

# Rps15a Imparts Chemoresistance by Regulating Cd44 Expression and Cell Stemness in Esophageal Squamous Cell Carcinoma

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## Research Article

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# Abstract

Chemotherapy is one of the effective ways to treat esophageal squamous cell carcinoma (ESCC), but the development of chemoresistance during chemotherapy lowers drug efficacy. Although previous studies have shown that the ribosomal protein S15A (RPS15A) involved in the progression and overall survival of malignancies, its function in chemoresistance is unknown. This study sought to elucidate the function of RPS15A in chemoresistance in ESCC. Our results show that knocking down or overexpressing RPS15A in ESCC cell lines can significantly change the sensitivity of chemotherapeutic drugs and affect cisplatin-induced apoptosis. Moreover, an increase in chemotherapeutic drug concentration leads to increased expression of RPS15A and CD44 proteins. When we utilized the ESCC cisplatin-resistant cell line to corroborate our findings, we found that the levels of RPS15A and CD44 proteins were substantially greater in daughter than parental cells. Subsequent experiments indicated that RPS15A modulated chemoresistance by controlling the expression of CD44 and the cell stemness in ESCC. Hence, our data suggest that RPS15A participates in the chemoresistance in ESCC by controlling the expression of CD44 and regulating cell stemness. Taken together, our study provides plausible mechanisms for RPS15A-mediated chemoresistance in ESCC cells and suggests that the inhibition of the RPS15A/CD44 pathway may be a potential target for improving chemotherapy efficacy.

## Introduction

Esophageal cancer currently accounts for a sixth of all cancer-related deaths across the world,[1] and esophageal squamous cell carcinoma (ESCC), for which effective treatment options are limited, is the most prevalent pathological type in China. Chemotherapy is the main treatment strategy for advanced unresectable/metastatic ESCCs.[2, 3] However, the emergence of acquired resistance to chemotherapy is the main cause of chemotherapy failure. The causes of acquired resistance are complex, so elucidation of the specific mechanisms by which acquired resistance occurs is urgently needed.

The highly conserved 40S ribosomal protein S15A (RPS15A) is vital for cellular growth, differentiation and apoptosis.[4] As an oncogene, RPS15A is involved in the progression and overall survival of malignancies such as hepatocellular carcinoma,[5] non-small cell lung cancer,[6] and gastric carcinoma.[7] RPS15A also plays an important role in the regulation of oncogenic signal transduction cascades, for example, Wnt/ $\beta$ -catenin,[5] p53[8] and Akt/IKK- $\beta$ /NF- $\kappa$ B.[7] These findings suggest that new cancer therapies could target RPS15A. It is unclear what, if any, role RPS15A plays in the progression of ESCC; therefore, this study explored the possible functional mechanism of RPS15A in ESCC.

CD44 is involved in various cellular processes,[9] especially in stemness maintenance and chemoresistance.[10] The activation of CD44 correlates with radiation resistance[11] and chemoresistance[12, 13]. Targeting CD44 has been shown to be effective in cancer treatment.[14, 15] With continuous chemotherapy, increased expression of CD44 contributes to chemoresistance and correlates with poor prognosis in ESCC.[16] Therefore, strategies that modulate the expression of CD44 may be beneficial in the treatment of ESCC.

We detected a significant association between the expression of RPS15A and chemoresistance when we modified the expression of RPS15A in ESCC cells with or without chemotherapy drugs. We also found that RPS15A could regulate the expression of CD44 to maintain ESCC cell stemness and impart chemoresistance. Our results highlight the importance of RPS15A in chemoresistance, which may lead to possible novel mechanisms for reversing chemoresistance in ESCC.

## Materials And Methods

### Cell lines and culture conditions

TE-1, Kyse510, Kyse450, Kyse140, Eca109, and Eca109/CDDP cells were grown in RPMI-1640 media (Gibco, 22400089, USA) containing 10% fetal bovine serum (Excell Bio, FND500, New Zealand), 100 U/mL penicillin/streptomycin (Gibco, 15140122, USA). Add 1 µg/mL CDDP (HANSOH PHARMA, China) to Eca109/CDDP cell medium to maintain its resistance to chemotherapy drugs.

### Flow cytometry analysis of cell apoptosis

Cells were incubated with CDDP for 24 hours, then harvested and washed with cold PBS prior to staining with AnnexinV Alexa Fluor647/PI (FXP023-100, 4A Biotech, China). Apoptosis was measured by flow cytometry using a FACSCanto analyzer (BD, USA).

### Cell viability analysis

The effect of chemotherapeutic drugs on the viability of ESCC cells was measured using CCK-8 (CK04-3000T, Dojindo, Japan). In brief, ESCC cells were plated into 96-well cell culture dishes (Excell Bio) at 3000 cells/per well 24 hours before the experiment and treated with varying amounts of CDDP or 5-Fu (Shanghai Xudong Haipu Pharmaceutical Co.,Ltd, China) for 24 hours or 48 hours and then incubated in CCK-8 for 4 hours. Absorbance (450 nm) was readed using an Elx800 microplate reader (BioTek, USA). Cells incubated with medium only were used as the negative control, with medium only wells used as the blank control.

### Cell transfection with overexpression and knockdown Lentiviral vectors

The human RPS15A gene sequence was obtained from GenBank (NM\_001019, transcript variant 2). Lentiviral vectors were constructed by Cyagen Biosciences. The target sequence that interfered with RPS15A is CCGGTTTCTCACTGTGATGAT with the EGFP-T2A-Puro plasmid. The over-expression RPS15A vector carrying the CDS sequence was derived from GenBank (NM\_001030009.1, CDS sequence is conservative) with the EGFP-T2A-Puro maker too. Cyagen Biosciences provided the transfection strategy manual. The fluorescence was observed 48 hours after lentiviral transfection, and the CCK-8 method was used to screen the concentration of Puromycin that did not affect the survival of the lentivirus-transfected cells. The knockdown and overexpression efficiency were then measured.

