SOX8 regulates cancer stem-like properties and cisplatin-induced EMT in tongue squamous cell carcinoma by acting on the Wnt/β-catenin pathway

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A sub-population of chemoresistant cells exhibits biological properties similar to cancer stem cells (CSCs), and these cells are believed to be a main cause for tumor relapse and metastasis. In our study, we explored the role of SOX8 and its molecular mechanism in the regulation of the stemness properties and the epithelial mesenchymal transition (EMT) of cisplatin-resistant tongue squamous cell carcinoma (TSCC) cells. We found that SOX8 was upregulated in cisplatin-resistant TSCC cells, which displayed CSC-like properties and exhibited EMT. SOX8 was also overexpressed in chemoresistant patients with TSCC and was associated with higher lymph node metastasis, advanced tumor stage and shorter overall survival. Stable knockdown of SOX8 in cisplatin-resistant TSCC cells inhibited chemoresistance, tumorsphere formation, and EMT. The Wnt/β-catenin pathway mediated the cancer stem-like properties in cisplatin-resistant TSCC cells. Further studies showed that the transfection of active β-catenin in SOX8 stable-knockdown cells partly rescued the SOX8 silencing-induced repression of stem-like features and chemoresistance. Through chromatin immunoprecipitation and luciferase assays, we observed that SOX8 bound to the promoter region of Frizzled-7 (FZD7) and induced the FZD7-mediated activation of the Wnt/β-catenin pathway. In summary, SOX8 confers chemoresistance and stemness properties and mediates EMT processes in chemoresistant TSCC via the FZD7-mediated Wnt/β-catenin pathway.

Tongue squamous cell carcinoma (TSCC) is a common oral cancer with a high rate of regional recurrence and lymphoid metastasis and a propensity to cause disfigurement and functional defects.1 Aggressive chemotherapy with cisplatin has greatly improved the 5-year survival rates of advanced TSCC patients, but a large proportion of TSCC patients...
eventually develop tumor relapse and become resistant to chemotherapy.\textsuperscript{2,3} Although the mechanism of acquired chemoresistance in TSCC patients remains unclear, increasing evidence suggests that drug-resistant cancer cells acquire features of cancer stem cells (CSCs) and undergo epithelial-mesenchymal transition (EMT).\textsuperscript{1–6} Complete Twist1 depletion reverses Adriamycin-induced EMT and invasiveness in breast cancer.\textsuperscript{7} In addition, cisplatin-selected bladder cancer cells displayed a strong self-renewal capacity and EMT characteristics.\textsuperscript{8} Therefore, chemotherapy-induced cancer stem-like properties and EMT in tumor cells are closely related to chemotherapy resistance, and examining the mechanisms that regulate chemotherapy-induced cancer stem-like features and EMT is necessary for the development of novel therapies.

The Sry-like high-mobility group box (SOX) genes regulate different aspects of development, and the complex participation of the SOX family in various aspects of oncology has attracted increasing attention.\textsuperscript{9} Accumulating evidence has indicated that SOX genes play an important role in drug resistance and CSCs or EMT. For example, SOX4 is a tumor promoter that contributes to drug resistance and progression in cervical cancer and regulates the EMT programme in breast cancer.\textsuperscript{10,11} More interestingly, SOX4 is also highly expressed during metastasis and is specifically associated with lymph node metastasis in TSCC.\textsuperscript{12} Conversely, the overexpression of SOX9 promotes self-renewal properties and in vivo tumorigenicity by facilitating symmetrical cell division in liver cancer.\textsuperscript{13} Moreover, the upregulation of SOX9 endows cancer cells with stemness features through the activation of the Wnt/β-catenin signalling pathway, and constitutive activation of the Wnt/β-catenin pathway in TSCC is essential for the maintenance of CSC self-renewal and the promotion of chemoresistance.\textsuperscript{14,15} However, whether SOX genes are responsible for conferring stem cell-like properties or EMT of chemoresistant TSCC remains unknown.

Here, we searched for SOX genes that were differentially expressed between two cisplatin-resistant cells and their parental TSCC cells, and we identified that SOX8 was significantly upregulated. Next, we investigated the role of SOX8 and its relationship with the Wnt/β-catenin pathway in regulating chemotherapy-induced stem-like features and EMT in TSCC cells and evaluated the function of SOX8 expression on the tumor growth and apoptosis of chemoresistant TSCC xenografts. Finally, we analyzed the correlation between SOX8 expression with clinicopathological status and survival outcomes in TSCC patients.

**Material and Methods**

**Patients and tissue specimens**

TSCC specimens (\( n = 103 \)) were collected from three independent centres, including Sun Yat-sen Memorial Hospital (\( n = 52 \)), West China Hospital (\( n = 18 \)), and the Affiliated Hospital of North Sichuan Medical College (\( n = 33 \)), between 2006 and 2012. Patient responses were classified as cisplatin-sensitive or cisplatin-nonsensitive according to previous studies.\textsuperscript{16} The tumor samples were examined by two independent pathologists, and tumor grade was defined according to WHO criteria (2004).\textsuperscript{16} Our study was approved by the ethics boards of the three hospitals, and all patients provided informed consent for participation.

