

Sox4 Regulates the Sensitivity of Canine Mammary Gland Tumor Cells to Cisplatin via the Wnt/ β -catenin Signaling Pathway

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Abstract

Background: The development of cisplatin resistance is one of the major causes of breast cancer treatment failure, and is associated with changes in Sox4 gene expression. In this study, a cisplatin-resistant cell line, CHMp^{CIS}, was constructed from the cell line CHMp, which was isolated from the primary lesion of a malignant canine mammary gland tumor (CMGT). Sox4 expression was evaluated to assess its roles in cisplatin sensitivity, proliferation and apoptosis, epithelial-mesenchymal transition (EMT), cancer stem cell (CSC) features, and activation of the Wnt/ β -catenin signaling pathway in CMGT cells.

Results: CHMp^{CIS} Cells exhibited changes in morphology, slower proliferation, and greater anti-apoptotic ability, EMT and CSC features, and the Wnt/ β -catenin pathway was activated in CHMp^{CIS} cells. In CMGT tissues, Sox4 expression was elevated. In CHMp^{CIS} cells, silencing Sox4 inhibited cisplatin resistance, EMT and CSC features, and Wnt/ β -catenin signaling activation. Then activating the Wnt/ β -catenin signaling pathway increased Sox4 expression levels.

Conclusions: Silencing Sox4 inhibited the above-mentioned cancer cell characteristics in CHMp^{CIS} cells compared with CHMp cells. In addition, activating the Wnt/ β -catenin signaling pathway increased Sox4 expression levels, as part of a positive feedback loop. These findings may provide new targets and therapeutic strategies for the clinical treatment of CMGT as well as a reference for human mammary gland tumor (HMGT) research.

Background

Breast cancer has the highest incidence and mortality among all women's cancers [1]. Breast cancer is also common in dogs, and the incidence of canine mammary gland tumors (CMGT) is approximately three times that of breast cancer among women in the same geographic area [2]. In both humans and dogs, breast cancer has high lethality, recurrence, and mortality, and the CMGT model is a reliable model for studying human mammary gland tumors (HMGT). In particular, spontaneous canine tumors are a model of human cancer biology and transformational cancer treatment. This model is a unique and underutilized resource and is expected to fill the gap between in vitro and in vivo research [3].

One of the most commonly used combination chemotherapy drugs for the clinical treatment of breast cancer is cisplatin. Cisplatin exerts anti-cancer effects through multiple mechanisms, but its most prominent mode of action is the successive induction of DNA damage, DNA damage responses, and mitochondrial apoptosis [4]. The development of resistance, including multi-drug resistance, greatly compromises the efficacy of cisplatin against breast cancer and can lead to chemotherapy failure and reduced patient survival. Numerous studies have shown that the development of cisplatin resistance arises from pleiotropic effects on cell growth-promoting pathways, apoptosis and development pathways, self-renewal pathways, and transcription pathways regulating epithelial-mesenchymal

transition (EMT), DNA damage repair and endocytosis [5]. These changes may be linked to overexpression of the Wnt/ β -catenin pathway [6, 7].

The Wnt/ β -catenin pathway regulates the process of cell development, and affects cell morphology, proliferation, differentiation, apoptosis, EMT and cancer stem cell (CSC) features in various tissue environments. Disorders of this pathway may also lead to breast tumor formation [8–11]. Sox4 (sex-determining region Y-box4) is a member of the Sox transcription factor family and a so-called "cancer characteristic genes" [12]. High Sox4 expression is associated with poor prognosis and may promote the development of cancer through a variety of mechanisms, including regulation of cellular proliferation, EMT, self-renewal, apoptosis, and signaling pathways such as Wnt, PI3K/Akt, Notch, and Hedgehog [13]. Sox4 is particularly important for Wnt signaling and can directly interact with β -catenin to activate gene expression [14, 15]. The present study explores the effects of Sox4 on CMGT progression and cisplatin resistance and the interaction between Sox4 and the Wnt/ β -catenin pathway.

Results

CHMp^{CIS} Cells Exhibit Changes in Morphology, Slower Proliferation, and Greater Anti-Apoptotic Ability

First, the CHMp parent cell line CHMp^{parent} was purified by the limiting dilution method to obtain 7 purified cells (CHMp^{1-16D}, CHMp^{2-10E}, CHMp^{3-2D}, CHMp^{3-3C}, CHMp^{4-4F}, CHMp^{4-9G}, CHMp^{5-5B}) from single-cell cultures as shown in Figure 1 A. CHMp4-9G had the highest relative protein expression of ER α and β -catenin and thus was named CHMp. The morphology of CHMp cells was obviously different from that of CHMp^{parent} cells (Figure 1 B). CHMp cells were larger and stretched.

The drug-resistant cell line CHMp^{CIS} was established from CHMp by the concentration gradient method. The IC₅₀ of cisplatin against CHMp and CHMp^{CIS} cells was 4.983 μ mol/L and 24.92 μ mol/L, respectively (Figure 1 E). The resulting resistance index (RI), 24.92/4.983=5.001, was greater than 3, indicating successful construction of the cisplatin-resistant cell line. Compared with CHMp cells, CHMp^{CIS} cells displayed raised pseudopods, aggregated growth, and slower proliferation (Figure 1 C, D). The effects of cisplatin on apoptosis were smaller for the drug-resistant cell line than for the non-drug-resistant cell line; flow cytometry analysis showed that in the presence of cisplatin, the apoptotic rates of CHMp^{CIS} and CHMp cells were 6.0% and 49.3%, respectively (Figure 1 F). The expression of the apoptotic protein Bax was remarkably reduced in CHMp^{CIS} cells, whereas the expression of the anti-apoptotic protein Bcl-2 and the multidrug resistance proteins MDR1 and MRP1 was remarkably increased (Figure 1 G). These results indicated that CHMp^{CIS} cells have enhanced anti-apoptotic ability in addition to drug resistance.

EMT and CSC Features Are Enhanced in CHMp^{CIS} Cells

Compared with CHMp cells, CHMp^{CIS} cells exhibited similar migration ability but distinctly stronger invasion ability in the absence of cisplatin. The addition of cisplatin obviously inhibited the migration and invasion ability of CHMp cells but had little effect on CHMp^{CIS} cells (Figure 2 A, B). In addition, the

expression of epithelial cadherin (E-cadherin) was lower in CHMp^{CIS} cells than in CHMp cells, whereas the expression of N-cadherin, a protein that provides greater connection flexibility, was increased in CHMp^{CIS} cells (Figure 2 C). These observations indicate enhanced EMT in CHMp^{CIS} cells. In the mammosphere experiment, CHMp^{CIS} cells formed more and larger II MS than CHMp cells; in addition, the majority of the mammospheres formed by CHMp^{CIS} cells, up to 70%, were large spheres (Figure 2 D). As shown in Figure 2 E, CHMp^{CIS} cells formed more clonal cell clusters than CHMp cells in the presence or absence of cisplatin; in fact, in the presence of cisplatin, CHMp cells were unable to form clonal cell clusters. Moreover, the expression of three key proteins related to cell stemness, Nanog, Oct4, and Sox2, was notably higher in CHMp^{CIS} cells than in CHMp cells (Figure 2 F). The above results demonstrate that CHMp^{CIS} cells have greater CSC features than CHMp cells.