### Colony formation assay

ESCC cells were treated with trypsin to form suspensions of single cells and then 40,000 cells/well were plated into 6-well cell dishes (NEST biotechnology, China) with varying concentrations of CDDP. Following 168 hours of incubation, Giemsa was used to stain the cells, and colonies were counted.

#### *In vitro* tumorsphere formation assays

ESCC cells were treated with trypsin to generate a suspension of single cells, washed twice in ice-cold PBS, and then 10,000 cells/well were placed into 6-well plates (3471, Corning). A total of 3 ml tumorsphere medium supplemented with B27 (12587010, Gibco), basic fibroblast growth factor (PHG0266, Gibco), and EGF (PHG0311L Gibco) were added to the plates for 1 week and the number of tumorspheres were counted. The data are presented as a percentage ratio of the amount of tumorspheres over the initial amount of cells seeded.

#### Side population (SP) assay

Single ESCC cells were incubated in 10 µg/mL Hoechst 33342 (FXP138-500, 4A Biotech) at room temperature for 90 minutes with gentle agitation every 10 minutes. The isotype control sample used was 100 µM verapamil (S4202, Selleck Chemicals). The PI staining solution was then added to the samples, and after gently mixing, the samples were incubated in the dark for 15 minutes. After passing the samples through a 70 µm cell strainer (352350, Corning) to get rid of clumped cells, the single cells were analyzed using flow cytometry.

#### Quantitative real-time PCR

Trizol reagent (DP424, Tiangen, China) was used to extract total RNA which was then reverse-transcribed into cDNA using the PrimerScript Master mix (RR036A, Takara, China) using the product manual. Gene expression was assessed by quantitative PCR (q-PCR) using TB Green™ premix Ex Taq™ (TaKaRa, RR820A) and a C1000 Thermal Cycler (CFX96 Real-Time System, Bio-Rad). The expression levels of β-actin or GAPDH mRNA were used as internal controls. The primer pairs we used are listed below:

RPS15A	F-5'-CTCCAAAGTCATCGTCCGGTT-3'	R-5'-TGAGTTGCACGTCAAATCTGG-3'
MDR1	F-5'-TCTATGGTTGGCAACTAACACT-3'	R-5'-CTCCTGAGTCAAAGAAACAACG-3'
CD44	F-5'-CGGACACCATGGACAAGTTT-3'	R-5'-GAAAGCCTTGCAGAGGTTCAG-3'
CD133	F-5'-TGGATGCAGAACTTGACAACGT-3',	R-5'-ATACCTGCTACGACAGTCGTGGT-3'
Oct-4	F-5'-CTGGGTTGATCCTCGGACCT-3'	R-5'-CCATCGGAGTTGCTCTCCA-3'
Sox2	F-5'-GCCGAGTGGAACCTTTTGTCTG-3'	R-5'-GGCAGCGTGTACTTATCCTTCT-3'
Nanog	F-5'-TTTGTGGGCCTGAAGAAAACCT-3'	R-5'-AGGGCTGTCCTGAATAAGCAG-3'
$\beta$ -Actin	F-5'-CCTCGCCTTTGCCGATCC-3'	R-5'-GGATCTTCATGAGGTAGTCAGTC-3';
GAPDH	F-5'-GAGTCAACGGATTTGGTCGT-3'	R-5'-GACAAGCTTCCCGTTCTCAG-3'

## Western blot analysis

Trident RIPA Lysis Buffer (GTX400005, GeneTex, USA) was used to prepare cell lysates from harvested cells. SDS-PAGE was used to separate the proteins, which were then transferred onto PVDF membranes (Millipore, MA, USA) as previously described.[17] The membranes were treated overnight at 4°C with primary antibodies, washed and incubated with secondary antibodies for 2 hours at 25°C. The fluorescence intensity of target proteins was detected using an Enhanced Chemiluminescence Minessence Kit (4 A Biotech, Beijing, China) with  $\beta$ -Actin or GAPDH as the loading control. Antibodies against p-gp (13342), MRP-1 (72202), Caspase-3 (14220), PARP (9532), c-Caspase-3 (9664), c-PARP (5625), Bcl-2 (3498), CD44 (37259), CD133 (64326), Oct-4 (75463), Sox2 (23064), Nanog (4903), Erk1/2 (4695), mTOR (2983), p38 (8690), AKT (4691),  $\beta$ -Actin (4970), GAPDH (5174), Anti-rabbit IgG HRP (8889) and Anti-mouse IgG HRP (8890) were obtained from Cell Signaling Technology, USA (CST; Beverly, MA). Anti-RPS15A antibody (AP4804a) was purchased from ABGENT, USA. All the antibodies were diluted according to the product manuals.

## Statistical analysis

The data are presented as means  $\pm$  standard deviations of triplicate experiments. Differences between two treatment groups were assessed using a two-tailed unpaired Student's t-test; three or more groups were evaluated using one-way multiple comparison ANOVAs.  $P < 0.05$  was regarded as statistically significant.

# Results

## RPS15A is highly expressed in ESCC cells

To assess the expression of RPS15A in ESCC, we measured both mRNA and protein expression in multiple human ESCC cell lines using RT-PCR and WB: Eca109, Eca9706, and TE-1 (Fig.1a, b) and

Ky510, Ky450, and Ky140 (Fig.S1a, S1b); human immortalization esophageal epithelial cell line (SHEE) and normal esophageal epithelial cells (HEEC) were used as controls. Our results show that both mRNA and protein levels of RPS15A were elevated in the ESCC cells compared to the levels in the esophageal epithelial cell lines. Then, we assessed RPS15A expression in ESCC patients using the GSE53625 dataset. The results show that there was significantly increased expression of RPS15A in ESCC compared to the surrounding tissues ( $p=0.0022$ ; Fig.S1c). These results suggest that RPS15A is highly expressed in ESCC.