**Cell culture and drug treatment**

The CAL27 and SCC9 cell lines were obtained from the American Type Culture Collection. The stable cisplatin-resistant cell lines, CAL27-res and SCC9-res, were established by clonal selection of CAL27 or SCC9 treated with cisplatin (Sigma, Carlsbad, CA) from \( 10^{-7} \) M to \( 10^{-5} \) M as described.\textsuperscript{17,18} The CAL27 and CAL27-res cells were cultured in DMEM (Gibco, Rockville, MD) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA). The SCC9 and SCC9-res cells were cultured in DMEM-F12 (Gibco, Rockville, MD) supplemented with 10% foetal bovine serum and 400 ng/mL hydrocortisone (Sigma-Aldrich, USA). Cisplatin (5 \( \mu \)M in CAL27 cells and 10 \( \mu \)M in SCC9 cells) is routinely added to the culture medium every other day and is removed prior to the experiments being performed.

**Immunohistochemistry**

Paraffin sections were dehydrated with xylene, rehydrated in descending concentrations of ethanol. Endogenous peroxidase was inhibited with 3% \( \text{H}_2\text{O}_2 \), and the slides were incubated with primary antibodies overnight at 4°C. Positive cells in five 200-μm fields were counted in each section under a microscope.

**RNA interference**

To establish SOX8 and β-catenin knockdown TSCC cells, recombinant lentivirus was generated by co-transfecting 293FT cells with shRNA from the lentiviral vector pLV3/H1/
GFP + Puro Vector (GenePharma, Shanghai, China) and packaging the plasmids pGag/Pol, pRev, and pVSV-G(GenePharma) using Lipofectamine 2000 (Life Technologies, USA). The shRNA sequences were listed in Supporting Information Table 2. Then we infect CAL27-res and SCC9-res cells with collected viral supernatant and selected with puromycin (Sigma-Aldrich). The β-catenin retroviral plasmids are a kind gift from Dr. Song (Sun Yat-sen University). For retroviral transfections, the constructs and packing vectors were co-transfected into Phx cells using Lipofectamine 2000. Target cells were transduced as described above. pCDNA3.1-FZD7, pCDNA3.1-SOX8, and pCDNA3.1-vector plasmids were obtained from GenePharma.

**Tumorsphere formation assay**

A total of 1000 cells were seeded into ultra-low-attachment 6-well plates (Corning, USA) and grown in DMEM/F12 medium ( Gibco, Rockville, MD) supplemented with 20 ng/mL EGF, 20 ng/mL bFGF (PeproTech, USA), and B27 (Invitrogen, Carlsbad, CA) for 14 days. Tumor colonies containing ≥20 cells were counted. To test the secondary capacity for tumorsphere formation, primary tumorspheres were dissociated to a single-cell suspension and resuspended in DMEM/F12 containing the above supplements and then cultured in ultra-low-attachment plates.

**Real time quantitative RT-PCR**

For real time quantitative RT-PCR, total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and then treated with RNase-free DNase (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Total RNA was converted to cDNA using a M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Then, real-time PCR analyses were carried out in triplicate for each sample using a standard LC480 SYBR PCR kit (Roche, Indianapolis, IN) on a LightCycler 480 (Roche). The primers listed in Supporting Information Table 3 were used for PCR amplification.

**Western blot analysis**

Total protein was extracted from tissue and cell samples using RIPA lysis buffer (Beyotime) supplemented with protease inhibitor mixture (Sigma-Aldrich). An equal amount of each protein sample was loaded onto a 10% SDS-PAGE gel for electrophoresis, transferred onto a PVDF membrane (Millipore Corporation, Bedford, MA), blocked with 5% (v/v) skim milk at RT for 1 hr and then incubated with primary specific antibodies overnight (Supporting Information Table 4). Peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Proteintech, USA) was used as a secondary antibody. Finally, the antigen-antibody reaction was visualized using enhanced chemiluminescence reagent (ECL, Thermo, Rockford).

**Flow cytometry assays**

To assay apoptosis, TSCC cells were treated with cisplatin (5 μM) for 24 hrs and then examined using an Annexin V-FITC Apoptosis Detection Kit I (556547, BD Pharmingen™) according to the manufacturer’s protocol. To analyse surface markers, 1 × 10⁵ cells were dissociated into a single-cell suspension and incubated with anti-CD44 antibody conjugated to FITC and anti-CD24 antibody conjugated to PE at 4°C in dark for 30 min (BD BioSciences). The cell suspension was washed with PBS to remove excess antibody, and data were collected and analysed on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ).

