel small-molecule stemness/STAT3 inhibitor, has been shown to efficiently inhibit tumor growth, metastasis, and recurrence in various cancers. Napabucasin also suppressed spheroid formation by CSCs in liver cancer by inhibiting several stemness marker expressions, including SOX2, Nanog, and c-Myc [34]. However, our study is the first to show that some SCLC cell lines develop drug resistance after cisplatin treatment. Furthermore, the cisplatin-resistant SCLC cells exhibited stronger SOX2 and c-Myc expression than did cisplatin-sensitive SCLC cells. To better understand the detailed mechanisms involved, we treated the cisplatin-resistant SCLC cells with napabucasin and observed that napabucasin markedly inhibited cell proliferation and arrested cell cycle in the S-phase in a dose-dependent manner. We then used annexin-V/PI double-staining assays and western blotting to evaluate the programmed cell death mechanism following napabucasin treatment; the results indicated that napabucasin significantly promoted apoptosis and increased the levels of cleaved PARP. Additionally, the absence of SOX2 expression in SCLC cells with SOX2 dysfunction significantly attenuated the efficiency of napabucasin treatment in a concentration-dependent manner.

These findings indicate the correlation between stemness marker downregulation and cell survival in cisplatin-resistant SCLC cells, consistent with the findings of a previous study, in which napabucasin inhibited proliferation and induced apoptosis in NSCLC CisR sublines [30].

Napabucasin treatment strongly decreased SOX2 expression in cisplatin-resistant SCLC cells, but the action mechanism remains unclear. Several studies have shown that SOX2 expresses at high levels in various tumors, such as SCLC [5], NSCLC [43], and breast cancer [44]. Additionally, SOX2 is a high-mobility transcription factor which is crucial for maintaining the unique characteristics of the CSC subpopulation in SCLC [39]. Bareiss *et al*. reported that ectopic expression of SOX2 could restore drug resistance in SOX2-knockdown ovarian cancer cells [45]. Our results further showed that SOX2 expression in SCLC suspension cells was higher than that in SCLC adhesion cells, and its levels were congruent with the levels of cisplatin resistance. This indicated that SOX2 overexpression was closely associated with drug resistance and tumor recurrence in SCLC cells. An earlier study reported that overexpression of SOX2 in SCLC can result in drug resistance *via* the downregulation of a cancer-related miRNA, has-miR-340-5p [46]. Rudin *et al.* showed that SOX2 knockdown using doxycycline-inducible shRNA effectively suppressed SOX2 protein levels and inhibited H446 and H720 cell proliferation [5]. In this study, we mainly showed that the dysfunctional SOX2 expression in SCLC cells is associated with interference in the napabucasin-mediated reduction of cell viability. Our data indicated that dysfunction in SOX2 expression in SCLC cells (sorted by flow cytometry) could induce resistance to napabucasin treatment. Furthermore, the induction of ectopic SOX2 expression in the cells with SOX2 expression dysfunction restored their napabucasin sensitivity. Together, these indicated that the presence or absence of SOX2 is potentially related to the regulation of napabucasin-mediated survival of cisplatin-resistant SCLC cells. To our knowledge, we were the first to present these findings by using Hoechst 33342 dye to sort and collect the SOX2 dysfunctional cells by flow cytometry in our experimental design. The aforementioned results indicate that SOX2 is involved in the regulation of napabucasin-mediated survival of cisplatin-resistant SCLC cells; therefore, the mechanisms by which napabucasin targets drug resistance may involve directly targeting the SOX2/survival signaling pathway.