To examine the function of RPS15A in ESCC, we used the lentiviral knockdown/overexpression system to assess the effect of RPS15A deletion or overexpression in Eca109, TE-1 and Ky510 cells. The infection efficiency is shown in Fig.S1d, and the efficiency of the RPS15A knockdown/overexpression was confirmed using RT-qPCR and WB (Fig.1c, d and Fig.S1e). Our results show that the designed overexpression lentiviral vector can upregulate the RPS15A mRNA level by  $2.350 \pm 0.192$ -fold compared with the control. Conversely, our interference sequence (CCG GTT TCT CAC TGT GAT GAT) reduced the RPS15A mRNA level by  $0.081 \pm 0.014$ -fold compared with the control, and the interference efficiency reached 91.9%.

### **RPS15A is crucial in ESCC sensitivity to chemotherapeutic intervention**

We then used fluorescence microscopy to evaluate the transfection efficiency and flow cytometry to analyze the percentage of transfected cells and observed some cell death when we knocked down RPS15A. We hypothesized that the absence of RPS15A may increase cells' sensitivity to chemotherapy drugs and therefore assessed the effect of RPS15A on chemosensitivity in ESCC cells. The cells were treated once with CDDP and 5-fluorouracil (5-FU), common chemotherapeutic agents used to treat ESCC, for 48 hours prior to assessment of cell viability using CCK-8 assay. In Eca109 cells, sensitivity to chemotherapeutic agents significantly increased when RPS15A was absent (Fig.2a, b); in contrast, the sensitivity to chemotherapeutic agents significantly decreased when RPS15A was overexpressed (Fig.2c, 2d). We also observed similar results in Ky510 cells (Fig.S2a, S2b). We performed colony formation assays to corroborate these findings and found that the knockdown of RPS15A enhanced ESCC cells' sensitivity to CDDP (Fig.S2c). In RPS15A-deleted Eca109 cells that were treated for 48 hours with  $0.4 \mu\text{g}/\text{mL}$  CDDP, WB data show that the levels of expression of apoptosis-related proteins, such as the cleaved form of Caspase-3, and PARP, increased significantly (Fig.S2d). Detection of apoptosis by flow cytometry, similarly, showed that after RPS15A deletion, cell apoptosis became more pronounced as the sensitivity to chemotherapeutic drugs increased (Fig.S3). RPS15A expression also regulated levels of both the multidrug resistance-associated protein 1 (MDR-1, p-gp) and multidrug resistance protein 1 (MRP1) proteins (Fig.2e). These results demonstrate that absence of RPS15A enhances the sensitivity of ESCC cells to chemotherapy drugs. Thus, RPS15A may be a key regulator in the resistance of ESCC cells to chemotherapy.

### **RPS15A modulates the chemoresistance of ESCC cells by regulating CD44 expression**

To explore the mechanism by which RPS15A regulates chemoresistance in ESCC cells, we used the CDDP-resistant cell line. First, we compared the ability to proliferate and to tolerate CDDP in CDDP-resistant Eca109/CDDP cells and the parental Eca109 cells using clone formation assay and CCK-8 assay, respectively. CDDP resistance increased while proliferation ability decreased in the Eca109/CDDP cells compared with Eca109 cells (Fig.S4). MDR1 gene expression level was higher in Eca109/CDDP than in Eca109 cells (Fig.3a); at the protein level, we observed similar results (Fig.3b). Simultaneously, we found that the level of both CD44 mRNA and protein was higher in Eca109/CDDP than those in Eca109 cells (Fig.3b, 3c). Interestingly, RPS15A and CD44 showed the same expression tendency (Fig.3b, 3c). We then examined the effect of CDDP on RPS15A and CD44 expressions and found that the concentration of CDDP was positively correlated with p-gp, RPS15A, and CD44 (Fig.3d). Concomitantly, RPS15A deletion significantly decreased p-gp and CD44 whereas RPS15A overexpression lead to a significant increase in the expression of p-gp and CD44 (Fig.3e). Consistent with the results presented above, the expression level of CD44 significantly decreased with the RPS15A deletion in CDDP-treated ESCC cells, which was accompanied by an increase in cell apoptosis (Fig.3f). Collectively, these results provide evidence that knockdown RPS15A enhances the ESCC cells' sensitivity to chemotherapy drugs reagents by regulating p-gp and CD44 expression.

### **RPS15A and CD44 are highly up-regulated in ESCC stem-like cells**

CD44, used as a marker of cancer stem cell (CSC), is upregulated in several cancers. An increasing number of recent reports suggest that CD44 participates in multiple cellular processes including chemoresistance. The CSC phenotype, on the other hand, is one of the key characteristics of chemoresistance in tumor cells, and this prompted us to explore the regulatory mechanism of RPS15A on CSC participation in chemoresistance. We first confirmed the existence of CSCs in ESCC by serum-free suspension cultures experiment and found that ESCC cells could form tumorspheres (Fig.4a and Fig.S5a), which we named ESCC stem cell-like cells (ESCLCs). Next, we evaluated the expression of a number of stem cell markers, including Nanog, OCT-4, Sox2, CD44, and CD133 using WB (Fig.4b and Fig.S5b) and RT-PCR (Fig.4c and Fig.S5c). Our results indicate that the stemness of tumorspheres significantly increased compared with the parent cells. Notably, the expression of RPS15A significantly increased in these ESCLCs (Fig.4b, 4c and Fig.S5b, S5c). We also found that the deletion of RPS15A significantly decreased the ESCC cells' tumorsphere formation ability; conversely, overexpression of RPS15A significantly increased that ability regardless of primary or secondary tumorsphere formation (Fig.4d, 4e and Fig.S5d, S5e). These findings suggest that RPS15A and CD44 are highly expressed in ESCC stem-like cells concurrently, and RPS15A plays a role in stemness maintenance in ESCLCs.

