

Original Article



SURF4 maintains stem-like properties via BIRC3 in ovarian cancer cells

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OPEN ACCESS

Received: Mar 9, 2019

Revised: Nov 9, 2019

Accepted: Dec 31, 2019

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ABSTRACT

Objective: As cancer stem cells (CSCs) are considered as the origin of tumor development, recurrence, and drug resistance, we aimed to explore the mechanism related to modulating stemness in CSCs, thus facilitating to search for new therapeutic strategy for ovarian cancer.

Methods: In this study, ovarian cancer stem cells (OCSCs) induced from cell line 3AO and A2780 were enriched in serum-free medium (SFM). The effect of SURF4 on CSC-like properties was evaluated by sphere-forming assays, re-differentiation assays, quantitative real-time polymerase chain reaction, flow cytometry, Western blotting, cell viability assays and in vivo xenograft experiments. The downstream molecule participating in SURF4 maintaining stemness was screened by RNA-sequencing and identified by the experiments of gene function.

Results: SURF4 was upregulated expressed in OCSCs. Knockdown of SURF4 reduced the expression of the related stem markers (SOX2 and c-MYC), inhibited self-renewal ability, and improved the sensitivity to chemotherapeutic drugs (paclitaxel and cisplatin) in OCSCs. SURF4 knockdown also inhibited tumorigenesis in nonobese diabetic/severe combined immunodeficiency mice. BIRC3 expression was controlled by SURF4, and BIRC3 showed the similar effect as SURF4 did, and BIRC3 overexpression partially recovered stem-like properties abolished by SURF4 knockdown.

Conclusion: Our findings suggest that SURF4 possesses the ability to maintain stemness of OCSCs via BIRC3, and may serve as a potential target in stem cell-targeted therapy for ovarian cancer.

Keywords: Ovarian Cancer; Cancer Stem Cell; SURF4; BIRC3

INTRODUCTION

Ovarian cancer is one of the three commonest malignancies and ranks the first lethality in female reproductive system malignancies. Up to 85% of patients are initially diagnosed at advanced stage, due to deep location of ovaries in the pelvis and no early diagnostic techniques [1]. Surgery is preferred option followed by platinum-based combination chemotherapy for the advanced ovarian cancer, but most patients relapse within 2 to 3 years [2]. Moreover, almost all of recurrent ovarian cancers are resistant to re-chemotherapy. The

Funding

The research was supported by the National Natural Science Foundation of China (Grant NO. 81772771) and Key Research and Development Program of Zhejiang Province (2019C03010).

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Author Contributions

Conceptualization: Y.Y.; Data curation: Y.Y., X.L., X.S., W.C., W.X., L.W., X.X.; Formal analysis: Y.Y., X.L., X.S., W.X., L.W.; Funding acquisition: X.X.; Investigation: Y.Y., X.L.; Methodology: Y.Y., X.L., W.C.; Resources: X.L., X.S., W.C.; Supervision: X.X.; Software: Y.Y., X.L., X.S.; Validation: X.L., X.S., W.C., W.X., L.W., X.X.; Visualization: X.L., X.S., W.C., W.X., L.W., X.X.; Writing - original draft: Y.Y.; Writing - review & editing: Y.Y.

median progression-free survival is only 16–22 months and the 5-year survival rate is merely 27% in patients with advanced stage disease [3]. Although many novel drugs and protocols have been applied in practice, the prognosis of advanced ovarian cancer patients has not been remarkably improved in past several decades.

Proposal of the theory of cancer stem cells (CSCs) may change this situation. CSCs are defined as heterogeneous tumor cell subsets which have self-renewal ability, indefinite proliferating power, and strong tumorigenic capacity [4]. CSCs were initially identified in acute leukemia in 1994 [5]. Since then, CSCs have been found in various types of tumors such as breast cancer [6], gastric cancer [7], colon cancer, melanoma [8], and others. Although the proportion of CSCs is extremely low in cancer tissues [9], they are considered as the origin of tumorigenesis, development, recurrence, metastasis, and drug resistance [10]. Therefore, the understanding the mechanisms by which CSCs maintain their stem-like properties in ovarian cancer would facilitate to search for effective therapeutic target for such cancer with poorest prognosis.

In our previous study, using LC-MS/MS label-free quantitative proteomics and bioinformatics, we analyzed the difference in gene expression between ovarian cancer stem cells (OCSCs) and their corresponding parental cells, and found that SURF4 was highly expressed in ovarian OCSCs [11]. It has been known that SURF4 is involved in constituting a transmembrane vector of the endoplasmic reticulum and Golgi transport cargo [12]. Recent studies reported the higher expression of SURF4 in tumor tissues and longer survival in patients with lower expression of SURF4 [13]. The study also showed that SURF4 overexpression enhanced cell proliferation, migration, and anchorage-independent growth *in vitro*, and promoted the tumor growth in mouse induced by NIH3T3 cells [13]. However, little is known about the effect of SURF4 in CSCs.

In this study, we performed function experiment of SURF4, and found a role of SURF4 in maintaining the stemness of OCSCs. To further illustrate the involved mechanism, we further searched for downstream molecules of SURF4 by using RNA-sequencing, bioinformatics analysis, and related function experiments, and discovered that BIRC3 as a downstream regulator participated in SURF4 maintaining stem-like properties of ovarian cancer cells. Our findings suggest that SURF4 may be a potential target in stem cell-targeted therapy for ovarian cancer.

MATERIALS AND METHODS

1. Cell culture, sphere-forming, and re-differentiation assay

The human epithelial ovarian cancer cell line, A2780, was purchased from Sigma. The human ovarian adenocarcinoma cell line, 3AO, was obtained from Women's Hospital, School of Medicine, Zhejiang University, which was tested and authenticated. 3AO and A2780 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (BI, Kibbutz Beit-Haemek, Israel), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, New York, NY, USA) at 37°C and 5% CO₂. The adherent cells were cultured in serum-free medium (SFM) composed of 10uL/mL B27 additive (Life Technologies, Carlsbad, CA, USA), 10 ng/mL, 1 mg/mL insulin (Sigma-Aldrich, Burlington, MA, USA), basic fibroblast growth factor, 20 ng/mL epidermal growth factor (Pepro-Tech, Rocky Hill, CT, USA), Dulbecco's modified Eagle's medium (DMEM/F12) (BI), to form spheroids after plating 5×10⁴ cells per well in ultra-low attachment 6-well culture plates (Corning, New York, NY, USA). The culture medium will be renewed every two or three days. After plating 400 or 600 cells per well in ultra-low

attachment 96-well culture plates (Corning), A2780 or 3AO cells were cultured in SFM at 37°C in 5% CO₂ for 7 days. The culture medium will be renewed every two or three days. The spheroids were cultured in RPMI-1640 medium with 10% FBS after plating in 6 Nunclon Delta plates (Thermo Scientific, Suzhou, China) to re-differentiate.