**Cell growth/survival assays**

Viable cells were measured via MTS assay as recommended by the manufacturer (Promega, Tokyo, Japan). Briefly, cells were cultured in a 96-well plate overnight at a concentration of 2,000 cells/mL per well and treated with the indicated concentrations of cisplatin (2, 4, 6, 8, and 10 μM) for 24 hrs. Then, 20 μL of MTS solution was added to each well, followed by a 1 hr incubation at 37°C. The reaction was quantitatively measured in a Microplate Reader (BioTek, Winooski, VT) at a wavelength of 490 nm. To assay cell growth, 2,000 cells/mL per well were measured using an MTS assay after 6 days culture as described above. To assay clonogenicity, 1,000 cells were seeded per well in a 6-well plate and then fixed and stained with 0.5% crystal violet solution after 7 days of culture. Colonies with a diameter >50 μm were counted.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) experiment was performed according to the manufacturer’s protocol (ChIP assay Kit, Millipore). The anti-Sox8 antibodies were used as below: Ab-1, sc-374445; Ab-2, sc-374446 (Santa Cruz Biotechnology) and normal mouse IgG (sc-2343, Santa Cruz). The pull-down purified DNA was amplified by RT-PCR.

**Boyden chamber assays**

For boyden chamber assays, 1 × 10⁵ cells in serum-free medium were seeded into the upper inserts of 24-well Boyden chambers (Corning, New York, NY) with (for invasion) or without (for migration) Matrigel (R&D, USA). DMEM supplemented with 10% FBS was added to the lower chambers. Cell mobility was detected after 36 hrs (for migration) or 48 hrs (for invasion) for CAL27 and CAL27-res cells and after 16 hrs (for migration) or 24 hrs (for invasion) for SCC9 and SCC9-res cells. The migrated and invadec cells were stained with 0.1% crystal violet and counted in five random fields.

**TCF/LEF reporter assay**

Activation of Wnt/β-catenin signalling was detected using a TCF reporter luciferase assay. Cells were transfected with a TCF reporter vector (TOPflash) (Millipore, Billerca, MA) or a Renilla luciferase reporter vector (pRL-TK) (40:1) using Lipofectamine 2000 (Invitrogen). TCF and Renilla luciferase activities were measured using a dual-luciferase reporter assay kit (BMG Labtech GmbH, Germany) 48 hrs after transfection.
The relative TOP/FOP activity (%) was calculated to show changes in Wnt/β-catenin activation.

In vivo subcutaneous xenograft inoculation
To perform the limited dilution assay, 5 × 10^3, 1 × 10^4, or 1 × 10^5 cells were subcutaneously injected into nude mice. Xenografts, lung, and liver were harvested and counted after 28 days (1 × 10^5 cells), 60 days (1 × 10^4 cells), or 70 days (5 × 10^3 cells). To assay the xenografts following cisplatin treatment, CAL27-res cells stably expressing shSOX8, shSOX8 CAL27-res+β-catenin or empty vector were subcutaneously injected (1 × 10^5 cells/mouse) into nude mice. When a xenograft reached 2 mm in diameter and was palpable, cisplatin (5 mg/kg) was administered via intraperitoneal injection every 3 days from Day 8 to 32. Tumor volume was calculated according to the following formula: TV (mm^3) = length × width^2 × 0.5. On Day 36, the xenografts were carefully removed, embedded in paraffin and analyzed via immunohistochemistry and TUNEL assay.

Statistical analysis
Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL). All data are expressed as the group mean ± standard deviation (SD). The χ^2 test was used to analyse relationships between related proteins. Kaplan-Meier survival curves were plotted, and the log-rank test was used. All experiments were performed at least three times. The results of the experiments are expressed as the mean± SD. p < 0.05 was considered statistically significant.

Results
Cisplatin resistant cells CAL27-res and SCC9-res display characteristics of cancer stem-like cells and have undergone EMT
We continuously treated CAL27 and SCC9 cells with 10^-7 M to 10^-5 M cisplatin to establish cisplatin-resistant cells as previously reported. CAL27-res and SCC9-res cells were resistant to clinically relevant doses of cisplatin (1–10 μM) and more anti-apoptotic than their parental cells after cisplatin treatment (Supporting Information Figs. 1a and 1b). CAL27-res and SCC9-res cells exhibited a decreased expression of the epithelial marker E-cadherin, but the mesenchymal markers vimentin and N-cadherin exhibited increased mRNA and protein levels (Supporting Information Figs. 2a and 2b; p < 0.01). Additionally, CAL27-res and SCC9-res cells acquired stronger invasiveness and migratory capacity examined by chamber assay, indicating that cisplatin-resistant cells had undergone EMT (Supporting Information Figs. 2c and 2d; p < 0.01). Next, we cultured cisplatin-resistant TSCC cells and their parental cells in sphere culture medium (SCM) to examine their self-renewal capacity. The cisplatin-resistant cells formed more spherical colonies than their parental cells from primary to tertiary spheres, demonstrating the self-renewing potential of cisplatin-resistant cells was maintained in vitro (Supporting Information Fig. 3a). Moreover, the mRNA and protein levels of stemness-associated genes, including BMI1, SOX2, OCT4, and ABCG2, were upregulated in CAL27-res and SCC9-res cells (Supporting Information Figs. 3b and 3c). It has been reported that CD44+/CD24− cells possess CSC-like features in oral squamous cell carcinoma. Our study showed the proportion of CD44 + CD24− cells in CAL27-res and SCC9-res cells (24.5 and 26%) was higher than that in CAL27 and SCC9 cells (10.9 and 13.3%) by flow cytometric analysis (Supporting Information Fig. 3d). In general, TSCC cells acquire cancer stem-like properties and features of EMT after cisplatin application.