### **RPS15A promotes chemoresistance in ESCC cells by altering cell stemness**

As our results have shown that RPS15A is important in maintaining stemness in ESCLCs, we decided to explore the effect of RPS15A on the CSC phenotype, which is one of the key chemoresistant characteristics of tumor cells. We first detected mRNA and protein levels of three stem cell markers Nanog (Fig.5a and Fig.S6a), OCT-4 (Fig.5b and Fig.S6b), SOX-2 (Fig.5c and Fig.S6c) by qRT-PCR and WB

(Fig.5d and Fig.S6d). As seen with the effect of RPS15A in regulating CD44 expression, Nanog, OCT-4, and SOX-2 were significantly reduced in the RPS15A knockdown and increased with RPS15A overexpression. We then investigated the modulation of RPS15A on the expression of key signal transducers involved in the maintenance of stemness (Fig.5e and Fig.S6e). We observed a similar trend in the master regulators for CSCs, such as Erk 1/2, mTOR, p38, and AKT. These results are consistent with the findings that RPS15A could significantly regulate the ability of ESCC cells to form spheres.

Since side population (SP) cells have CSC-like characteristics and display elevated chemoresistance, we investigated the effect of RPS15A in these cells as well. We analyzed SP cells which was stained with Hoechst 33342, using flow cytometry. Our results show that both the proportion and number of SP cells significantly decreased when RPS15A was knocked down and significantly increased when RPS15A was overexpressed (Fig.6). Collectively, these results demonstrate that RPS15A modulates chemotherapeutic resistance in ESCC cells by regulating cell stemness.

## Discussion

Chemoresistance is a major issue in cancer treatment as it leads to failure of chemotherapy. ESCC, an especially aggressive cancer, is largely resistant to current treatments.[18] In this study, we explored how RPS15A imparts chemoresistance to the ESCC cells. Changes in the expression of ribosomal proteins have been used in some studies to differentiate cancerous from normal cells; such differences may have predictive or prognostic value.[4, 19] Studies have shown that dysregulation of the expression and translation of ribosomal proteins leads to tumor progression,[20] and chemoresistance.[21] It has been reported that RPS6 inhibition could prevent anti-HER2 resistance to chemotherapeutic drugs in gastric cancer patients,[22] and dysregulation of the phosphorylation of RPS6 induced drug resistance in skin cancer.[23] In this study, we found that RPS15A sensitized ESCC cells to chemotherapeutic drugs by regulating CD44 expression. Furthermore, we show that RPS15A participates in the maintenance of stemness in ESCC cells. These factors are functionally important in the chemoresistance of ESCC cells.

In the present study, data from WB and RT-qPCR confirmed that the expression of RPS15A in ESCC cells was significantly greater than in the esophageal epithelial cells. We observed some cell death after RPS15A knockdown, which could be due to increased apoptosis caused by lack of RPS15A expression as reported before.[24] Interestingly, with CDDP and 5-Fu, two chemotherapy drugs used as the first-line therapy for ESCC, the ESCC chemosensitivity to both CDDP and 5-Fu increased after RPS15A knockdown. Conversely, RPS15A overexpression protected ESCC cells against chemotherapeutic agents. As there has not been any clinically proven therapeutic target for ESCC, our results suggest that RPS15A may be a novel treatment target for the inhibition of chemoresistance in ESCC.

Excretion of the anticancer agents from the cells by the drug extrusion pump is currently thought to be the main reason for chemoresistance in ESCC.[25] Our data support this notion as the expressions of ABCB1 and ABCC1 were regulated by RPS15A. Our results further demonstrate that RPS15A has a function in the multidrug resistance (MDR) of ESCC cells. Apart from the drug extrusion pump, many other factors are

involved in the development of chemoresistance; specifically, DNA damage repair (DDR) is another important mechanism.[26] An increase in excision repair cross complementation group 1 (ERCC1) protein is associated with an enhanced clearance of DNA-platinum adducts and increased resistance to CDDP.[27] Although the effect of RPS15A on ERCC1 levels was not enhanced in our study, we confirmed that RPS15A can participate in the drug resistance of ESCC cells by regulating DDR. We also found that the deletion of RPS15A increases the cleavage of poly ADP-ribose polymerase (PARP, Fig.3f and Fig.S2d), which is important for DDR and cell apoptosis.[28] Therefore, RPS15A might be involved in the chemoresistance of ESCC through different mechanisms.

CD44, a CSC marker, is crucial in the regulation of self-renewal, the inception of tumors, metastasis, and chemotherapy resistance and is widely overexpressed in a variety of cancerous cells.[29] CD44 is involved in tumor cell resistance to chemotherapy mainly through the regulation of cancer cells' stemness and cell adhesion mediated drug resistance.[29, 30] Hyper-expression of CD44 and p-gp has been linked to resistance to chemotherapeutic drugs resistance and tumor progression.[31, 32] Here, we found that in CDDP resistant cell line Eca109/CDDP, RPS15A, CD44, and p-gp had significantly higher expression. Moreover, RPS15A, CD44, and p-gp expression was positively associated with increasing concentration of CDDP; p-gp and CD44 significantly decreased with the RPS15A deletion and increased with the RPS15A overexpression. Regulation of p-gp by RPS15A could further affect the number and proportion of stem-like cancer cell side-population (SP) cells (Fig.6) through the efflux mechanism for chemoresistance.[33] In CDDP-treated ESCC cells, the expression level of CD44 significantly decreased and apoptosis increased concurrently upon RPS15A deletion (Fig.3). Our findings are similar to a previous report that showed that CD44 inhibition reduced the adhesion of multiple myeloma cells and reversed the resistance to lenalidomide.[34] Taken together, our findings indicate that regulation of CD44 expression by RPS15A may be crucial in ESCC chemoresistance.