The Wnt/ β -catenin Pathway Is Activated in CHMp^{CIS} Cells

As shown in Figure 3 A, in the absence of cisplatin, the Wnt/ β -catenin pathway inhibitor FH535 decreased the viability of CHMp cells in a concentration-dependent manner but had little effect on the viability of CHMp^{CIS} cells. Cisplatin also reduced the viability of CHMp cells, and no obvious effect of FH535 was observed in the presence of cisplatin. By contrast, the initial addition of cisplatin did not remarkably reduce the viability of CHMp^{CIS} cells, but as the concentration of FH535 increased, cell viability decreased dramatically under the dual effects of cisplatin and FH535. When the Wnt/ β -catenin pathway activator Wnt3a was added, the cell viability of CHMp^{CIS} and CHMp cells was obviously restored.

In addition, FH535 significantly reduced the migration and invasion ability of CHMp but not CHMp^{CIS} cells (Figure 3 D). In the presence of 10 μ mol/L FH535, the apoptotic rates of CHMp^{CIS} and CHMp cells were 8.1% and 33.9%, respectively, indicating a much smaller effect of FH535 on the apoptosis of CHMp^{CIS} cells compared with CHMp cells (Figure 3 D). FH535 weakened the sphere-forming ability of both CHMp^{CIS} and CHMp cells, as evident by comparing Figure 3 E with Figure 2 D, but the sphere-forming ability of CHMp^{CIS} cells remained stronger than that of CHMp cells. FH535 also markedly inhibited the clonal cluster-forming ability of CHMp cells, whereas CHMp^{CIS} cells formed the same number of clonal clusters, albeit smaller in size, in the presence of FH535 as in its absence. In CHMp^{CIS} cells, exposure to FH535 increased the expression of the key Wnt/ β -catenin pathway proteins β -catenin and Wnt3a but decreased the expression of Gsk3 β , a protein involved in the degradation of β -catenin. Accordingly, the expression of P-Gsk3 β and P- β -catenin increased and decreased, respectively. The above results indicate that the Wnt/ β -catenin pathway is active in CHMp^{CIS} cells.

Sox4 Expression Is Elevated in CMGT Tissues

The analysis of Sox4 protein and mRNA expression showed that Sox4 expression was significantly higher in CMGT tissues (CMGTT) than in adjacent tissues (CAMGTT) (Figure 4 A B). Combined with previous results from our experimental group [16], these results suggest that Sox4 participates in and regulates the disease process of CMGT.

Low Expression of Sox4 Inhibits Cisplatin Resistance, EMT and CSC Features

Evaluation of Sox4 gene knockdown (Figure 5 A) showed that the second gene sequence had the most obvious effect on Sox4 expression. Therefore, subsequent experiments were carried out using siSox4-2 cells. Cisplatin treatment slowed the proliferation of siSox4-2 cells (Figure 5 B); compared with siNC cells, Bax expression was elevated, and Bcl-2, MDR1, and MRP1 expression were decreased (Figure 5 C), indicating increased cisplatin sensitivity and weaker drug resistance. As shown in Figure 5 D, E, F, the migration and invasion capabilities of siSox4-2 cells were substantially reduced compared with siNC cells, with increased E-cadherin expression, weaker N-cadherin expression, enhanced adhesion, and weaker EMT features. Stemness testing showed that the μ MS and clonal cell clusters formed by siSox4-2 were small and few in number (Figure 5 G, H), with far inferior sphere-forming and clone-forming ability compared with siNC cells. The expression levels of Nanog, Oct4 and Sox2 were also much lower in siSox4-2 cells than siNC cells, indicating weaker CSC features.

Low Sox4 Expression Inhibits Wnt/ β -catenin Signaling Activation

Knockdown of the Sox4 gene increased the expression of the Wnt/ β -catenin pathway-related protein Gsk3 β but decreased the expression of β -catenin and Wnt3a (Figure 6 A, B). Adding Wnt3a augmented the expression of Sox4 (Figure 6 C). Thus, reducing Sox4 expression inhibits the Wnt/ β -catenin signaling pathway, which in turn regulates Sox4 expression.

Discussion

Cisplatin resistance remains an important issue in the clinical treatment of breast cancer. Here, we demonstrated that MDR1 and MRP1 expression, anti-apoptotic ability, EMT, CSC features, and activation of the Wnt/ β -catenin signaling pathway were enhanced in cisplatin-resistant CMGT cells compared with parent CMGT cells. Moreover, the expression of Sox4 was elevated in CMGT tissues compared with adjacent tissues. Cisplatin resistance, apoptosis- and drug resistance-related protein expression, EMT and CSC features were all related to the expression of Sox4 in CHMp^{CIS} cells. Sox4 silencing significantly reversed cisplatin resistance, related biological characteristics and Wnt/ β -catenin pathway activity in CHMp^{CIS} cells. Finally, activating the Wnt/ β -catenin signaling pathway increased Sox4 expression. Taken together, these findings indicate that Sox4 plays a key role in the development of cisplatin resistance by increasing autocrine Wnt signaling, thereby enabling escape from apoptosis and enhancing EMT and CSC features.

In ovarian cancer cells, cisplatin resistance is associated with upregulated expression of the anti-apoptotic protein Bcl-2, activation of Fas, blockade of Caspase-3 and Caspase-8 activation, reduced levels of the pro-apoptotic protein Bax, and increased cell proliferation due to elevated Wnt/ β -catenin expression [17, 18]. There is evidence that the multi-drug resistance gene MDR1 is also a target of TCF/ β -catenin [19]. MDR1 and MRP1 are cisplatin-pumping proteins, and their overexpression is considered one of the main mechanisms of cisplatin resistance. The expression of β -catenin is positively correlated with

upregulation of MDR1 gene expression [20, 21]. Consistent with our findings, the Wnt/ β -catenin pathway enhances the EMT and CSC features of human tongue squamous cell carcinoma and breast cancer cells [22, 23].