The expression of SOX8 is upregulated in TSCC with cisplatin-resistance and correlated with poor prognosis
To explore the roles of the SOX family in cisplatin-resistant TSCC cells, we measured the mRNA levels of SOX family genes in cisplatin-resistant cells and their parental cells. SOX2, SOX8, and SOX9 were significantly upregulated in CAL27-res and SCC9-res cells compared to their parental cells (fold change >2, p < 0.01), but no significant differences were found in the mRNA expression levels of the other SOX family genes (Fig. 1a). We found that mRNA and protein levels of SOX8, which showed the most significant differences between cisplatin-resistant cells and the parental cells, were increased in both CAL27-res and SCC9-res cells (Fig. 1b). Then, we analyzed the clinical significance of SOX8 expression in chemosensitive and chemo-insensitive TSCC specimens to determine its clinical significance. SOX8 was mainly localized in TSCC nuclei and was significantly upregulated in TSCC samples, while it was virtually absent in normal oral epithelium samples. Interestingly, we found a higher number of SOX8-positive cells in chemo-insensitive patients than in chemosensitive specimens, and this number was significantly higher than that found in normal oral tissues (Fig. 1c, p < 0.01). In addition, statistical analysis showed high SOX8 expression was positively associated with high tumor metastasis rate and reduced sensitivity to cisplatin, but no significant associations were found between SOX8 expression and age, sex, or clinical stage (Table 1). Kaplan-Meier analysis indicated that high SOX8 expression in TSCC patients was significantly associated with a reduced overall survival rate (Fig. 1d). Collectively, these data indicate that SOX8 is not only overexpressed in chemo-insensitive TSCC patients but also correlated with a poor prognosis, indicating that SOX8 may have an important function in TSCC chemoresistance.

SOX8 knockdown reduces cisplatin-induced stemness properties and EMT in cisplatin-resistant TSCC cells
To investigate the significance of SOX8 in cisplatin-resistant TSCC cells, we first designed three shRNAs targeting SOX8 using a lentiviral-based approach. The shSOX8#3 construct caused an obvious reduction of SOX8 in CAL27-res and SCC9-res cells (Figs. 2a and 2b) and was therefore used to generate stable cell lines. Then, we examined whether SOX8 knockdown in cisplatin-resistant tongue cancer cells inhibited CSC-like
features. SOX8 knockdown in CAL27-res and SCC9-res cells significantly reduced the expression of the stem cell transcription factors SOX2, OCT4, and BMI1 as well as ABCG2 (Figs. 2c and 2d; \( p < 0.01 \)) and prevented cisplatin-induced cell death (Fig. 2e; \( p < 0.001 \)). Furthermore, SOX8 knockdown significantly decreased tumorsphere-formation efficiency (Fig. 2f; \( p < 0.001 \)) and the proportions of CD44+CD24− cells in CAL27-res cells (from 24.2% to 12.9%) and SCC9-res cells (from 26.1% to 14%), which suggested SOX8 downregulation repressed self-renewal capacity in vitro (Fig. 2g; \( p < 0.05 \)).

We next investigated whether SOX8 knockdown may reverse the mesenchymal features and cell growth in chemoresistant TSCC cells. MTS and colony-formation assays both indicated that CAL27-res and SCC9-res cells with stable shRNA-mediated SOX8 knockdown showed a significantly lower capacity for proliferation than the control cells (Figs. 3a and 3b; \( p < 0.01 \)). Meanwhile, SOX8 knockdown in cisplatin-resistant cells inhibited migration and invasion and reversed the mesenchymal markers, as indicated by the downregulation of vimentin and \( N \)-cadherin and the upregulation of \( E \)-cadherin (Figs. 3c and...
Table 1. Correlation among clinicopathologic status and the expression of SOX8 in TSCC patients

| Characteristic       | SOX8 (%)                   |
|----------------------|-----------------------------|
|                      | No. of high expression | No. of low/-expression |
| Sex                  |                            |
| Male                 | 24(41.4)                   | 34(58.6)                |
| Female               | 18(40.0)                   | 27(60.0)                |
| Age                  |                            |
| <50                  | 18(47.4)                   | 20(52.6)                |
| ≥50                  | 24(36.9)                   | 41(63.1)                |
| Node metastasis      |                            |
| N0                   | 12(25.5)                   | 35(74.5)                |
| N+                   | 30(53.6)                   | 26(46.4)                |
| Clinical stage       |                            |
| III                  | 19(41.3)                   | 27(58.7)                |
| IV                   | 23(40.4)                   | 34(59.6)                |
| Status               |                            |
| Survival             | 2(10.0)                    | 18(90.0)                |
| Death                | 40(48.2)                   | 43(51.8)                |
| Cisplatin            |                            |
| Sensitive            | 10(30.3)                   | 23(69.7)                |
| Non-sensitive        | 32(45.7)                   | 38(54.3)                |

3df). Conversely, SOX8 overexpression in parental cells promoted mesenchymal features and the capacity of migration and invasion (Figs. 3e and 3f). Together, these data indicate that SOX8 knockdown inhibited cisplatin resistance in CAL27-res and SCC9-res cells, possibly by suppressing the adoption of a CSC-like and mesenchymal phenotype in these cells.