2. siRNA, plasmid transfection, and lentivirus infection

Small-interfering RNAs (siRNAs) against SURF4 and BIRC3, along with RNAi negative controls, were purchased from Genepharma (Shanghai, China). Transfection were performed on cells with the siRNAs (50 nM) using Lipofectamine™ RNAiMAX (ThermoFisher, Waltham, MA, USA) according to the manufacturer's protocol. The siRNA sequences are listed in the **Table 1**.

The full-length open reading frames with FLAG tag of human BIRC3 (NM_182962) were digested by XhoI and KpnI, then ligated into GV141 vector (GENECHEM, Shanghai, China). DNA sequencing was applied to verify sequences of all the constructs. The primer sequences are listed in the **Table 1**. Cells were transfected with plasmid using X-treme GENE HP DNA transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol.

The short-hairpin RNAs (shRNA) for SURF4 oligonucleotides were cloned into hU6-MCS-CMV-puromycin lentivirus expression vectors (GENECHEM, Shanghai, China) between the AgeI and EcoRI sites and then transfected into cells according to instructions. After 72 hours of transfection, the cells were selected using 2 µg/mL puromycin for 4 days. All construct sequences were confirmed using DNA sequencing. The shRNA oligonucleotides are listed in **Table 1**.

3. RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

RNA extraction kit (TaKaRa, Dalian, China) was applied to extract the total RNA. The products were reverse-transcribed into cDNA using the reverse transcription cDNA kit (TaKaRa). SYBR® Premix Ex Taq™ (TaKaRa) and applied biosystems 7900HT fast real-time PCR system (Life Technologies) were used to carry out PCR reactions. The relative mRNA expression was calculated through the 2- $\Delta\Delta C_t$ method and normalized to β -actin expression. Primer sequences are listed in the **Table 1**.

Table 1. Sequences used in this study

Sequences	
Primers for reverse transcriptase polymerase chain reaction	
β -actin	Forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' Reverse: 5'-GGCTGTTGCATACTTCTCATGG-3'
SURF4	Forward: 5'-CCTTTAAGGCTTGGCCTACG-3' Reverse: 5'-GGGCCAGGTTCCATCAAA-3'
BIRC3	Forward: 5'-AAGCTACCTCTCAGCCTACTTT-3' Reverse: 5'-CCACTGTTTTCTGTACCCGGA-3'
c-MYC	Forward: 5'-GTCAAGAGGCGAACACACAAC-3' Reverse: 5'-TTGGACGGACAGGATGTATGC-3'
SOX2	Forward: 5'-GCCGAGTGGAAACTTTGTGCG-3' Reverse: 5'-GGCAGCGTACTTATCCTTCT-3'
Primers for plasmid sequencing	
pcDNA3.1	Forward: ACGGGCCCTCTAGACTCGAGCGCCACCATGAACATAGTAGAAAACAGCATATTC Reverse: AGTCACTTAAGCTTGTACCGATGAAAGAAATGTACGAACGTACCCTTG
Sequences used for siRNAs	
SURF4	Sense 5'-CCUUCGUCUCCUCAACUUTT-3'
BIRC3	Sense 5'-GCCUUGAUGAGAAGUUCUTT-3'
Lentivirus target sequence	
SURF4	GTCCTCGTCTTCTCAACTT

4. Western blot analysis

Cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) with Phenylmethanesulfonyl Fluoride (Beyotime). The cell lysates were denatured before separated on sodiumdodecyl sulfate polyacrylamide gel (SDS-PAGE) and then shifted onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% non-fat milk dissolved in Tris buffered saline supplemented with Tween 20 (TBST) for 1 hour, and immunoblotted with primary antibodies overnight at 4°C. The membranes were washed thrice using TBST for 10 minutes each time and immunoblotted with secondary antibodies for 1 hour at room temperature and then washed thrice with TBST for 10 minutes each time. The protein bands were detected using FDbio-Dura ECLkit (Hangzhou Fude Biotechnology Co., Ltd., Hangzhou, China) in Image quant LAS400 mini (GE Healthcare, Munich, Germany). Primary antibodies against SURF4 (Abcam, Cambridge, MA, USA), BIRC3 (Sigma), c-MYC (CST, Danvers, MA, USA), and SOX2 (Sigma), were used. β -actin (Multi Sciences, Hangzhou, China) was used as the loading control.

5. RNA-sequencing analysis

Total RNAs were extracted from spheroids induced from 3AO cells transfected with siSURF4 or control siRNA which were cultured in SFM for 7 days. High-quality RNA from siSURF4 and control groups assessed by the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) were prepared for transcriptome sequencing. The samples were handled by TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and performed on a cBot Cluster Generation System. The generated library preparations were sequenced on an Illumina Hiseq platform followed by the generation of 125 bp/150 bp paired-end reads.

The clean data were obtained from the raw data of FASTQ format by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data through in-house perl scripts. Then, the Q20, Q30, and GC contents of the clean data were analyzed. Paired-end clean reads were applied to the reference genome through STAR. HTSeq v0.6.0 was applied to calculate the reads numbers mapped to each gene. Differential expression analysis of these groups was performed using the DESeq2 R package (1.10.1). The Benjamini and Hochberg's approach were used to adjust the resulting p-values for controlling the false discovery rate. Genes with an adjusted p-value < 0.05 which were distinguished by DESeq2, were assumed to be differentially expressed.

6. Cell viability assays

Cells were transfected with siRNA or plasmid for 48 hours, cultured in SFM culture condition for 7 days. Then 6,000 cells per well cells were seeded in 96-well plates followed by exposing to paclitaxel or cisplatin at various concentrations for 48 hours after seeded into the plates. Then 10 μ L CCK8 (Dojindo, Shanghai, China) was added to each well and cells were incubated at 37°C for 2–4 hours. Varioskan Flash microplate reader (Thermo Scientific) was used to measure OD values at 450 nm. The cell viability was determined from the absorbance values compared with the control group.

7. Flow cytometry analysis

Spheroids induced from 3AO cells were filtered through 40 μ m mesh to remove cell fragments, centrifuged and digested using trypsin. 1×10^6 cells were resuspended in 100 μ L PBS after washed and counted. Then the cell suspension was stained with either anti-CD44-FITC (eBiosciences, Vienna, Austria), anti-CD24-APC (eBiosciences) antibodies or isotype control antibodies (eBiosciences), and incubated for 30 minutes at room temperature

according to the manufacturer's protocol. FC 500 series flowcytometer (Beckman Coulter, Brea, CA, USA) was used to detect the processed cells. And the subsequent data was analyzed through the CXP 2.1 software.