Sox4 is an important developmental transcription factor that regulates stemness, differentiation, progenitor cell development and multiple developmental pathways, including PI3K, Wnt and TGF β signal transduction [13]. Sox4 also inhibits terminal differentiation. These functions are strongly associated with the development of malignant tumors, and Sox4 gene upregulation has been observed in a variety of cancers [24, 25]. Increased Sox4 activity promotes cancer cell survival, proliferation [26], migration [15], and self-renewal [27]. High Sox4 expression is associated with poor tumor prognosis in patients, and thus Sox4 is a pan-cancer prognostic biomarker [28]. However, in dogs, the prognostic value of Sox4 expression has not been examined. In the present study, silencing Sox4 reduced β -catenin activation, resulting in a weakened invasive phenotype. Similarly, silencing Sox4 has been shown to prevent cancer progression before prostate intraepithelial neoplasia becomes cancerous [29].

The induction of β -catenin/TCF activity by Sox4 depends on the stability of the β -catenin protein and not the induction of β -catenin transcription [30]. We showed that Sox4 knockdown suppressed the Wnt/ β -catenin signaling pathway, thereby restraining the proliferation, migration, invasion, and self-renewal of CMGT cells. Sox4 gene deletion also reduces active β -catenin levels in prostate cancer [31]. The mechanism by which Sox4 deletion inhibits β -catenin activation remains to be determined, but there are several possibilities. First, Sox4 may directly interact with TCF and β -catenin to stabilize the β -catenin/TCF complex [14]. Second, Sox4 may stimulate β -catenin activity indirectly by maintaining active AKT. Crosstalk is known to occur between the PI3K-AKT and Wnt/ β -catenin pathways, as AKT phosphorylation and inhibition of GSK3 β increase the activity of Wnt pathway mediators and β -catenin [32]. Third, it has been proposed that Sox4 stabilizes β -catenin by inducing the transcription of CK2, thereby protecting it from degradation. Finally, in endometrial cancer, Sox4 has been found to promote Wnt/ β -catenin signal transduction through direct transcription to activate the expression of TCF4, leading to the activation of Wnt target genes [28].

Activation of Wnt/ β -catenin signaling pathway further regulates Sox4 gene expression through a positive feedback loop, thereby promoting tumor progression. Regulation of Sox4 transcriptional activity and expression occurs not only at the mRNA level but also via post-translational modifications (PTMs) and protein-protein interactions. In prostate cancer, C-myc and CUL4B act directly on Sox4 to promote progression [33, 34], forming a positive feedback loop. The transcriptional activity and target gene specificity of Sox4 are also controlled by synergistic interactions with different transcription factors and cofactors. Syntenin-1 regulates Sox4 protein stability and transcriptional activity by interacting with syndecans [35], ephrin [36], Frizzled [37], etc. Other mutations in cancer cells, including in receptor signaling pathways and DNA damage pathways, may also affect Sox4 transcriptional activity [28].

Taken together, these observations emphasize the relevance of the interaction of Sox4 with the Wnt/ β -catenin signaling pathway in CMGT and cisplatin-resistance. The development of cisplatin resistance

increases the expression of Sox4, leading to Wnt signal activation, enhanced cancer cell migration and invasion capabilities, and cancer stem cell population enrichment. Targeting Sox4 may facilitate the differentiation of stem cells and increase the sensitivity of cells to cisplatin. Thus, treatment methods that reduce Sox4 expression may prevent cancer recurrence in CMGT and provide a theoretical basis for the treatment of HMGT.

Conclusion

Silencing Sox4 inhibited the Wnt/ β -catenin signaling pathway and ameliorated the significant increases in cisplatin resistance, anti-apoptotic ability, EMT and CSC features in CHMp^{CIS} cells compared with CHMp cells. In addition, the Wnt/ β -catenin signaling pathway regulated the expression level of Sox4 in a positive feedback manner. These findings may provide new targets and therapeutic strategies for the clinical treatment of CMGT as well as a reference for human mammary gland tumor (HMGT) research.

Methods

Clinical Subjects and Specimens

Specimens were collected from a total of 7 cases of CMGT recruited at Northeast Agricultural University Animal Hospital. Tissues were identified and classified. All procedures were performed with consent from the animals' owners and in accordance with the Experimental Animal Control law of Northeast Agricultural University. All animal care and handling procedures were approved by the Ethics Committee of the Animal Hospital of Northeast Agricultural University.

Cell Lines and Cell Culture

The CHMp cell line was isolated from the primary lesion of a 12-year-old female dog with a malignant breast tumor by the Department of Veterinary Science, University of Tokyo, Japan [38]. CHMp cells were cultured in pink high-glucose DMEM (Meilunbio, China, MA0212), supplemented with fetal bovine serum (FBS) (Tianhang, China, 11011-8611) and penicillin-streptomycin-amphotericin B solution (Beyotime, China, C0224-100ml). Cell suspensions were prepared by digesting logarithmic-phase.

CHMp cells with 0.25% EDTA-trypsin (Beyotime, China, C0201-100ml). The cells were then diluted with complete culture medium containing 20% FBS and inoculated into 96-well plates. An inverted microscope was used to identify wells containing single cells, and then the plates were incubated at 37°C and viewed once every 24h. The surviving cells were expanded and exposed to a drug concentration gradient plus drug maintenance method (final cisplatin concentration of 20 μ mol/L) for 10 months to obtain the CHMp^{CIS} cell line.

Western Blot Analysis

Cell proteins were extracted with the BCA Protein Assay Kit (Beyotime Biotechnology, P0012S) and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to an NC membrane, which was blocked and then incubated sequentially with primary and secondary antibodies. Finally, ECL Luminescent Solution (Beyotime Biotechnology, P0018FS) was added for imaging. Quantification was performed using Image J software.

Cell Counting Kit-8 (CCK8) Assay

Logarithmic-phase CHMp and CHMp^{CIS} cells were adjusted to 3×10^4 cells/mL and transferred to 96-well plates at 100 μ L/well for culture at 37°C. After 24 h, the culture medium was replaced with medium containing a gradient of cisplatin concentrations, and the cells were again cultured at 37°C for 24 h. The culture medium was then replaced with CCK8 detection solution (Abcam, UK, ab228554), and the cells were incubated at 37°C for 30 minutes. Finally, OD values were recorded using a microplate reader. The half maximal inhibitory concentration (IC₅₀) of cisplatin was calculated according to relative cell survival.

In separate assays, logarithmic-phase CHMp and CHMp^{CIS} cells were adjusted to 1×10^4 cells/mL and transferred to 96-well plates at 100 μ L/well for culture at 37°C. On days 1, 2, 3, 4, or 5, the culture medium was replaced with CCK8 detection solution, the OD value was detected, and the value-increasing curves were plotted.