As CD44 is a surface marker for CSCs in a number of cancers including ESCC,[35, 36] and CSCs in ESCC play a key role in therapy resistance,[25, 37] we hypothesized that RPS15A may play a role in modulating cells' stemness in ESCC. Hence, we examined the effect of RPS15A on ESCC stem cell-like cells (ESCLCs). In addition to CD44, we selected CD133 which has been utilized as a marker of CSC in a number of carcinomas,[37, 38] as another ESCLC marker. CD133 was significantly associated with the overall survival rate of ESCC patients due to its involvement in tumor recurrence and therapy resistance.[39, 40] In this study, we detected high levels of both CD44 and CD133 expression in ESCLCs. CSCs have also been referred to as "sphere-forming cells",[41] and our data confirm that ESCC cells formed tumorspheres in serum-free suspension culture experiments and the stemness of tumorsphere cells increased compared to the parent cells. A previous study compared the cancer drug resistance using CDDP and doxorubicin in ESCC tumorsphere cells and showed that cancer drug resistance had increased.[42] As expected, in this study, the absence of RPS15A significantly reduced the tumorsphere formation ability of the ESCC cells, and then increased their sensitivity to chemotherapeutic drugs. We also showed that the expression of Nanog, Oct-4 and Sox-2 significantly decreased with the RPS15A deletion and increased with the RPS15A overexpression. These results suggest that RPS15A may be involved in chemoresistance by regulating the stemness of ESCC cells.

A large number of recent studies have shown the links between PI3K/Akt/mTOR signaling pathway and CSC biology, including chemoresistance.[43] In advanced hepatocellular carcinoma, activation of the PI3K/Akt/mTOR cascade is crucial in modulating the generation of liver CSCs in acquired resistance to Sorafenib.[44] In breast CSCs, PD-L1 promotes OCT4 and Nanog expression following sustained PI3K/Akt/mTOR activation.[45] In this study, we found that the RPS15A overexpression upregulated the key signaling proteins in the PI3K/Akt/mTOR pathway, which further explains the role of RPS15A in maintaining ESCC cells' stemness phenotypes. Identification and further elucidation of specific markers and signaling molecules that ESCC stem cells might lead to seminal therapeutic strategies for the elimination of cancerous stem cells in ESCC. Our data show that a subpopulation of ESCC cells that exhibit ESCCs properties may contribute to chemoresistance and that RPS15A could impart therapeutic resistance by impinging the stemness properties of ESCC cells.

In summary, elevated expression of CD44 in a subset of cancerous cells leads to enhanced chemoresistance in ESCC. In this study, we demonstrated that RPS15A modulates chemosensitivity in ESCC by regulating CD44 expression. Finding new CSC targets or formulating drugs that can modulate the current molecular targets could lead to better clinical outcomes in ESCC patients, and our findings provide a possible new adjuvant ESCC treatment strategy that targets RPS15A. Overall, our study reveals a unique perspective on drug resistance in ESCC and may provide a novel therapeutic new molecular target to overcome chemoresistance.

## References

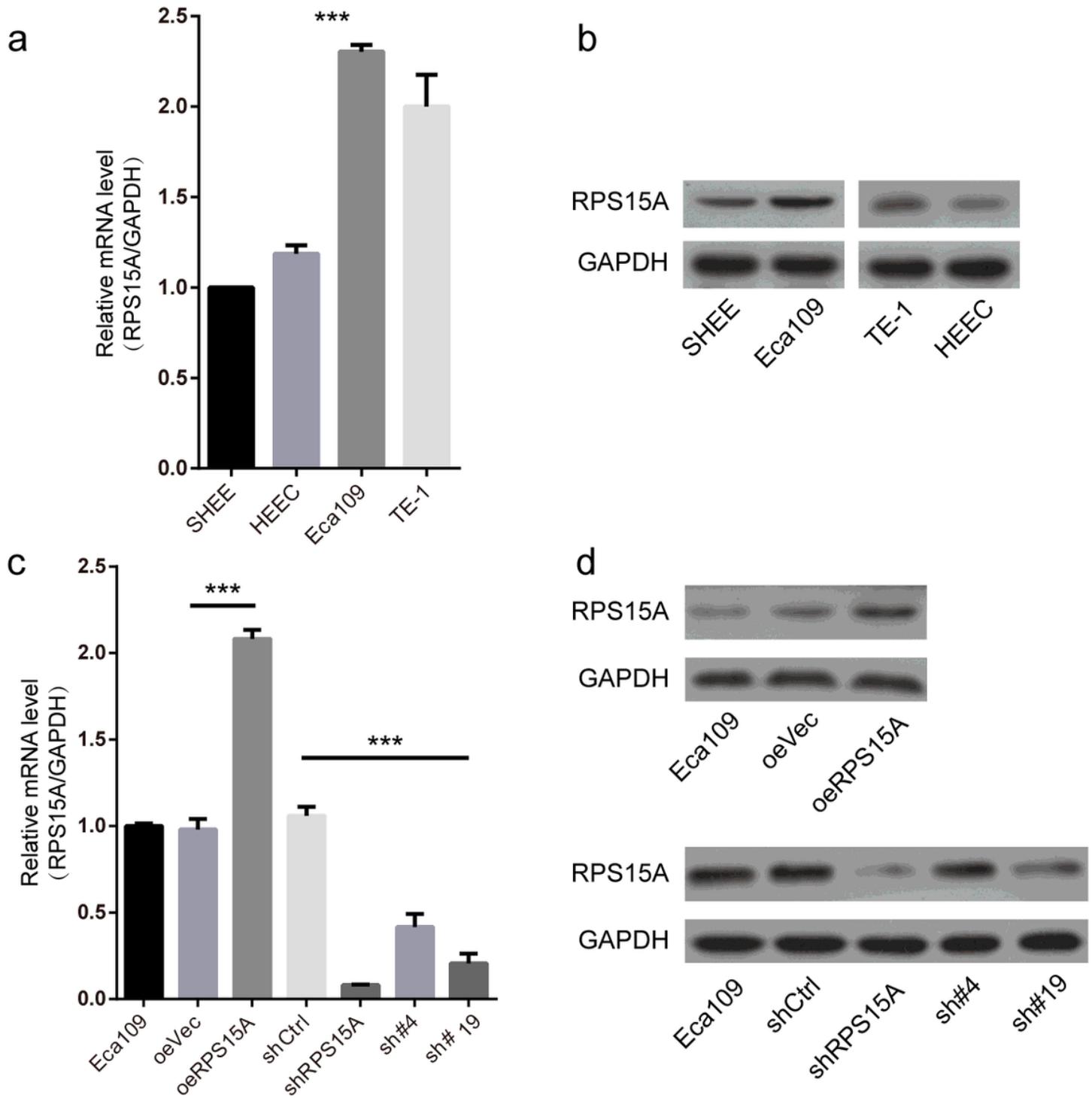
1. Thrumurthy SG, Chaudry MA, Thrumurthy SSD, Mughal M. Oesophageal cancer: risks, prevention, and diagnosis. *BMJ*. 2019;366:l4373. doi:10.1136/bmj.l4373.
2. Ilson D, Lordick F. Definitive or neoadjuvant chemoradiotherapy for squamous cell oesophageal cancer? *Lancet Oncol*. 2018;19(10):1285-6. doi:10.1016/S1470-2045(18)30662-4.
3. van Rossum PSN, Mohammad NH, Vleggaar FP, van Hillegersberg R. Treatment for unresectable or metastatic oesophageal cancer: current evidence and trends. *Nat Rev Gastroenterol Hepatol*. 2018;15(4):235-49. doi:10.1038/nrgastro.2017.162.
4. Wang W, Nag S, Zhang X, Wang MH, Wang H, Zhou J et al. Ribosomal proteins and human diseases: pathogenesis, molecular mechanisms, and therapeutic implications. *Med Res Rev*. 2015;35(2):225-85. doi:10.1002/med.21327.
5. Guo P, Wang Y, Dai C, Tao C, Wu F, Xie X et al. Ribosomal protein S15a promotes tumor angiogenesis via enhancing Wnt/beta-catenin-induced FGF18 expression in hepatocellular carcinoma. *Oncogene*. 2018;37(9):1220-36. doi:10.1038/s41388-017-0017-y.
6. Ning Q, Pang Y, Shao S, Luo M, Zhao L, Hu T et al. MicroRNA-147b suppresses the proliferation and invasion of non-small-cell lung cancer cells through downregulation of Wnt/beta-catenin signalling via targeting of RPS15A. *Clin Exp Pharmacol Physiol*. 2020;47(3):449-58. doi:10.1111/1440-1681.13203.