8. ALDEFLUOR assay

ALDEFLUOR assay kit (STEMCELL Technologies Inc., Vancouver, Canada) was performed on spheroid cells induced from A2780 cells following the manufacturer's protocol. After filtered and trypsinized, cells were re-suspended in ALDEFLUOR assay buffer containing ALDH enzyme substrate BODIPY-aminoacetaldehyde (BAAA) and control buffer containing the specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB) serving as a negative control, then incubated at 37 °C for about 40 minutes. FC 500 series flow cytometer (Beckman Coulter, Brea, CA, USA) was used to detect the processed cells. And the subsequent data was analyzed through the CXP 2.1 software.

9. In vivo xenograft experiments

The animal experiment was performed according to Animal Research Reporting In Vivo Experiments guidelines for the use of laboratory animals and were approved by the ethics committee of Zhejiang Chinese Medical University. Nonobese diabetic/severe combined immunodeficiency mice were purchased from the Shanghai SLAC Laboratory Animal Co., LTD (Shanghai, China) and divided into each treatment group randomly. For experiments, 1×10^7 A2780 cells transfected with shSURF4 or shNC were resuspended in 100 μ L PBS and injected into the left flank of mice. Tumor volume was examined by caliper measurement every 3 days and calculated according to the following formula: $V = a \times b^2 \times 0.5$, where V, volume (mm^3); a, the largest diameter (mm); b, smallest diameter (mm).

10. Statistical analysis

Statistical comparison between groups were carried out using two-tailed Student's t-tests unless otherwise indicated. The SPSS software, version 20.0 (SPSS Inc., IBM, Armonk, NY, USA) or with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to perform statistical tests. A p-value of less than 0.05 was considered as significant.

RESULTS

1. Up-regulated SURF4 expression in OCSC-like cells

In a previous proteomic study of ours, we found up-regulated expression of SURF4 protein in 3AO spheroids that derived from parental ovarian cancer cell line 3AO [11]. In order to confirm it, we cultured ovarian cancer cells, 3AO and A2780, in the serum-free culture condition to induce cells into spheroid clusters, and observed the change of SURF4 mRNA and protein expression in the process of spheroid formation of cells. As shown in **Fig. 1A**, we found that the expression of SURF4 mRNA and protein showed an increasing trend when adherent cells gradually became suspensive, denser and bigger spheroids with stronger shading ability. Consistently, the expression levels of stem-related markers, SOX2 and c-MYC, were increased during this process. In contrast, we also observed the change of SURF4 mRNA and protein expression when 3AO and A2780 spheroids were re-differentiated in medium with 10% serum. As shown in **Fig. 1B**, the expression of both SURF4 mRNA and protein showed decreasing trend following spheroids growth from floating into adherent state. Consistently, the expressions of stem-related markers, SOX2 and c-MYC, were decreased during this process. Our results suggest that SURF4 expression is up-regulated in OCSC-like cells.