Flow Cytometry

Cells adjusted to 1×10^5 cells/mL were seeded in a 6-well plate and cultured in a 37°C incubator. After 48 h, the cell culture solution was collected, and the cells were harvested by digestion with trypsin and centrifugation. Apoptosis was detected by flow cytometry with Annexin V-FITC/PI (Bioss, China, BA00101).

Transwell Assay

Cells adjusted to 5×10^4 cells/mL in serum-free culture medium were transferred to the upper chamber of a Transwell apparatus at 100 μ L/well (Corning, USA, 3422). During the migration experiment, Matrigel was not added to the upper chamber, whereas for the invasion experiment, the upper chamber was coated with Matrigel in advance. Complete medium containing 20% fetal bovine serum was added to the lower chamber at 600 μ L per chamber and the entire apparatus was placed in a 37°C incubator. After migration for 24 h/invasion for 48 h, the cells on the lower surface of the upper chamber were fixed with paraformaldehyde, stained with crystal violet, washed with distilled water, and dried in a fume hood. Cells on transparent plates were photographed under an inverted microscope ($\times 200$ magnification). For counting, 5 fields were randomly selected from each group of cell samples.

Mammosphere Formation Assays

Cells were transferred to DMEM/F-12 (Meilunbio, China, MA0214) containing phenol red and supplemented with B-27 supplement medium (Gibco, USA, 17504044), human EGF recombinant protein (Gibco, USA, PHG0311), and human FGF-basic (FGF-2/bFGF) recombinant protein (Gibco, USA, 13256-029). The cells were adjusted to 5×10^4 cells/mL and inoculated into an ultra-low-adhesion 6-well plate. The mammospheres were cultivated until most had a diameter of $>200 \mu\text{m}$ and were subsequently passaged at 1×10^4 cells/mL to cultivate second-generation mammospheres (MS). Mammospheres with a diameter $>75 \mu\text{m}$ and large mammospheres with a diameter $>200 \mu\text{m}$ were then counted.

Clone Formation Assay

Cells were seeded in a 6-well culture plate at 500 cells/well. The medium was replaced with fresh medium on day 5. On day 10, the cells were fixed in 4% paraformaldehyde and stained with crystal violet, and the number of colonies was recorded.

Cell Transfection

Transfection kits, siR-NC and siR-SOX4 were purchased from Ribobio. Transfection was performed according to the manufacturer's instructions.

Reverse-transcription Quantitative PCR (QRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA, 15596026). The total RNA concentration was measured with an ultra-micro ultraviolet spectrophotometer (Thermo, USA, ND-ONE-W), and the RNA was reverse-transcribed using a cDNA first-strand synthesis kit (Tiangen, China, KR118) with TB Green II dye (TaKaRa, Japan, RR820A). Finally, a Light Cycler R 480 System (Roche, Basel, Switzerland) was used for quantitative analysis. Each gene was analyzed at least 3 times in each sample, and the average value was calculated. The data were analyzed by the $2^{-\delta\delta\text{CT}}$ method. See Supplementary Table 1 for primer sequences.

Immunofluorescence

Cells were seeded onto sterile coverslips placed in 6-well plates, cultured to 80–90% confluence, washed with PBS, fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (Solarbio, China, T8200), blocked with BSA (Solarbio, China, T8020), and incubated sequentially with primary and secondary antibodies and DAPI (Solarbio, China, C0060) in the dark. Anti-fluorescence quenching agent was added, and images were acquired with a fluorescence microscope.

Statistical Analysis

Data are presented as the mean \pm SD. Statistical comparisons between treated and control groups were performed using Student's t-test in GraphPad Prism 5, and multiple groups were compared by two-way ANOVA. Differences that are not significant ($P > 0.05$) are not marked; significant differences ($0.01 < P < 0.05$) are marked with "*" / "#", and extremely significant differences ($P < 0.01$) are marked with "**" / "##".

Abbreviations

ANOVA: Analysis of variance

BSA: Bovine serum albumin

Bcl2: B-cell lymphoma 2

Bax: Bcl2-associated X

cDNA: Complementary DNA

CSC: Cancer stem cell

CMGT: Canine mammary gland tumor

CMGTT: Canine mammary gland tumor tissues

CAMGTT: Canine adjacent mammary gland tumor tissues

CCK8: Cell counting kit-8

DMEM: Dulbecco's modified eagle medium

EMT: Epithelial-mesenchymal transition

Era: Estrogen receptor α

EDTA: Ethylene diamine tetraacetic acid

FBS: Fetal bovine serum

Gsk3 β : Glycogen synthesis kinase 3 β

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HMGT: Human mammary gland tumor

IgG: Immunoglobulin G

mRNA: Messenger RNA

MDR1: Multi-drug resistance 1

MRP1: Multidrug resistance-associated protein

NC membrane: Nitrocellulose filter membrane

Oct4: Octamer binding transcription factor 4

OD: Optical density

PBS: Phosphate buffered saline

PTMs: post-translational modifications

QRT-PCR: Reverse-transcription Quantitative PCR

RI: Resistance index

Sox2: Sex-determining region Y-box2

Sox4: Sex-determining region Y-box4

SDS: Sodium dodecyl sulfate

SD: Standard deviation

Declarations

Ethics approval and consent to participate

The animal study was reviewed and approved by Northeast Agricultural University, Harbin, Heilongjiang Province. Written informed consent was obtained from the owners for the participation of their animals in this study.

Consent for publication

Not applicable

Availability of data and materials

All of the data generated or analysed during this study are available from the first author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Hu M, Huang S, Xu E and Liu Y contributed conception and design of the study. Hu M, Huang S, Xu E, Tong D, Jin S and Guan T conducted experiments. Hu M performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Authors' information (optional)