The Wnt/β-catenin pathway regulates cisplatin-resistant tongue CSCs

As the Wnt/β-catenin pathway regulates TSCC cell proliferation and differentiation, we explored whether the increase in the TSCC CSC population that results from cisplatin pressure is regulated by Wnt/β-catenin signaling. We first detected the expression of two key targets of Wnt/β-catenin signaling, p-GSK3β and β-catenin. We observed an upregulation of p-GSK3β and β-catenin in cisplatin-resistant TSCC cells, whereas the total GSK3β level remained unchanged (Fig. 4a).

A TOP/FOP Flash reporter assay also showed Wnt pathway in CAL27-res and SCC9-res cells was transactivated (Fig. 4b).

We next designed two shRNAs targeting β-catenin to establish stable cell lines. As shown in Figures 4c and 4d, knockdown of β-catenin resulted in a decreased expression of β-catenin and c-MYC, but an increased expression of Dkk1, in CAL27-res and SCC9-res cells (p < 0.01). β-catenin knockdown led to reduced TOP/FOP luciferase activity in cisplatin-resistant cells (Fig. 4e).

Moreover, we observed that knockdown of β-catenin in cisplatin-resistant cells enhanced their sensitivity to cisplatin (Fig. 4f, p < 0.01). Knockdown of β-catenin in cisplatin-resistant cells inhibited tumoursphere formation efficiency in vitro and decreased the tumourformation rate in nude mice compared to the control group (Fig. 4g, p < 0.01, Supporting Information Table 5). The above results suggest that Wnt/β-catenin pathway activation mediates cisplatin resistance in TSCC CSCs.

**Regulatory interaction between SOX8 and the Wnt/β-catenin signaling pathway**

SOX8 is a regulator of the Wnt/β-catenin signaling pathway,21 and consistent with this information, we observed reduced β-catenin staining in both CAL27-res and SCC9-res shSOX8 cells compared to their corresponding parental cells. A decrease of C-myc levels and an increase of Dkk1 levels were observed in chemoresistant cells infected shSOX8 compared to uninfected cells (Supporting Information Figs. 4a and 4b; p < 0.01). In addition, silencing of SOX8 decreased Wnt/β-catenin pathway activation according to the results of a TOP/FOP Flash reporter assay (Supporting Information Fig. 4c). To further clarify the above findings, we performed rescue experiments by stably expressing constitutively active β-catenin plasmids in SOX8-knockdown cells. As shown in Supporting Information Figures 4d and 4e, β-catenin expression increased in the SOX8 knockdown cells after transfection with active β-catenin plasmids. Additionally, there was increased TOP/FOPFlash reporter activity in SOX8-silenced clones (CAL27-res and SCC9-res) following the forced expression of β-catenin (Supporting Information Fig. 4f, p < 0.01). However, the forced expression of β-catenin only partially rescued the effect of SOX8 silencing on tumoursphere formation in vitro, indicating a rescue of self-renewal ability even if SOX8 knockdown (Supporting Information Fig. 4g).

Based on these results, we conclude that the Wnt/β-catenin pathway mediates the downstream effects of SOX8 on chemoresistance in TSCC.