7. Liu C, He X, Liu X, Yu J, Zhang M, Yu F et al. RPS15A promotes gastric cancer progression via activation of the Akt/IKK-beta/NF-kappaB signalling pathway. *J Cell Mol Med.* 2019;23(3):2207-18. doi:10.1111/jcmm.14141.
8. Chen J, Wei Y, Feng Q, Ren L, He G, Chang W et al. Ribosomal protein S15A promotes malignant transformation and predicts poor outcome in colorectal cancer through misregulation of p53 signaling pathway. *Int J Oncol.* 2016;48(4):1628-38. doi:10.3892/ijo.2016.3366.
9. Mattheolabakis G, Milane L, Singh A, Amiji MM. Hyaluronic acid targeting of CD44 for cancer therapy: from receptor biology to nanomedicine. *J Drug Target.* 2015;23(7-8):605-18. doi:10.3109/1061186X.2015.1052072.
10. Zoller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? *Nat Rev Cancer.* 2011;11(4):254-67. doi:10.1038/nrc3023.
11. Jia L, Yang J, Hao X, Zheng M, He H, Xiong X et al. Validation of SAG/RBX2/ROC2 E3 ubiquitin ligase as an anticancer and radiosensitizing target. *Clin Cancer Res.* 2010;16(3):814-24. doi:10.1158/1078-0432.CCR-09-1592.
12. Bourguignon LY, Earle C, Wong G, Spevak CC, Krueger K. Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. *Oncogene.* 2012;31(2):149-60. doi:10.1038/onc.2011.222.
13. Bourguignon LY, Peyrollier K, Xia W, Gilad E. Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. *J Biol Chem.* 2008;283(25):17635-51. doi:10.1074/jbc.M800109200.
14. Choi KY, Han HS, Lee ES, Shin JM, Almquist BD, Lee DS et al. Hyaluronic Acid-Based Activatable Nanomaterials for Stimuli-Responsive Imaging and Therapeutics: Beyond CD44-Mediated Drug Delivery. *Adv Mater.* 2019;31(34):e1803549. doi:10.1002/adma.201803549.
15. Roy S, Kar M, Roy S, Padhi S, Kumar A, Thakur S et al. Inhibition of CD44 sensitizes cisplatin-resistance and affects Wnt/beta-catenin signaling in HNSCC cells. *Int J Biol Macromol.* 2020;149:501-12. doi:10.1016/j.ijbiomac.2020.01.131.
16. Okamoto K, Ninomiya I, Ohbatake Y, Hirose A, Tsukada T, Nakanuma S et al. Expression status of CD44 and CD133 as a prognostic marker in esophageal squamous cell carcinoma treated with neoadjuvant chemotherapy followed by radical esophagectomy. *Oncol Rep.* 2016;36(6):3333-42. doi:10.3892/or.2016.5133.
17. Zhou P, Li Z, Xu D, Wang Y, Bai Q, Feng Y et al. Cepharanthine Hydrochloride Improves Cisplatin Chemotherapy and Enhances Immunity by Regulating Intestinal Microbes in Mice. *Front Cell Infect Microbiol.* 2019;9:225. doi:10.3389/fcimb.2019.00225.
18. Li MY, Fan LN, Han DH, Yu Z, Ma J, Liu YX et al. Ribosomal S6 protein kinase 4 promotes radioresistance in esophageal squamous cell carcinoma. *J Clin Invest.* 2020. doi:10.1172/JCI134930.