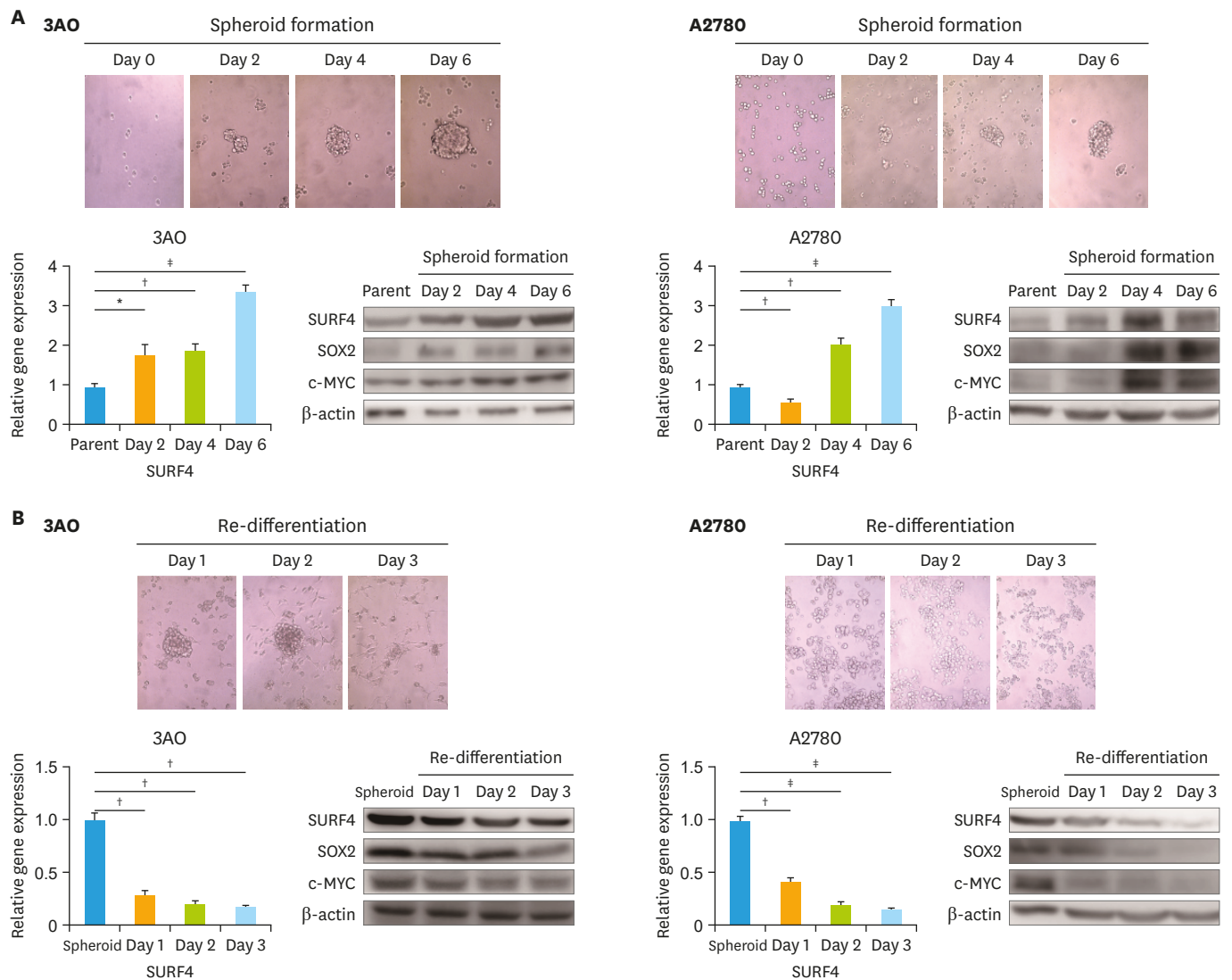


Fig. 1. SURF4 expression is up-regulated in ovarian cancer stem cell-like cells. (A) Relative expression levels of SURF4, SOX2, and c-MYC mRNA and protein at day 0, 2, 4, and 6 during spheroid formation from parental 3AO and A2780 cells to spheroids growing in serum-free medium with growth factors, measured by RT-PCR (3AO-SURF4 $p=0.018$ [day 2], 0.004 [day 4], 0.0007 [day 6]; A2780-SURF4 $p=0.0068$ [day 2], 0.005 [day 4], 0.0006 [day 6]) and Western blotting, respectively. (B) Relative expression levels of SURF4, SOX2, and c-MYC mRNA (3AO-SURF4 $p=0.004$ [day 1], 0.002 [day 2], 0.002 [day 3]; A2780-SURF4 $p=0.002$ [day 1], 0.0005 [day 2], 0.0005 [day 3]) and protein at day 1, 2, and 3 when 3AO and A2780 spheroids were re-differentiated in medium with 10% serum, measured by RT-PCR and Western blotting, respectively. Data represents the mean \pm standard error of three independent experiments (Scale bar, 50 μ m). RT-PCR, reverse transcriptase polymerase chain reaction. The level of significance is indicated by * $p<0.05$, † $p<0.01$, ‡ $p<0.001$.

2. SURF4 positively modulates stem-like properties in ovarian cancer cells

Since SURF4 expression was found to be up-regulated in spheroid cells, SURF4 may act as a potential modulator in stem-like property of ovarian cancer cells. Thus, we conducted a specific siRNA to suppress SURF4 expression in 3AO and A2780 cells, which were cultured to form spheroids in serum-free medium with growth factors for 7 days. As **Fig. 2** showed, SURF4 protein level was successfully inhibited by specific siRNA. As we expected, SURF4 knockdown inhibited the expression of stem-related markers, SOX2 and c-MYC, in spheroids (**Fig. 2A**), decreased the proportion of CD44⁺CD24⁻ cells from 82.4% to 56.8% in 3AO spheroids and ALDH⁺ cells from 79.8% to 43.21% in A2780 spheroids (**Fig. 2B**), weakened the spheroid forming ability of cells (**Fig. 2C**), increased the sensitivity of cells to paclitaxel

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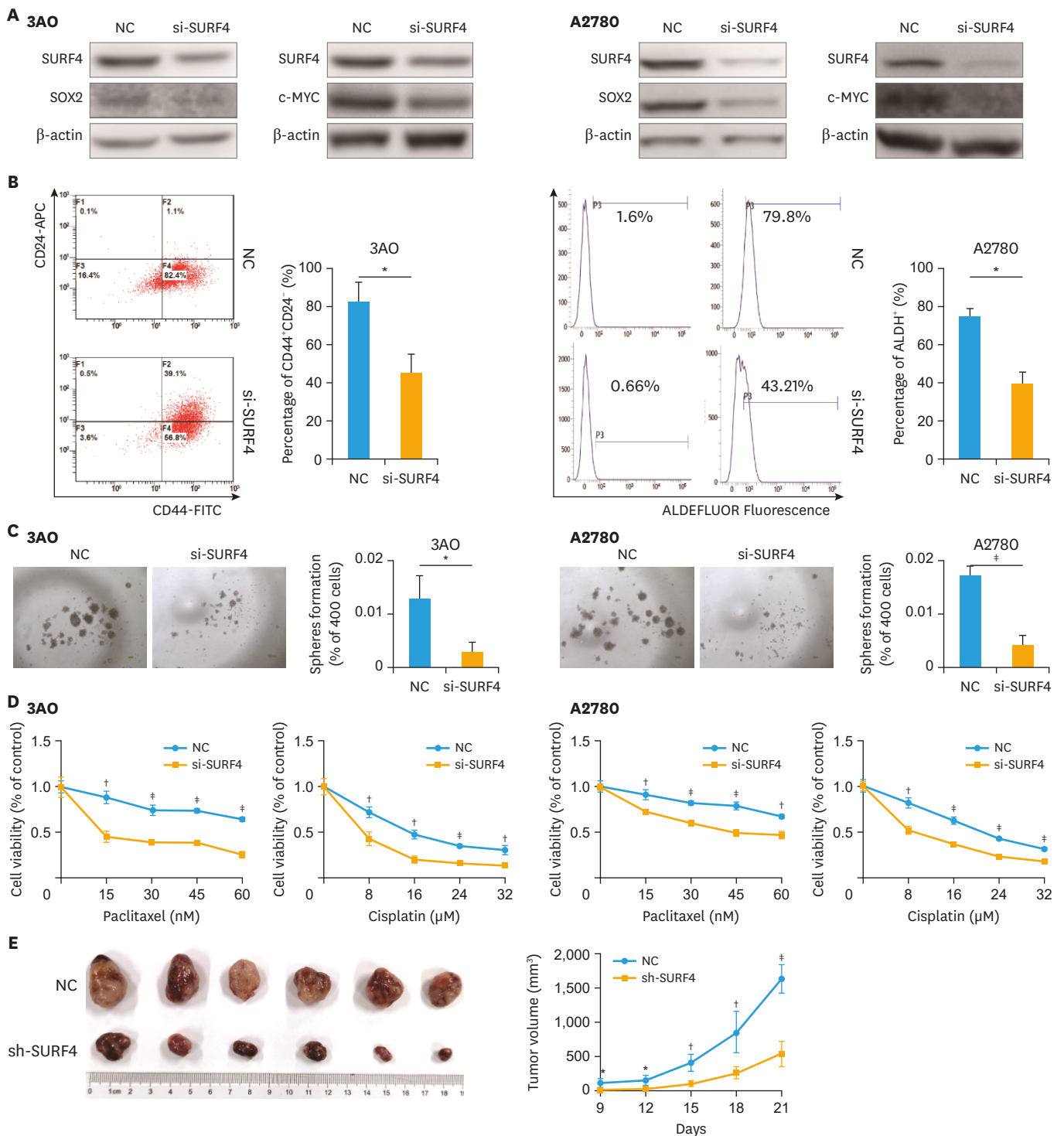