**SOX8 promotes the Wnt/β-catenin pathway by binding to the promoter of Frizzled-7**

Based on our findings, we further explored the possible role of the SOX8-mediated Wnt/β-catenin pathway. Previous studies have reported that SOX proteins can transactivate the FZD family (key receptors in Wnt signaling),14,22,23 we attempted to examine the relationship between SOX8 and the FZD family. We found FZD7 mRNA levels in SOX8 stable-knockdown cells were reduced by 82% (p < 0.001) among all FZD family members (Fig. 5a). Furthermore, the silencing of SOX8 in CAL27-res and SCC9-res cells efficiently decreased FZD7 protein levels (Fig. 5b). Using JASPAR programme for transcription factor binding analysis, we found one possible binding site of SOX8 in the 3.5-kb region upstream of FZD7 (jaspar.genereg.net; Supporting Information Table 6). We performed ChIP assays in CAL27-res cells to detect the interaction of SOX8 with the FZD7 promoter region. Using two independent SOX8-specific
Figure 2. SOX8 is essential for the chemoresistance and stem-like properties of cisplatin-resistant cells. Western blot (a) and qPCR (b) showed the expression of SOX8 in cisplatin-resistant cells after infection with lentivirus containing different shRNAs against SOX8. **p < 0.01. Western blot (c) and qPCR. (d) Shows that transfection of SOX8 inhibits stem cell-related genes assessed in cisplatin-resistant cells. **p < 0.01. (e) MTS assay shows that cell viability is downregulated in resistant cells after knockdown of SOX8. **p < 0.01. (f) Tumorsphere formation assay shows that knockdown of SOX8 reduced the number and size of the tumorspheres in both CAL27-res and SCC9-res cells compared to control group at Day 14, respectively. **p < 0.01, Scale bar: 200 μm. (g) Flow cytometric analysis shows that CD44+/CD24- cells in SOX8 knockdown group are higher than that in scrambled-control group. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 3. SOX8 regulates cell growth, migration and invasion in cisplatin-resistant cells and their parental cells. (a) MTS assay and (b) clone formation assay shows that knockdown of SOX8 in resistant cells inhibits cell growth at Day 6. **p < 0.01. (c) Western blot shows SOX8 knockdown enhances expression of E-cadherin (E-cad) and inhibits expression of vimentin (Vim) in CAL27-res and SCC9-res cells. (d) Knockdown of SOX8 inhibits cell migration ability and invasiveness in both CAL27-res and SCC9-res cells, when compared to control group. **p < 0.01, Scale bar: 100 μm. (e) Western blot showed that SOX8 overexpression inhibits expression of E-cadherin (E-cad) and increases expression of vimentin (Vim) in CAL27 and SCC9 cells. (f) Overexpression of SOX8 enhances cell migration ability and invasiveness in both CAL27 and SCC9 cells, when compared to control group. **p < 0.01, Scale bar: 100 μm. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 4. Wnt/β-catenin signaling participates in cisplatin-resistant CSCs. (a) Wnt/β-catenin pathway is upregulated in cisplatin-resistant cells displayed by Western blot. (b) The transcriptional activity is enhanced in cisplatin-resistant cells compared to their parental cells by luciferase assay. **p < 0.01. (c) β-catenin and C-myc is downregulated and DKK1 is upregulated in resistant cells when SOX8 is repressed examined by qPCR. **p < 0.01. (d) C-myc is downregulated and DKK1 is upregulated in resistant cells when SOX8 is repressed. (e) The transcriptional activity is reduced in cisplatin-resistant cells transfected with sh-β-catenin compared to control cells by luciferase assay. **p < 0.01. (f) The number of survival cells under cisplatin pressure were decreased in TSCC resistant cells transfected with β-catenin shRNA via MTS assay. **p < 0.01. (g) Knockdown of β-catenin reduces the number and size of the tumorspheres comparing with control group in both CAL27-res and SCC9-res cell lines at Day 14, respectively. **p < 0.01; Scale bar: 200 μm. [Color figure can be viewed at wileyonlinelibrary.com]
mouse antibodies (sc-374445 and sc-374445, Santa cruz, CA), we found that SOX8 binds to the FZD7 gene promoter region (Fig. 5c). We further used the luciferase reporter assay to examine the direct binding of FZD7 promoter region. A significant decrease in signals was detected in all SOX8-silenced cisplatin-resistant cells, but FZD7 overexpression rescued the reduction in TCF/LEF luciferase activity induced by SOX8 knockdown in CAL27-res and SCC9-res cells (Fig. 5d). Similar results were obtained by Western blot assay for β-catenin expression (Fig. 5e). Collectively, these findings indicate that SOX8 promotes the activation of Wnt/β-catenin pathway through the transcriptional regulation of FZD7.

**SOX8 knockdown decreases the capacity of xenograft formation and tumor metastasis initiation in CAL27-res cells**

We subcutaneously injected five groups of stably transfected CAL27-res cells into nude mice to investigate whether the
Figure 6. SOX8 deletion decreases the capacity of xenograft formation and tumor metastasis initiation in CAL27-res cells. BALB/c-nu mice bearing shSOX8 CAL27-res cells expressed activated β-catenin or vector and scrambled CAL27-res cells were treated with saline or cisplatin. (a) Representative photomicrographs of tumors from each group at Day 36. (b) Tumor growth curves for CAL27-res tumors treated with saline or cisplatin. *p < 0.05. (c) Tumor weight for CAL-27res xenografts treated with saline or cisplatin. *p < 0.05. (d) SOX8, OCT4, PCNA, E-cadherin, and vimentin are analyzed using immunohistochemistry. The quantification of apoptosis rate in each group is detected by TUNEL assays. Scale bar: 100 μm. (e) HE staining of lung metastases in each group are shown. Scale bar: 100 μm. [Color figure can be viewed at wileyonlinelibrary.com]
SOX8-β-catenin axis affected the apoptosis and cisplatin sensitivity of TSCC cells in vivo. As shown in Figures 6a-c, SOX8 knockdown attenuated xenograft growth and increased its sensitivity to cisplatin. However, stable transfection of activated β-catenin in shSOX8 cells enhanced their tumor growth and resistance to cisplatin. Furthermore, a reduced number of PCNA+ cells and an increased number of apoptotic cells were found in SOX8-knockdown xenograft specimens compared to the negative control group, but the apoptosis cells was repressed and the number of PCNA+ cells markedly increased after cisplatin treatment when activated β-catenin was stably expressed in shSOX8 xenografts (Fig. 6d).