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Figures

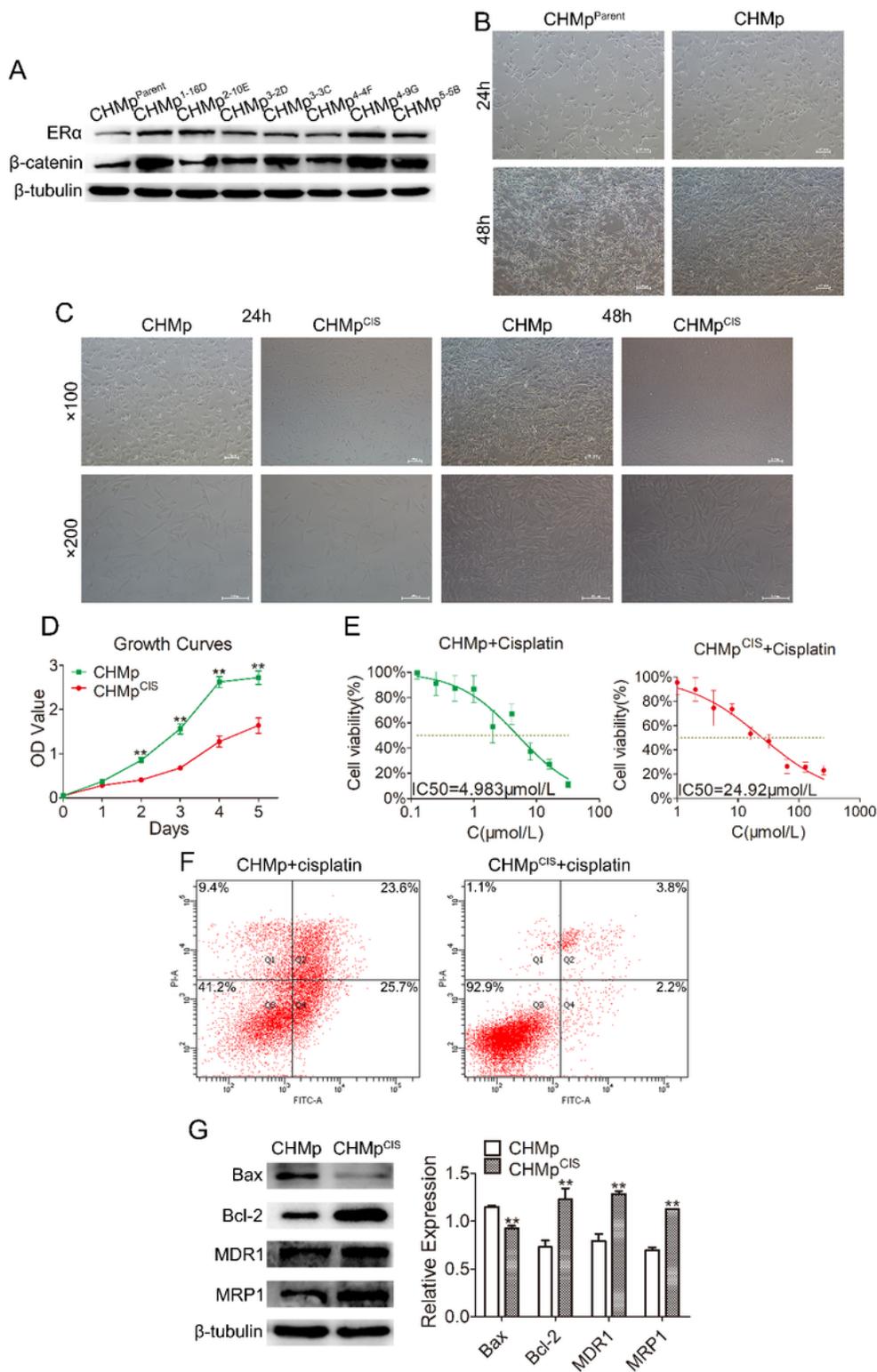


Figure 1

CHMpCIS cells exhibit changes in morphology, slower proliferation, and enhanced anti-apoptotic ability. A Relative expression levels of CMGT-related proteins in the CHMp parent cell line CHMpparent and 7 monoclonal cell lines. B Differences in morphology between CHMpparent cells and the monoclonal cell line CHMp. C Differences in morphology between CHMp cells and the cisplatin-resistant cell line CHMpCIS. D Comparison of the proliferation ability of CHMp and CHMpCIS cells (n=6). E The IC50 of

cisplatin against CHMp and CHMpCIS cells (n=6). F The effect of cisplatin on the apoptosis of CHMp and CHMpCIS cells. G Relative expression levels of apoptosis- and drug resistance-related proteins in CHMp and CHMpCIS cells (n=3). 0.01<*P<0.05, **P<0.01.