Fig. 2. SURF4 positively modulates stem-like properties in ovarian cancer cells. 3AO and A2780 cells were transfected with SURF4 siRNA for 48 hours, and cultured in serum-free culture condition for 7 days. (A) The expression of SURF4 and stem-related markers, SOX2 and c-MYC, were detected by Western blotting analysis. (B) The proportion of CD44⁺CD24⁻ phenotype in 3AO spheroids and ALDH⁺ phenotype in A2780 spheroids was analyzed by FCM (3AO $p=0.044$; A2780 $p=0.011$) (left: representative images, right: quantitative analysis). (C) Representative images (left) and quantitative analysis (right) of spheroid formation from parental cells (sphere >50 μm) were shown (3AO $p=0.033$; A2780 $p=0.0008$; Scale bars, 50 μm). (D) The sensitivity of cells to paclitaxel and cisplatin was analyzed by CCK8 (3AO-Paclitaxel $p=0.001, 0.0006, <0.0001, <0.0001$; 3AO-Cisplatin $p=0.007, 0.001, <0.0001, 0.004$; A2780-Paclitaxel $p=0.007, 0.0003, 0.0006, 0.001$; A2780-Cisplatin $p=0.002, 0.0005, <0.0001, <0.0001$). (E) The effect of SURF4 knockdown on the growth of ovarian cancer cells in vivo was analyzed by inoculating A2780 cells into nude mice ($p=0.036, 0.029, 0.004, 0.009, 0.0002$). Data represents the mean±standard error of three independent experiments. The level of significance is indicated by * $p<0.05$, † $p<0.01$, ‡ $p<0.001$ (not significant, $p>0.05$).

and cisplatin (**Fig. 2D**). Then we investigated the effect of SURF4 knockdown on ovarian tumorigenicity. As shown in **Fig. 2E**, in vivo tumor growth was significantly impaired upon SURF4 knockdown. These results suggest that SURF4 positively modulates stem-like properties in ovarian cancer cells.

3. BIRC3 as a downstream molecule for SURF4

As SURF4 played a modulating role in stem-like properties of ovarian cancer cells, we attempted to explore the downstream molecule of SURF4. We detected the differentially expressed mRNA using RNA-sequencing technique, and found 233 differentially expressed genes (<0.05 -Padj), including 58 up-regulated genes and 175 down-regulated genes, by DESeq2 software analysis (**Fig. 3A**). Of those, BIRC3, was shown to be particularly down-regulated (0.000147 -Padj) and has been demonstrated to be associated with stemness of human adipose-derived mesenchymal stem cells [14] and chemoresistance in chronic lymphocytic leukemia [15]. Thus, we used qPCR and Western blotting to determine the indicated gene expression in 3AO and A2780 cells. Results showed that the expression levels of BIRC3 mRNA and protein were inhibited after SURF4 knockdown (**Fig. 3B**). But, SURF4 expression remained unchanged when BIRC3 knockdown or over-expression (**Fig. 3C**). The results suggest that BIRC3 is a downstream molecule of SURF4 and may participate in SURF4 modulating stemness in ovarian cancer cells.

4. BIRC3 positively modulates stem-like properties in OCSC-like cells

To verify that BIRC3 participates in SURF4 modulating stem-like properties in ovarian cancer cells, we inhibited BIRC3 expression by specific siRNA in 3AO and A2780 spheroids cultured in serum-free medium, and found that the relative expression levels of stem-related proteins, SOX2 and c-MYC, were reduced (**Fig. 4A**), the proportion of CD44⁺CD24⁻ and ALDH⁺ cells were decreased, from 79.22% to 48% and 79.8% to 36.96%, respectively (**Fig. 4B**), the capability in spheroid formation was diminished (**Fig. 4C**), and the sensitivity of cells to paclitaxel and cisplatin was increased (**Fig. 4D**) in both spheroids with BIRC3 knockdown. Our results suggest that BIRC3 positively modulates stem-like properties, as SURF4 did, in OCSC-like cells.

5. BIRC3 participates in the SURF4-mediated modulation of stem-like properties in ovarian cancer cells

Further, we conducted a rescue experiment. We enforcedly over-expressed BIRC3 after SURF4 knockdown in 3AO and A2780 spheroids, and found that BIRC3 overexpression reversed, at least partially, stem-like properties that were reduced by SURF4 knockdown. As **Fig. 5** showed, inhibited BIRC3, SOX2, and c-MYC were over-expressed, de novo (**Fig. 5A**). The proportion of CD44⁺CD24⁻ cells in 3AO spheroids varied from 81.11% to 39.16% following SURF4 knockdown, then rose to 54.5% by BIRC3 overexpression, similarly, ALDH⁺ cells in A2780 spheroids varies from 64.62% to 39.23% after SURF4 knockdown, then turned to 57.19% by BIRC3 overexpression (**Fig. 5B**). Moreover, weakened spheroid formation ability of 3AO and A2780 cells caused by SURF4 knockdown was enhanced again by BIRC3 overexpression (**Fig. 5C**). Finally, SURF4 knockdown elevated the sensitivity of 3AO and A2780 spheroids to chemotherapeutic drugs, but BIRC3 overexpression attenuated partially the sensitivity resulted from SURF4 knockdown (**Fig. 5D**). Our results suggest that BIRC3, as a downstream molecule, participates in the SURF4-mediated modulation of stem-like properties in ovarian cancer cells.