19. Lessard F, Brakier-Gingras L, Ferbeyre G. Ribosomal Proteins Control Tumor Suppressor Pathways in Response to Nucleolar Stress. *Bioessays*. 2019;41(3):e1800183. doi:10.1002/bies.201800183.
20. Ebright RY, Lee S, Wittner BS, Niederhoffer KL, Nicholson BT, Bardia A et al. Deregulation of ribosomal protein expression and translation promotes breast cancer metastasis. *Science*. 2020;367(6485):1468-73. doi:10.1126/science.aay0939.
21. Huang Z, Zhang Y, Li H, Zhou Y, Zhang Q, Chen R et al. Vitamin D promotes the cisplatin sensitivity of oral squamous cell carcinoma by inhibiting LCN2-modulated NF-kappaB pathway activation through RPS3. *Cell Death Dis*. 2019;10(12):936. doi:10.1038/s41419-019-2177-x.
22. Gambardella V, Gimeno-Valiente F, Tarazona N, Martinez-Ciarpaglini C, Roda D, Fleitas T et al. NRF2 through RPS6 Activation Is Related to Anti-HER2 Drug Resistance in HER2-Amplified Gastric Cancer. *Clin Cancer Res*. 2019;25(5):1639-49. doi:10.1158/1078-0432.CCR-18-2421.
23. Gao MZ, Wang HB, Chen XL, Cao WT, Fu L, Li Y et al. Aberrant modulation of ribosomal protein S6 phosphorylation confers acquired resistance to MAPK pathway inhibitors in BRAF-mutant melanoma. *Acta Pharmacol Sin*. 2019;40(2):268-78. doi:10.1038/s41401-018-0020-z.
24. Kong L, Wei Q, Hu X, Chen L, Li J. Ribosomal protein small subunit 15A (RPS15A) inhibits the apoptosis of breast cancer MDA-MB-231 cells via upregulating phosphorylated ERK1/2, Bad, and Chk1. *J Cell Biochem*. 2020;121(1):587-95. doi:10.1002/jcb.29304.
25. Islam F, Gopalan V, Wahab R, Smith RA, Lam AK. Cancer stem cells in oesophageal squamous cell carcinoma: Identification, prognostic and treatment perspectives. *Crit Rev Oncol Hematol*. 2015;96(1):9-19. doi:10.1016/j.critrevonc.2015.04.007.
26. Motegi A, Masutani M, Yoshioka KI, Bessho T. Aberrations in DNA repair pathways in cancer and therapeutic significances. *Semin Cancer Biol*. 2019;58:29-46. doi:10.1016/j.semcancer.2019.02.005.
27. Li B, Jiang J, Assaraf YG, Xiao H, Chen ZS, Huang C. Surmounting cancer drug resistance: New insights from the perspective of N(6)-methyladenosine RNA modification. *Drug Resist Updat*. 2020;53:100720. doi:10.1016/j.drug.2020.100720.
28. Ame JC, Spenlehauer C, de Murcia G. The PARP superfamily. *Bioessays*. 2004;26(8):882-93. doi:10.1002/bies.20085.
29. Ganesh S, Iyer AK, Morrissey DV, Amiji MM. Hyaluronic acid based self-assembling nanosystems for CD44 target mediated siRNA delivery to solid tumors. *Biomaterials*. 2013;34(13):3489-502. doi:10.1016/j.biomaterials.2013.01.077.
30. Vitale D, Kumar Katakam S, Greve B, Jang B, Oh ES, Alaniz L et al. Proteoglycans and glycosaminoglycans as regulators of cancer stem cell function and therapeutic resistance. *FEBS J*. 2019;286(15):2870-82. doi:10.1111/febs.14967.
31. Miletto-Gonzalez KE, Chen S, Muthukumaran N, Saglimbeni GN, Wu X, Yang J et al. The CD44 receptor interacts with P-glycoprotein to promote cell migration and invasion in cancer. *Cancer Res*. 2005;65(15):6660-7. doi:10.1158/0008-5472.CAN-04-3478.
32. Chen H, Hao J, Wang L, Li Y. Coexpression of invasive markers (uPA, CD44) and multiple drug-resistance proteins (MDR1, MRP2) is correlated with epithelial ovarian cancer progression. *Br J*