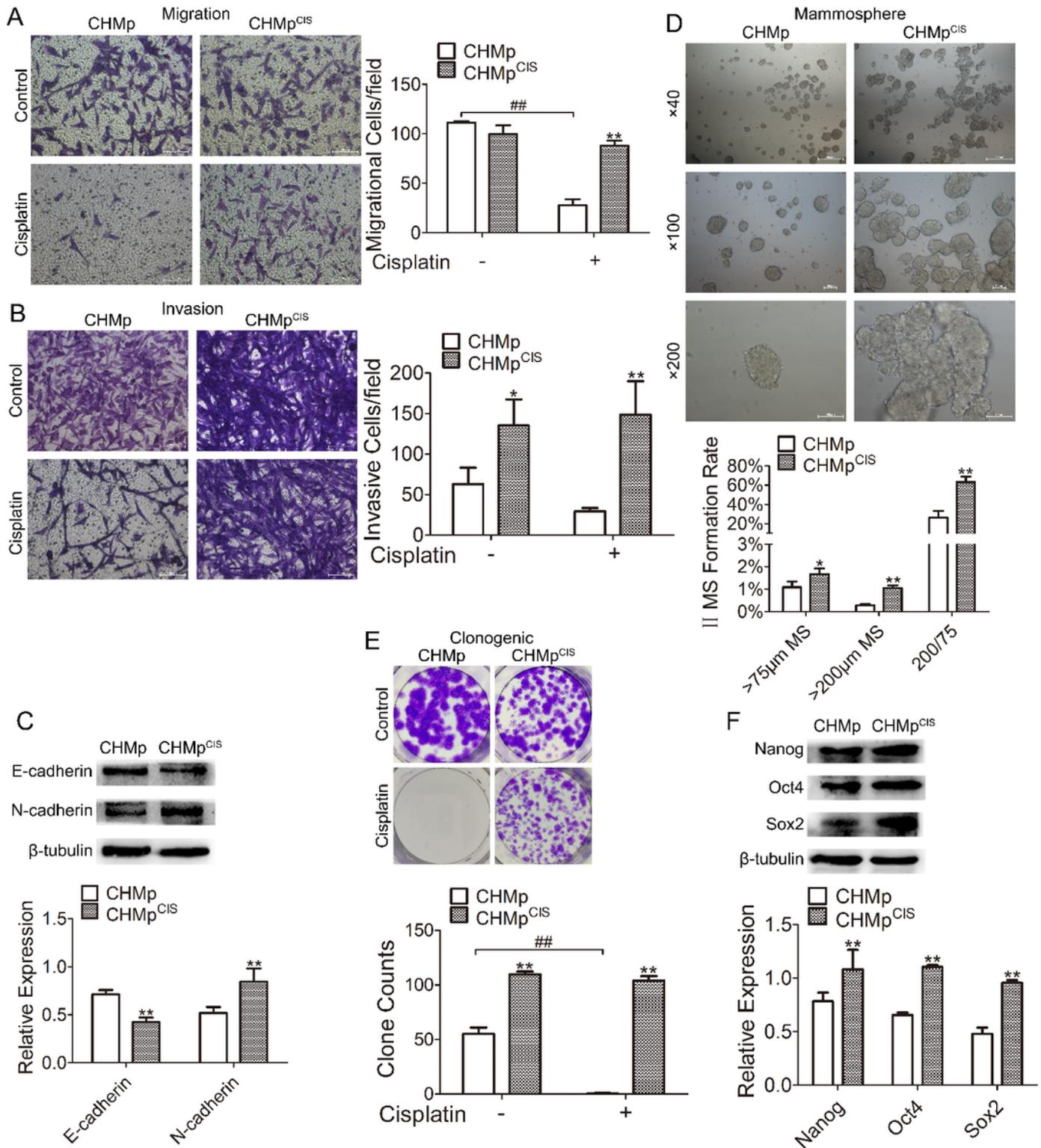


Figure 2

EMT and CSC features are enhanced in CHMpCIS cells. A Comparison of the migration ability of CHMp and CHMpCIS cells and the influence of cisplatin (×200 magnification) (n=3). B Comparison of the

invasion ability of CHMp and CHMpCIS cells and the influence of cisplatin ($\times 200$ magnification) ($n=3$). C Relative expression levels of EMT-related proteins in CHMp and CHMpCIS cells ($n=3$). D Comparison of the mammosphere-forming ability of CHMp and CHMpCIS cells ($\times 40$, $\times 100$, $\times 200$ magnification) ($n=3$). E Comparison of the clonal cell cluster-forming ability of CHMp and CHMpCIS cells ($n=3$). F Relative expression levels of CSC-related proteins in CHMp and CHMpCIS cells ($n=3$). $0.01 < * / \# P < 0.05$, $** / \#\# P < 0.01$.

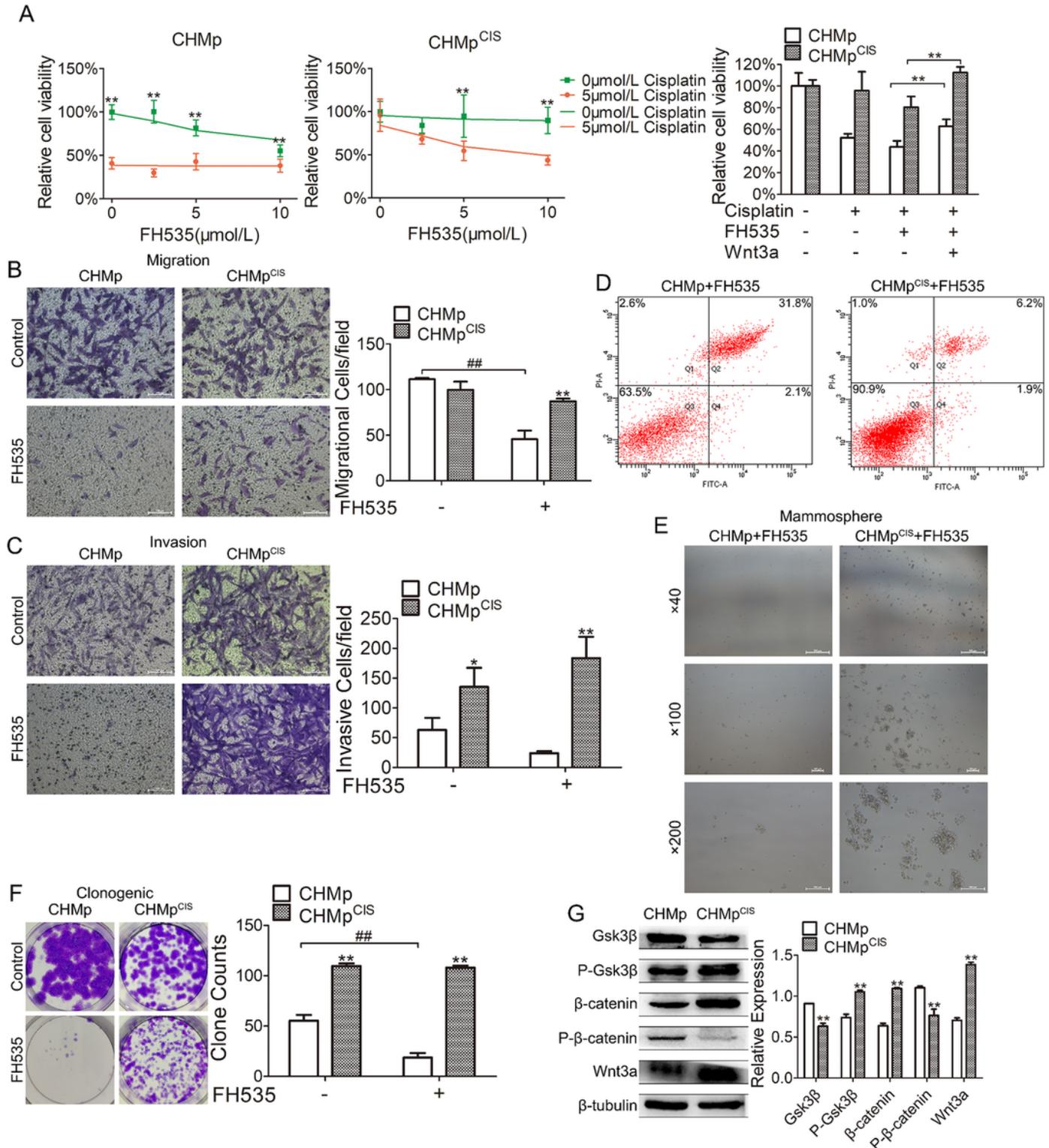


Figure 3

Wnt/ β -catenin pathway activation in CHMpCIS cells. A The effect of inhibiting or activating the Wnt/ β -catenin pathway on the viability of CHMp and CHMpCIS cells in the presence or absence of cisplatin (n=6). B The effect of inhibiting the Wnt/ β -catenin pathway on the migration ability of CHMp and CHMpCIS cells ($\times 200$ magnification) (n=3). C The effect of inhibiting the Wnt/ β -catenin pathway on the invasion ability of CHMp and CHMpCIS cells ($\times 200$ magnification) (n=3). D The effect of inhibiting the Wnt/ β -catenin pathway on the apoptosis of CHMp and CHMpCIS cells. E The effect of inhibiting the Wnt/ β -catenin pathway on the formation of CHMp and CHMpCIS mammospheres ($\times 40$, $\times 100$, $\times 200$ magnification). F The effect of inhibiting the Wnt/ β -catenin pathway on the ability of CHMp and CHMpCIS cells to form clonal cell clusters (n=3). G Relative expression levels of Wnt/ β -catenin pathway-related proteins in CHMp and CHMpCIS cells (n=3). 0.01 < * / # P < 0.05, ** / ## P < 0.01.