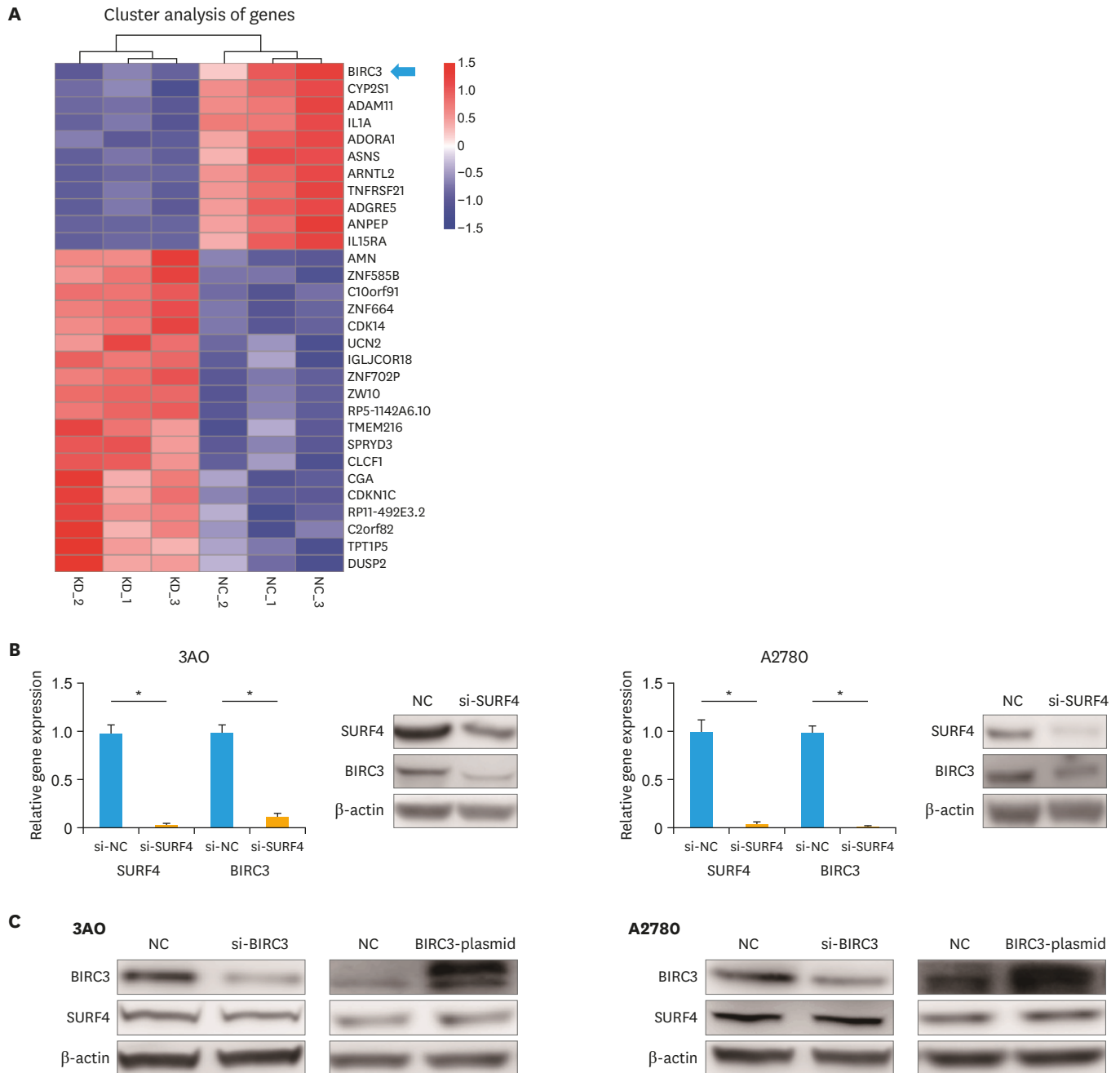


Fig. 3. BIRC3 is a downstream molecule for SURF4. (A) RNA-sequencing data from the analysis between 3AO spheroids with SURF4 knockdown and ones without (NC). The cluster map generated by DESeq2 software analysis displayed a series of differentially expressed genes (Padj<0.05). Down-regulated genes are shown as green color and up-regulated gene are shown as red color. The intensity of the green or red color is consistent with the degree of alteration, referring to the color strip beside the figure. The arrow points to BIRC3. (B) BIRC3 mRNA and protein expression in 3AO and A2780 spheroids after SURF4 knockdown, detected by quantitative real-time polymerase chain reaction (3AO p=0.002, 0.002; A2780 p=0.004, 0.001) and western blotting, respectively. (C) The expression of SURF4 protein in 3AO and A2780 spheroids after BIRC3 knockdown or BIRC3 overexpression. Data represents the mean±standard error. of three independent experiments. The level of significance is indicated by *p<0.01.

DISCUSSION

The flow cytometry (FCM) is one of the commonly used methods for isolating, enriching and identifying CSCs through detecting specific markers on cell surface [16]. CD24,

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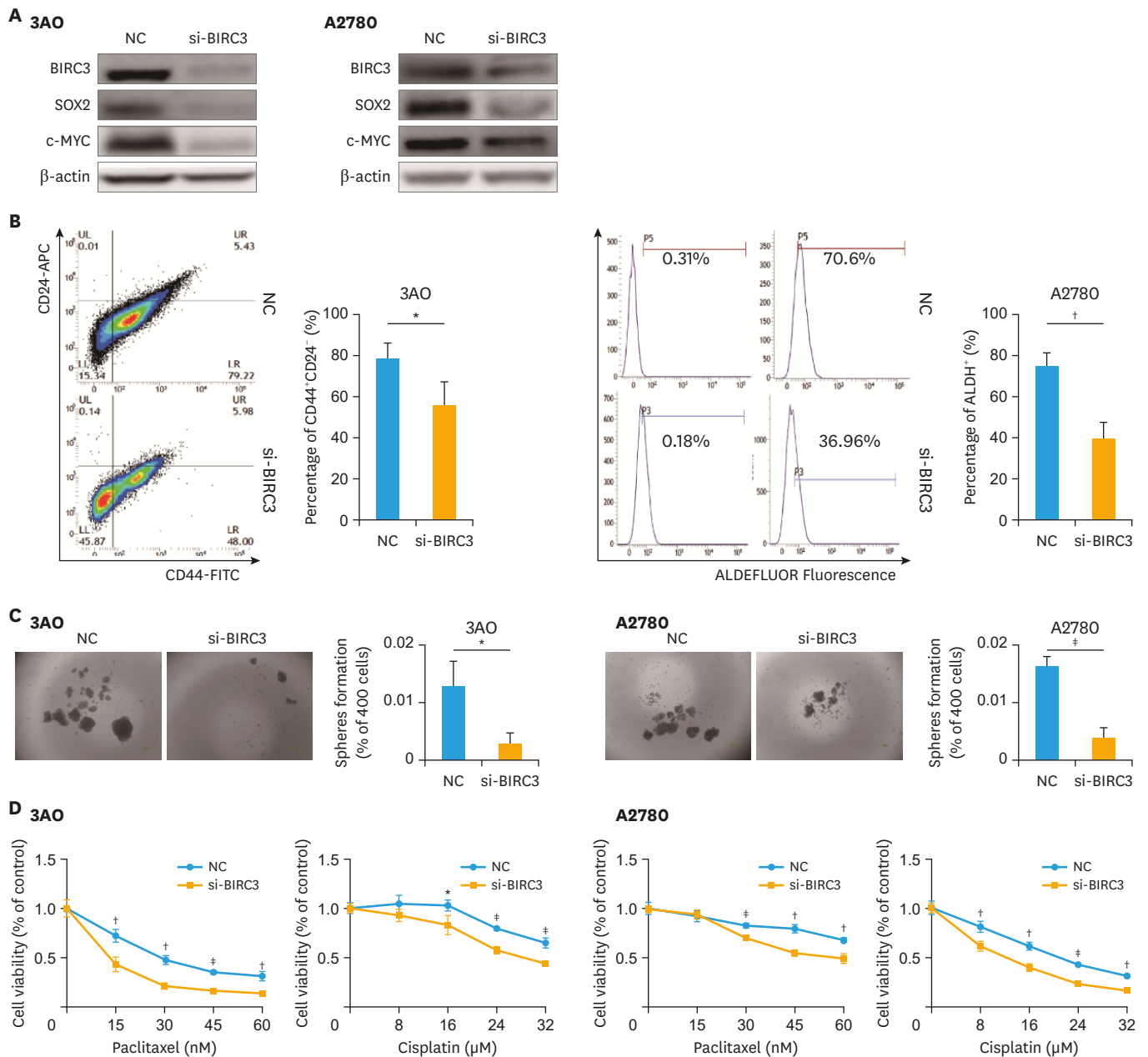