A definite feature of CSCs is efficient tumorigenicity.24–27 When 5 × 10^3, 1 × 10^4, and 1 × 10^5 CAL27-res cells were inoculated into immunodeficient mice, three, four and five mice in each Group (five mice) generated tumors, respectively (Supporting Information Table 7). On the contrary, mice inoculated with 5 × 10^3, 1 × 10^4 CAL27 cells developed no tumor, while tumors developed in only one out of five mice inoculated with 1 × 10^5 CAL27 cells. Therefore, cisplatin resistant CAL27-res cells were at least 20-fold more tumorigenic than CAL27 cells. Furthermore, xenograft formation capacity was also detected in SOX8 knockdown CAL27-res cells with or without active β-catenin plasmids. One out of five mice injected with 1 × 10^4 SOX8 knockdown resistant cells and two out of five mice similarly injected with 1 × 10^5 cells generated tumors, while no tumor developed in mice inoculated with 5 × 10^3 cells. Nevertheless, activation of Wnt pathway rescued its strong tumorigenicity of SOX8 stable knockdown CAL27-res cells (Supporting Information Table 8). An important stem marker OCT4 was detected in xenograft samples. OCT4 was downregulated in xenografts with shSOX8 cells and upregulated in β-catenin overexpressing cells (Fig. 6d). These results suggest CAL27-res have stronger potent tumorigenic capability and the SOX8-β-catenin axis contributed to the high self-renewing capacity in vivo.

It has been hypothesized that only cancer cells with CSC properties can initiate metastases.24 Six weeks after injection with 1 × 10^5 CAL27-res cells, lung metastases were observed in four out of five mice using microscopy, but lung metastases were invisible in lung specimens of mice inoculated with 1 × 10^5 CAL27 cells (Supporting Information Table 7). Moreover, no mice with 1 × 10^5 shSOX8 CAL27-res cells developed microscopic lung metastases. However, three out of five mice injected with β-catenin overexpressing shSOX8 cells developed lung metastases (Fig 6e; Supporting Information Table 8). IHC staining for vimentin and E-cadherin also showed SOX8-β-catenin axis contributed to EMT in vivo (Fig. 6d). These data suggest that SOX8 mediates cisplatin sensitivity and regulates CSC-like and EMT features through the Wnt/β-catenin pathway in vivo.

**Discussion**

In this study, we demonstrated that SOX8 upregulation was crucial for chemotherapy-induced CSC enrichment, a mesenchymal phenotype and the chemoresistance of TSCC cells, and knockdown of SOX8 expression inhibited cancer stem-like properties, reversed mesenchymal features and repressed the tumor metastasis of chemoresistant TSCC via activation of the Wnt/β-catenin pathway. In addition, SOX8 bound to the promoter region of FZD7 to enhance the activity of the Wnt/β-catenin pathway. Furthermore, SOX8 levels were positively associated with lymph node metastasis and chemotherapeutic resistance of TSCC patients, and high SOX8 expression indicated a poor prognosis for TSCC patients.

Dysregulation of SOX genes have been well studied in numerous human neoplasms, and various SOX genes that act as oncogenes or tumor suppressors are involved in tumor formation and progression.9,23,28 Here, we identified SOX8 as a functional oncogene that is involved in the maintenance of cancer stem-like capacities, the mesenchymal phenotype and chemoresistance in TSCC cells. SOX8 belongs to the SOX subfamily and is an important transcription factor that is mainly involved in regulating mammalian testis and nervous system development.29,30 Previous studies in hepatocellular carcinoma and gliomas have demonstrated that SOX8 promotes cell growth through the activation of Wnt/β-catenin signaling.31,32 Xie et al. also reported that SOX8, as a target of miR-124b, is overexpressed in lung cancer and closely associated with a poor prognosis.33 In addition, we found that SOX8 expression correlated with lymph node metastasis rate and poor survival time in 103 TSCC patients and was higher especially in chemoresistant TSCC patients than in chemosensitive patients, indicating its potential value in chemoresistance. Through detecting SOX8 expression in TSCC patients after surgery, we could determine the patient’s sensitivity to chemotherapy, leading to a reduced probability of recurrence and metastasis after chemotherapy.