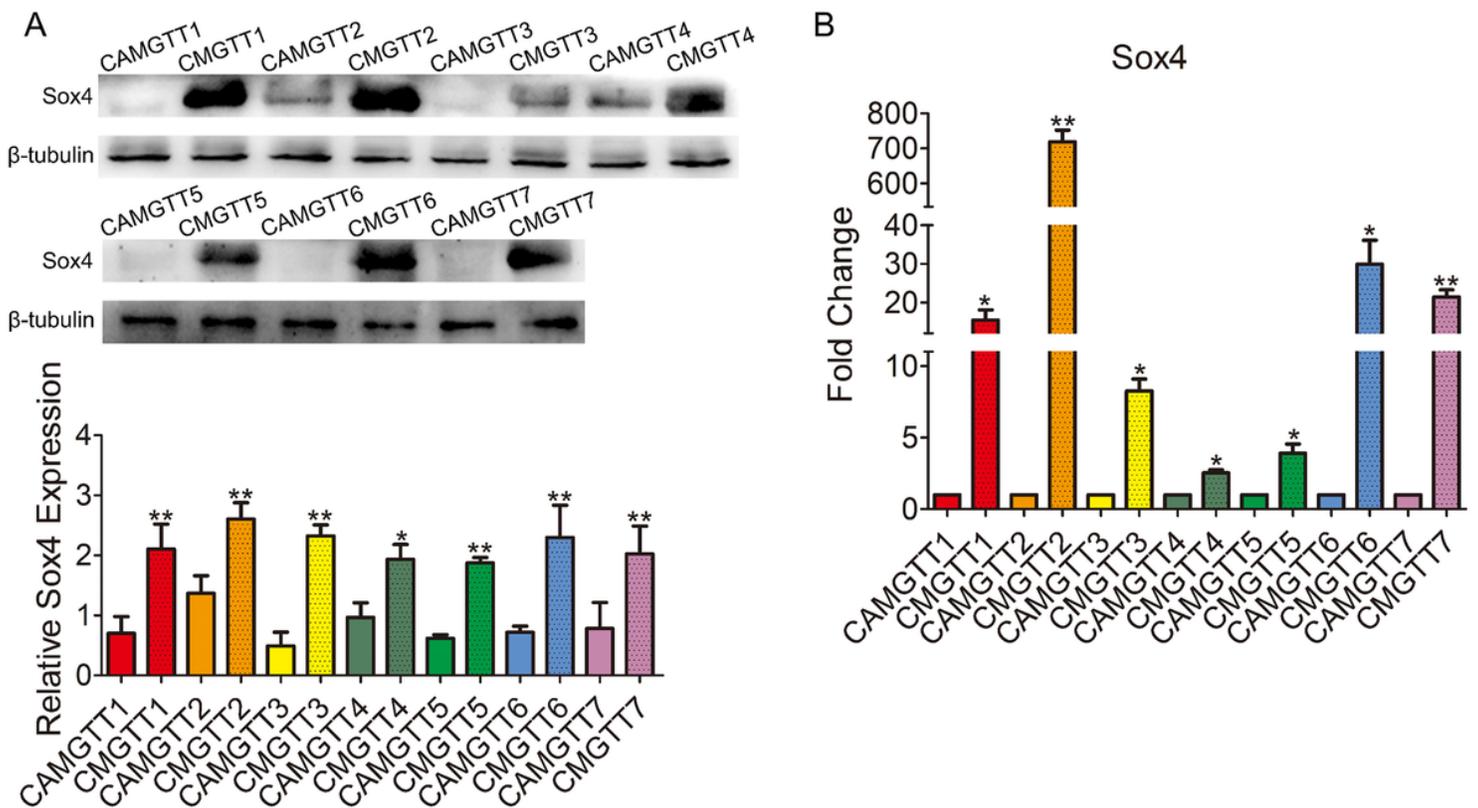


Figure 4

Sox4 expression is elevated in CMGT tissues. A Relative expression levels of Sox4 in CMGTT and CAMGTT from CMGT cases (n=3). B Relative expression levels of Sox4 mRNA in CMGTT and CAMGTT (n=3). 0.01 < * P < 0.05, ** P < 0.01.