Fig. 4. BIRC3 positively modulates stem-like properties in ovarian cancer stem cell like cells. 3AO and A2780 spheroids were transfected with BIRC3 siRNA for 48 hours, and cultured in SFM culture condition for 7 days. (A) The expression of BIRC3 and stem-related markers, SOX2 and c-MYC, were detected by western blotting analysis. (B) The proportion of CD44⁺CD24⁻ phenotype in 3AO spheroids and ALDH⁺ phenotype in A2780 spheroids was analyzed by FACS (3AO p=0.031; A2780 p=0.007). (C) Representative images (left) and quantitative analysis (right) of spheroid formation (sphere >50 μm) were shown (3AO p=0.013; A2780 p=0.0004; Scale bars, 50 μm). (D) The sensitivity of cells to paclitaxel and cisplatin was detected by CCK8 (3AO-Paclitaxel p=0.007, 0.001, <0.0001, 0.004; 3AO-Cisplatin p=0.114, 0.034, 0.0007, 0.0003; A2780-Paclitaxel p=0.664, 0.0005, 0.001, 0.003; A2780-Cisplatin p=0.009, 0.002, 0.0002, 0.001). Data represents the mean±standard error. of three independent experiments. The level of significance is indicated by *p<0.05, †p<0.01, ‡p<0.001 (not significant, p>0.05).

CD44, CD133, and other markers are usually used to identify CSCs [17,18], but different CSCs express respective markers. For instance, the characteristics of breast CSCs is ESA⁺CD44⁺CD24^{-/low} [19], while leukemia stem cells exhibit CD34⁺CD38⁻ phenotype [20]. Our previous studies identified that CD44⁺CD24⁻ cells derived from 3AO cells exhibited stem-like properties [17], while other studies found that ALDH⁺ cells induced from A2780 cells showed

Cancer stemness maintained by SURF4

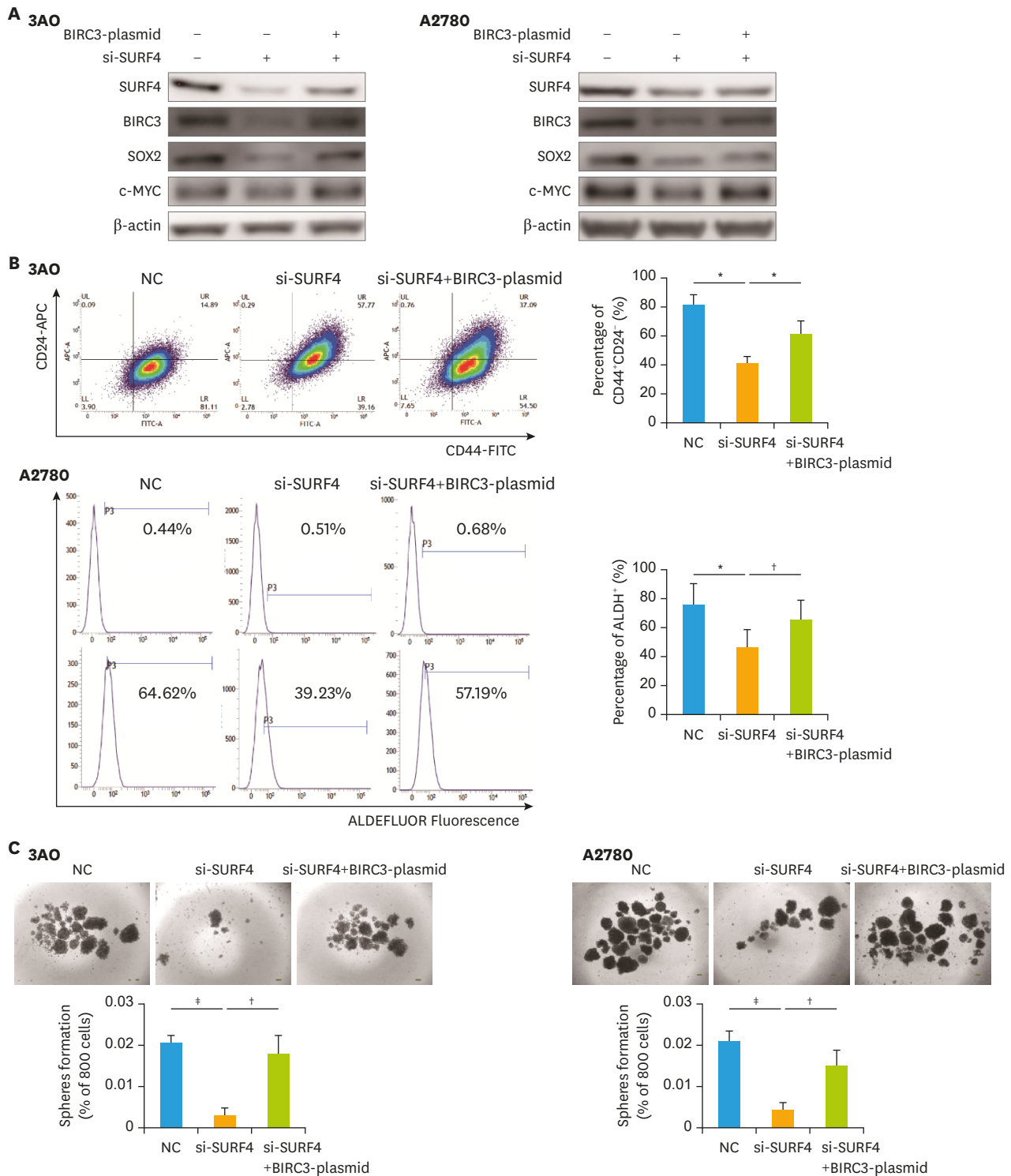


Fig. 5. BIRC3 participates in SURF4-mediated modulation of stem-like properties in ovarian cancer cells. 3AO and A2780 spheroids were transfected with SURF4 siRNA, SURF4 siRNA plus BIRC3 overexpression plasmid, or without anyone as control for 48 hours and cultured in SFM culture condition for 7 days. (A) The indicated stem-markers, SOX2 and c-MYC, were detected by western blotting. (B) The proportion of CD44⁺CD24⁺ phenotype in 3AO spheroids and ALDH⁺ phenotype in A2780 spheroids were detected by FACS (3AO $p=0.010$, 0.014 ; A2780 $p=0.034$, 0.001). (C) Sphere formation (sphere >50 μm) was assessed (3AO $p=0.0001$, 0.003 ; A2780 $p=0.0002$, 0.005 ; Scale bars, 50 μm .) (D) The sensitivity of cells to paclitaxel and cisplatin was analyzed by CCK8 (3AO-Paclitaxel $p=0.021$, 0.037 , 0.021 , 0.0003 ; 3AO-Cisplatin $p=0.017$, 0.0003 , 0.006 , 0.011 ; A2780-Paclitaxel $p=0.003$, 0.113 , 0.021 , <0.0001 , 0.002 ; A2780-Cisplatin $p=0.012$, 0.0008 , 0.002 , <0.0001). Data represents the mean \pm standard error. of three independent experiments. The level of significance is indicated by * $p<0.05$, † $p<0.01$, ‡ $p<0.001$.