Our findings that SOX8 enhances the expression of FZD7, a transmembrane receptor for the Wnt pathway, suggest a correlation between SOX8 and the Wnt/β-catenin signalling. Wnt/β-catenin signalling in HNSCC has been shown to control self-renewal and differentiation of cancer cells,33 and aberrant activation of Wnt pathway leads to EMT in epithelial tongue cancer cells34 and acquisition of cancer stem-like properties.35 As a key receptor of the Wnt pathway, FZD7 is highly expressed in oesophageal squamous cell carcinoma, breast cancer and in oral squamous cell carcinoma, and it is associated with the activation of Wnt signalling in these cancers.36,37 For instance, FZD7 activates the Wnt/β-catenin signaling in oesophageal squamous cell carcinoma and enhances cell growth and metastasis and inhibits multidrug resistance.35 In addition, FZD7 enhances the activity of the Wnt/β-catenin signaling in oral cancer cells.37 Herein, we showed that FZD7 overexpression in tongue cancer cells due to increased SOX8 expression led to abnormal activation of Wnt pathway and therefore induced CSCs and EMT in cisplatin-resistant cells. Moreover, we identified FZD7 as a direct target of SOX8, and SOX8 enhanced the activity of
FZD7-mediated Wnt/β-catenin signaling in chemoresistant TSCC cells, along with the EMT phenotype with reduced E-cadherin and increased vimentin and N-cadherin and cancer stem-like features with increased SOX2, BMI1, OCT4, and ABCG2.

Numerous studies have shown that the EMT process and/or CSCs confer tumor drug resistance. For instance, depletion of Twist1 in chemoresistant breast cancer cells can partially reverse sensitivity to chemotherapeutic agents.7 Similarly, Slug or Snail overexpression also increases resistance to chemotherapy-induced cell death.36 In our study, we identified SOX8-FZD7-Wnt/β-catenin as another important pathway regulating EMT and chemoresistance in TSCC. Therefore, EMT in tongue cancer cells may result in chemoresistance either through Twist1, Slug, or Snail, which directly controls the EMT process, or through the SOX8-FZD7-Wnt/β-catenin pathway. Tumor cells with a more aggressive phenotype display strong self-renewal capacity and tumorigenicity under average chemotherapy pressure, which are similar characteristics to those harboured by CSCs that enable them to escape drug-induced cell death.36,40 For example, Oct4 overexpression contributes to tamoxifen resistance and ALDH1 upregulation in hormone receptor-positive breast cancer.41 Silencing SOX2 expression also attenuates the resistance to cisplatin in glioblastoma multiforme.42 Our study demonstrated that chemoresistant TSCC cells acquired abilities similar to CSCs, which were accompanied by an increase in the CSC markers ABCG2, SOX2, OCT4, and BMI1 as well as strong self-renewal capacity and tumorigenicity in vivo. In addition, recent studies have shown a clear relationship between EMT and the acquisition of stem cell-like characteristics,43 and cancer cells that have undergone EMT may acquire stem cell-like properties.43,44

The SOX proteins have emerged as important modulators of Wnt/β-catenin signaling in various diseases and numerous studies indicate diverse regulating mechanisms including direct protein-protein interactions, binding to the promoters of Wnt signaling genes, and the recruitment of co-factors to regulate the stability of the target proteins.15 For instance, SOX6, SOX9, and SOX17 directly combine with β-catenin in the region where TCF promoters also combine with β-catenin.15 SOX17 regulates Wnt pathway via direct interaction with TCF3, TCF4, and LEP1 as well as β-catenin.15 Alternatively, the histone de-acetylase HDAC1 can be recruited by SOX6 to β-catenin complexes.46 Additionally, ChIP assays have shown that SOX9, a member of the SOX8 group, directly binds to promoters of TCF4 and LRP6 and enhances the activation of Wnt signaling in breast cancer.23 We have demonstrated that silencing of SOX8 inhibited the expression of β-catenin and its downstream genes and reduced the transcriptional activity of Wnt signaling in resistant TSCC cell lines. Moreover, we identified that SOX8 has a DNA-binding site in the promoter region of FZD7, a vital receptor of Wnt signaling. Our research is the first to explicitly show that SOX8 promotes CSC properties, chemoresistance and EMT in TSCC by acting on the FZD7-mediated Wnt/β-catenin pathway.

In summary, we identified SOX8 as the most differentially expressed protein between two cisplatin-resistant cells and their parental TSCC cells, and the ectopic expression of SOX8 promoted chemoresistance, CSC properties and EMT features in chemoresistant TSCC cells. The Wnt/β-catenin pathway mediated the cancer stem-like properties in cisplatin-resistant TSCC cells. SOX8 could bind to the promoter region of FZD7 and activated FZD7-mediated Wnt/β-catenin pathway to regulate chemoresistance, stem-like properties and EMT. Furthermore, SOX8 was upregulated in chemoresistant TSCC patients and significantly associated with high lymph node metastasis and poor prognosis.

Acknowledgments
This work was supported by National Natural Science Foundation of China (81672676, 81472521, and 81272951 to J.-S.L., 81602379 to Z.-Y.L., 81402251 to S. F.); by Specialized Research Fund for the Doctoral Program of Higher Education (2011017110068 to J.-S.L.); by Science and Technology Project of Guangzhou City (20121400078 to J.-S.L.); China Postdoctoral Science Foundation (2016M590839 to Z.-Y. L.); by Grant [2013]163 from Key Laboratory of Malignant Tumor Molecular Mechanism and Translational Medicine of Guangzhou Bureau of Science and Information Technology; Grant K1809001 from the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes.

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