In a previous study [47], STAT3 was shown to serve as a transcriptional factor that promotes cell proliferation and the cell cycle, suppresses apoptosis, and generates chemoresistance in several human cancers. *STAT3* silencing by siRNA or inhibitor treatment was shown to cause downregulation of downstream genes, including those encoding SOX2, c-Myc, Mcl-1, and survivin, in lung cancer [48-50]. A pervious study had showed that MYC activates Notch signaling pathway to promote tumor progression [51]. Other previous studies demonstrated that SCLC patients with high Myc expression had shorter survival than those with low Myc expression, and high Myc expression SCLC cells are more aggressive and resistant to drugs in phenotypes [52-54]. Together, Myc had been suggested a potential therapeutic target in SCLC. Our results showed that napabucasin decreased c-Myc expression in SCLC cells, and suggested that napabucasin is a potential drug for the treatment of SCLC. Survivin is one of the members in apoptosis inhibitor family proteins acting as an apoptosis suppressor and crucial for promoting tumor initiation, cancer progression, and drug resistance [26]. In our previous study, we showed that survivin knockdown may enhance the radiosensitivity of lung cancer cells [28]. Chen *et al.* showed that the silencing of SOX2 leads to the apoptosis of NSCLC cells *via* the activation of MAP4K4 and the consequent loss of survivin expression [20]. Moreover, Feng *et al.* indicated that the SOX2/survivin anti-apoptotic pathway could protect neural stem cells from apoptosis induction [55]. Additionally, Mcl-1, a member of the Bcl-2 family, can increase cell survival and suppress apoptosis by interfering with the release of cytochrome C from mitochondria into the cytosol [56]. The siRNA-mediated knockdown of c-Myc directly decreased the expression of Mcl-1 at the protein and transcript levels in gastric cancer cells [57]. Our results showed that SOX2, c-Myc, Mcl-1, and survivin were downregulated in a dose-dependent manner in response to napabucasin treatment. These findings implied that downexpression of SOX2 and c-Myc directly suppresses cell viability and triggers cell growth arrest and

apoptosis by regulating the anti-apoptotic pathways following napabucasin treatment.

In summary, we clearly demonstrated that napabucasin mediated the downregulation of the stemness markers SOX2 and c-Myc to significantly inhibit cell proliferation and induce the arrest of S-phase cell cycle and apoptosis in cisplatin-resistant SCLC cells. Furthermore, napabucasin showed therapeutic potential in SCLC xenograft tumors *in vivo*. These results indicate the potential of napabucasin for the treatment of cisplatin-resistant SCLC in clinical settings.

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Disclosure of conflict of interest

None.

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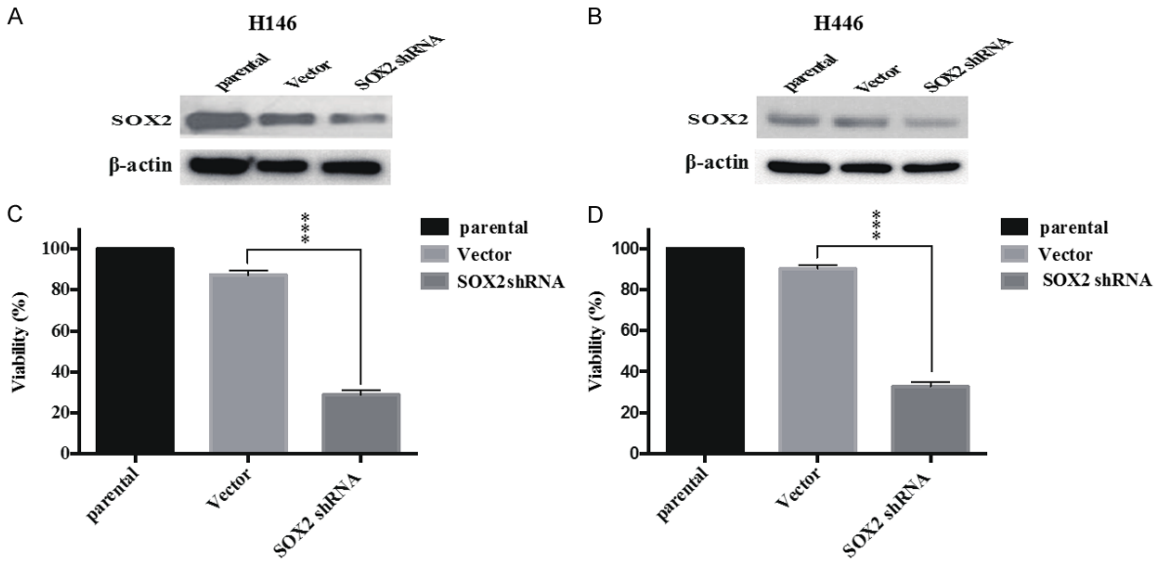

Supplementary Figure 1. Silencing SOX2 expression suppressed the viability of cisplatin-resistant SCLC cells. A and B. SOX2 protein decreased in cisplatin-resistant SCLC cells by lentivirus-expressing SOX2 shRNA infection for 48 h. C and D. Knockdown of SOX2 by shRNA significantly inhibited cell viability in cisplatin-resistant SCLC cells for 72 h. ***P < 0.001 compared with the Vector.