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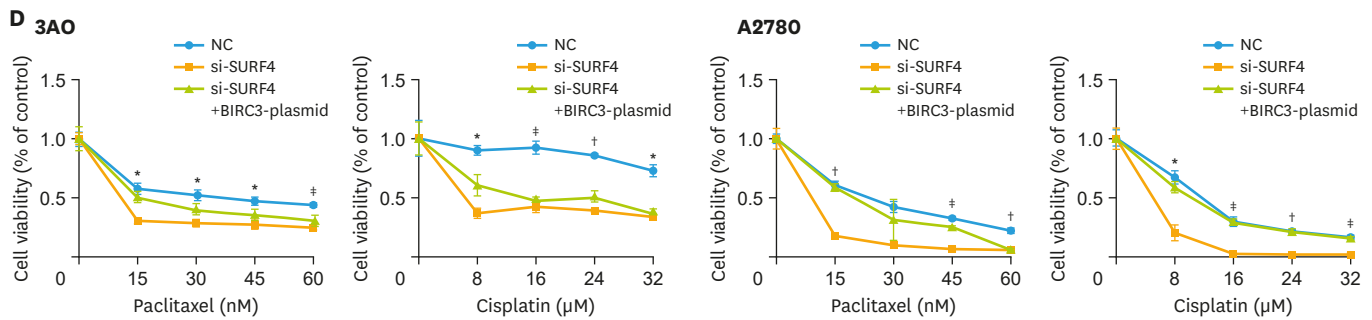


Fig. 5. (Continued) BIRC3 participates in SURF4-mediated modulation of stem-like properties in ovarian cancer cells. 3AO and A2780 spheroids were transfected with SURF4 siRNA, SURF4 siRNA plus BIRC3 overexpression plasmid, or without anyone as control for 48 hours and cultured in SFM culture condition for 7 days. (A) The indicated stem-markers, SOX2 and c-MYC, were detected by western blotting. (B) The proportion of CD44⁺CD24⁻ phenotype in 3AO spheroids and ALDH⁺ phenotype in A2780 spheroids were detected by FACS (3AO p=0.010, 0.014; A2780 p=0.034, 0.001). (C) Spheroid formation (sphere>50 μm) was assessed (3AO p=0.0001, 0.003; A2780 p=0.0002, 0.005; Scale bars, 50 μm.) (D) The sensitivity of cells to paclitaxel and cisplatin was analyzed by CCK8 (3AO-Paclitaxel p=0.021, 0.037, 0.021, 0.0003; 3AO-Cisplatin p=0.017, 0.0003, 0.006, 0.011; A2780-Paclitaxel p=0.003, 0.113, 0.021, <0.0001, 0.002; A2780-Cisplatin p=0.012, 0.0008, 0.002, <0.0001). Data represents the mean±standard error. of three independent experiments. The level of significance is indicated by *p<0.05, †p<0.01, ‡p<0.001.

stem-like traits [2]. Therefore, using different markers is needed to enrich and identify CSCs from different tumors. In this study, we cultured 3AO and A2780 cells in serum-free medium to induce cells with CD44⁺CD24⁻ and ALDH⁺ phenotype, respectively, and then confirmed that both types of cells possess stem-like properties by means of the spheroid-formation assay, re-differentiation assay, and the detection of the related stem markers (SOX2, c-MYC), thus laying the foundation for further follow-up experiments.

Previous studies have illustrated that SURF4 is highly expressed in tumor tissues and associated with the prognosis of cancer patients [13], but the role of SURF4 in CSCs remains largely unclear. Therefore, we examined the expression of SURF4 in the process of spheroid-formation of 3AO and A2780 cells and re-differentiation of 3AO and A2780 spheroids, and found that the expression of SURF4 and stem markers (SOX2, c-MYC) showed consistently changed tendency during two opposite processes. Further, we down-regulated the expression of SURF4 in OCSCs and ovarian cancer cells, and found that the expression of the related stem markers, self-renewal ability, resistance to chemotherapeutic drugs and *in vivo* tumorigenicity were attenuated, suggesting that SURF4 functions in maintaining the stem-like properties of OCSCs.

To further determine downstream molecular pathways involved in SURF4 regulating stemness, we used RNA-sequencing assay and confirmatory experiment to examine differentially expressed genes, and found that the BIRC3 expression was decreased after SURF4 downregulation in OCSCs. As a member of the inhibitors of apoptosis proteins (IAP) family, BIRC3 not only modulates apoptosis, but also associates with immunity regulation, inflammatory pathway, mitogenic kinase signaling, cell invasion and metastasis, as well as cell proliferation [21,22]. Our function experiments showed that the expression of the related stem markers, self-renewal ability, and resistance to chemotherapeutic drugs were reduced following the knockdown of BIRC3 in OCSCs. What is more, we found that some deprived stemness properties induced by SURF4 knockdown, such as reduced expression of stem markers, inhibited self-renewal ability, and decreased chemoresistance, were partially reversed by BIRC3 overexpression, suggesting that BIRC3 participates in the process of SURF4 modulating stem-like properties in ovarian cancer cells.

Taken our results together, SURF4 was highly expressed in OCSCs. Knockdown of SURF4 reduced the expression of the related stem markers, inhibited self-renewal ability, and

improved the sensitivity to chemotherapy in OCSCs and tumor growth induced by ovarian cancer cells. BIRC3 showed the similar effect as SURF4 did, and BIRC3 overexpression partially recovered stem-like properties abolished by SURF4 knockdown. As CSCs are considered to be the primary source leading to the genesis, development, invasion, metastasis, recurrence, and chemo-resistance of cancers in a series of CSC studies, the results from studies on CSCs could be translated into useful approaches in clinical, particularly in the development of drug that control or eliminate CSC populations, therefore leading to much more effective cancer therapies. Our findings suggest that SURF4 possesses the ability to maintain stemness of OCSCs via BIRC3, and may serve as a potential target in stem cell-targeted therapy for ovarian cancer.

ACKNOWLEDGMENTS

We thank our colleagues for providing valuable discussions. We also appreciate the experts reading our manuscript and the editors giving our article a chance to be published.

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