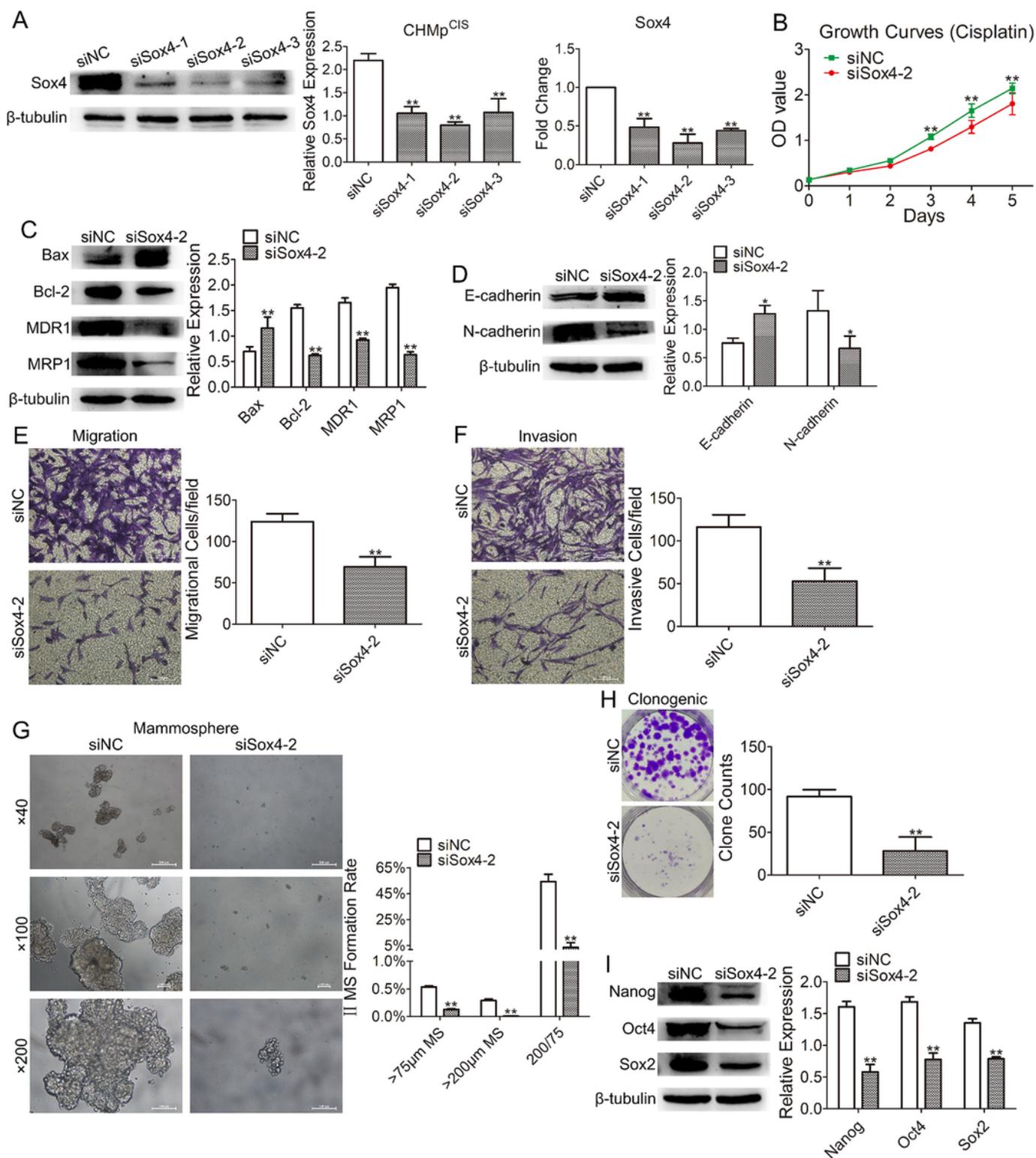


Figure 5

Knockdown of Sox4 expression inhibits cisplatin resistance, EMT and CSC features. A Relative expression levels of Sox4 and Sox4 mRNA in siNC, siSox4-1, siSox4-2, and siSox4-3 cells (n=3). B Comparison of the proliferation ability of siNC and siSox4-2 cells exposed to cisplatin (n=6). C Relative expression levels of apoptosis- and drug resistance-related-proteins in siNC and siSox4-2 cells (n=3). D Relative expression levels of EMT-related proteins in siNC and siSox4-2 cells (n=3). E Comparison of the

migration ability of siNC and siSox4-2 cells ($\times 200$ magnification) (n=3). F Comparison of the invasion ability of siNC and siSox4-2 cells ($\times 200$ magnification) (n=3). G Comparison of the mammosphere-forming ability of siNC and siSox4-2 cells ($\times 40$, $\times 100$, $\times 200$ magnification) (n=3). H Comparison of the clonal cell cluster-forming ability of siNC and siSox4-2 cells (n=3). I Relative expression levels of CSC-related proteins in siNC and siSox4-2 cells (n=3). $0.01 < *P < 0.05$, $**P < 0.01$.

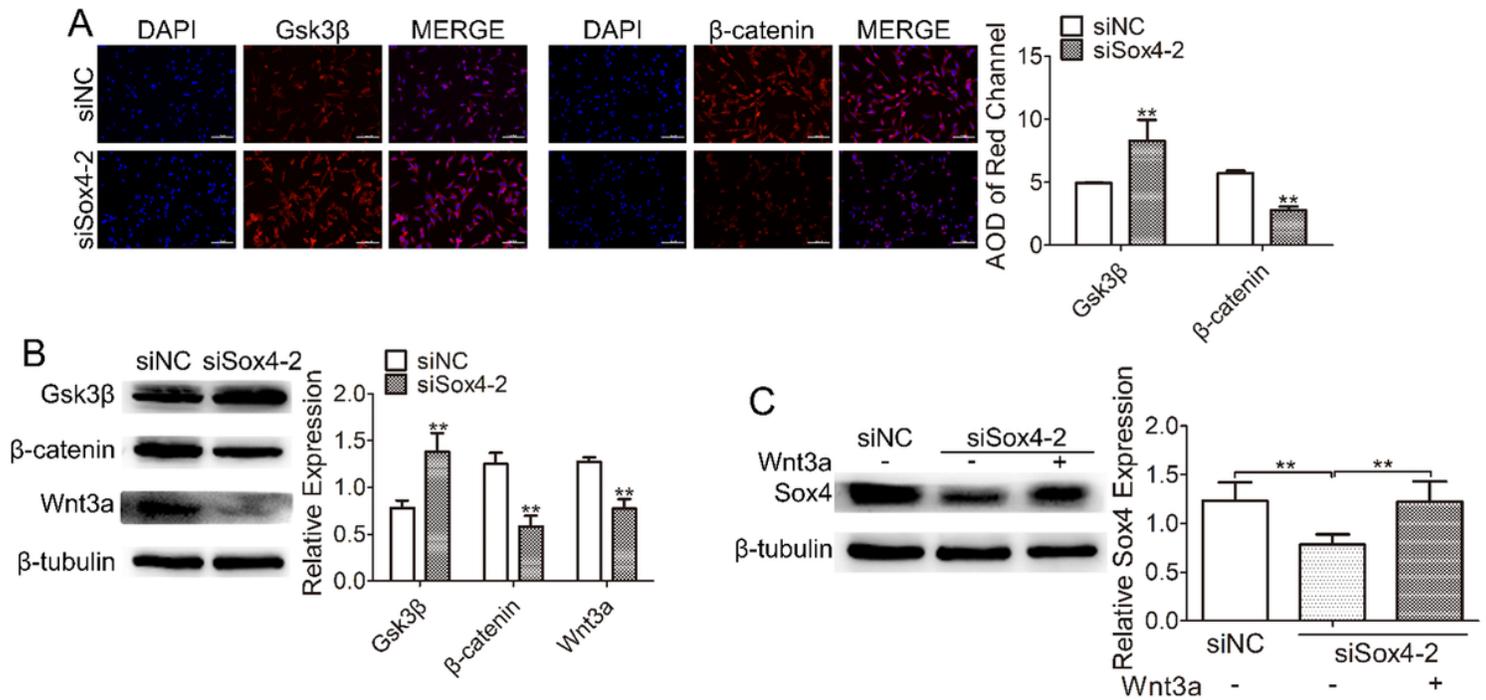


Figure 6

Knockdown of Sox4 expression inhibits Wnt/ β -catenin signal activation. A, B Relative expression levels of Wnt/ β -catenin pathway-related proteins in siNC and siSox4-2 cells (n=3). C Relative expression levels of Sox4 in siSox4-2 cells in the presence or absence of Wnt/ β -catenin pathway activation compared with siNC cells (n=3). $0.01 < *P < 0.05$, $**P < 0.01$.

Supplementary Files

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- [SupplementaryTable1.xlsx](#)