- Cancer. 2009;101(3):432-40. doi:10.1038/sj.bjc.6605185.
33. Baeten JT, Waarts MR, Pruitt MM, Chan WC, Andrade J, de Jong JLO. The side population enriches for leukemia-propagating cell activity and Wnt pathway expression in zebrafish acute lymphoblastic leukemia. *Haematologica*. 2019;104(7):1388-95. doi:10.3324/haematol.2018.206417.
  34. Spaan I, Raymakers RA, van de Stolpe A, Peperzak V. Wnt signaling in multiple myeloma: a central player in disease with therapeutic potential. *J Hematol Oncol*. 2018;11(1):67. doi:10.1186/s13045-018-0615-3.
  35. Natsuizaka M, Whelan KA, Kagawa S, Tanaka K, Giroux V, Chandramouleeswaran PM et al. Interplay between Notch1 and Notch3 promotes EMT and tumor initiation in squamous cell carcinoma. *Nat Commun*. 2017;8(1):1758. doi:10.1038/s41467-017-01500-9.
  36. Yan Y, Zuo X, Wei D. Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med*. 2015;4(9):1033-43. doi:10.5966/sctm.2015-0048.
  37. Harada K, Pool Pizzi M, Baba H, Shanbhag ND, Song S, Ajani JA. Cancer stem cells in esophageal cancer and response to therapy. *Cancer*. 2018;124(20):3962-4. doi:10.1002/cncr.31697.
  38. Xu WW, Li B, Zhao JF, Yang JG, Li JQ, Tsao SW et al. IGF2 induces CD133 expression in esophageal cancer cells to promote cancer stemness. *Cancer Lett*. 2018;425:88-100. doi:10.1016/j.canlet.2018.03.039.
  39. Lin CH, Li HY, Liu YP, Kuo PF, Wang WC, Lin FC et al. High-CLDN4 ESCC cells harbor stem-like properties and indicate for poor concurrent chemoradiation therapy response in esophageal squamous cell carcinoma. *Ther Adv Med Oncol*. 2019;11:1758835919875324. doi:10.1177/1758835919875324.
  40. Hang D, Dong HC, Ning T, Dong B, Hou DL, Xu WG. Prognostic value of the stem cell markers CD133 and ABCG2 expression in esophageal squamous cell carcinoma. *Dis Esophagus*. 2012;25(7):638-44. doi:10.1111/j.1442-2050.2011.01298.x.
  41. Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV et al. Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer*. 2012;12(11):767-75. doi:10.1038/nrc3368.
  42. Saygin C, Matei D, Majeti R, Reizes O, Lathia JD. Targeting Cancer Stemness in the Clinic: From Hype to Hope. *Cell Stem Cell*. 2019;24(1):25-40. doi:10.1016/j.stem.2018.11.017.
  43. Xia P, Xu XY. PI3K/Akt/mTOR signaling pathway in cancer stem cells: from basic research to clinical application. *Am J Cancer Res*. 2015;5(5):1602-9.
  44. Kahraman DC, Kahraman T, Cetin-Atalay R. Targeting PI3K/Akt/mTOR Pathway Identifies Differential Expression and Functional Role of IL8 in Liver Cancer Stem Cell Enrichment. *Mol Cancer Ther*. 2019;18(11):2146-57. doi:10.1158/1535-7163.MCT-19-0004.
  45. Almozyan S, Colak D, Mansour F, Alaiya A, Al-Harazi O, Qattan A et al. PD-L1 promotes OCT4 and Nanog expression in breast cancer stem cells by sustaining PI3K/AKT pathway activation. *Int J Cancer*. 2017;141(7):1402-12. doi:10.1002/ijc.30834.

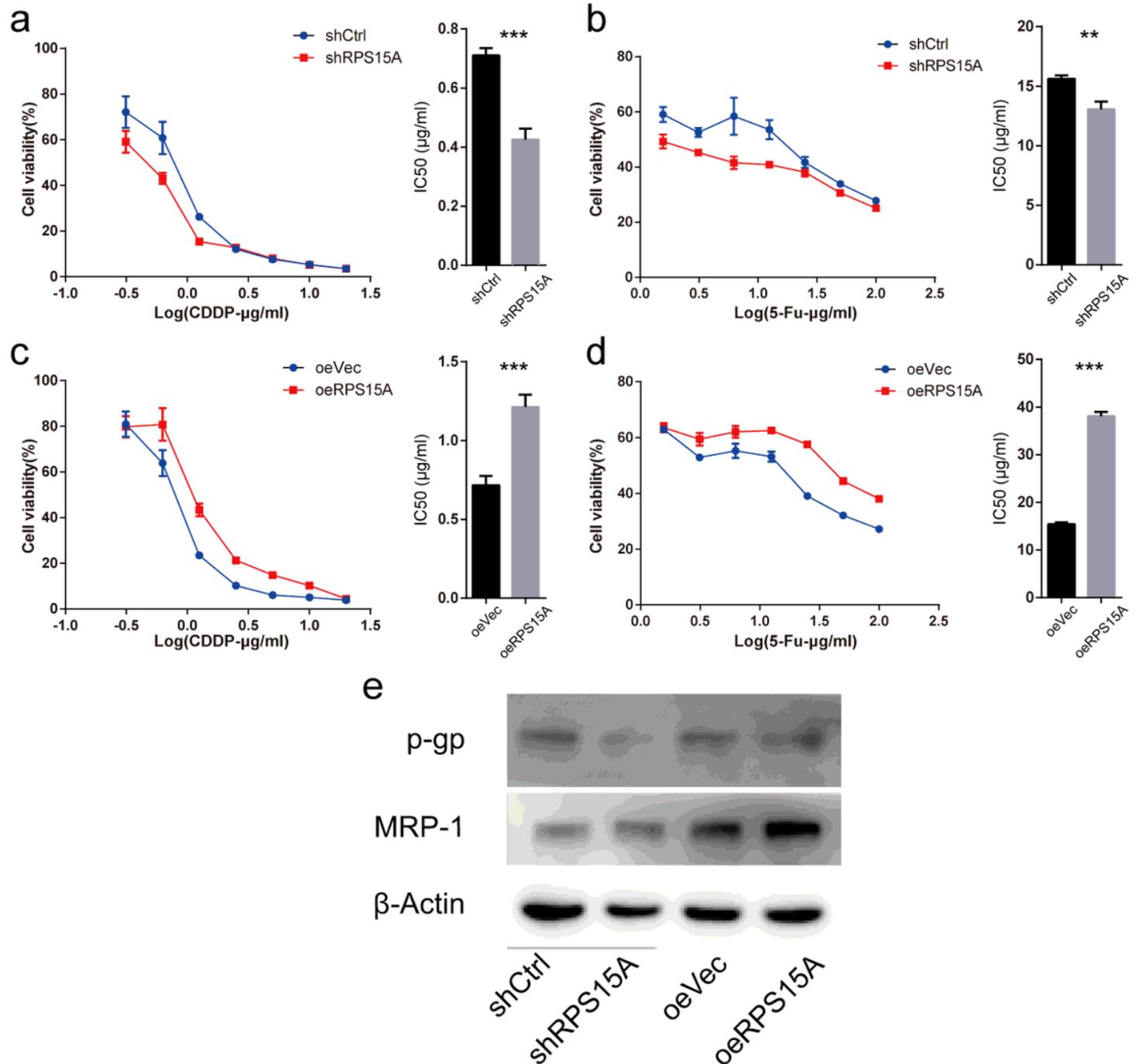
# Figures



**Figure 1**

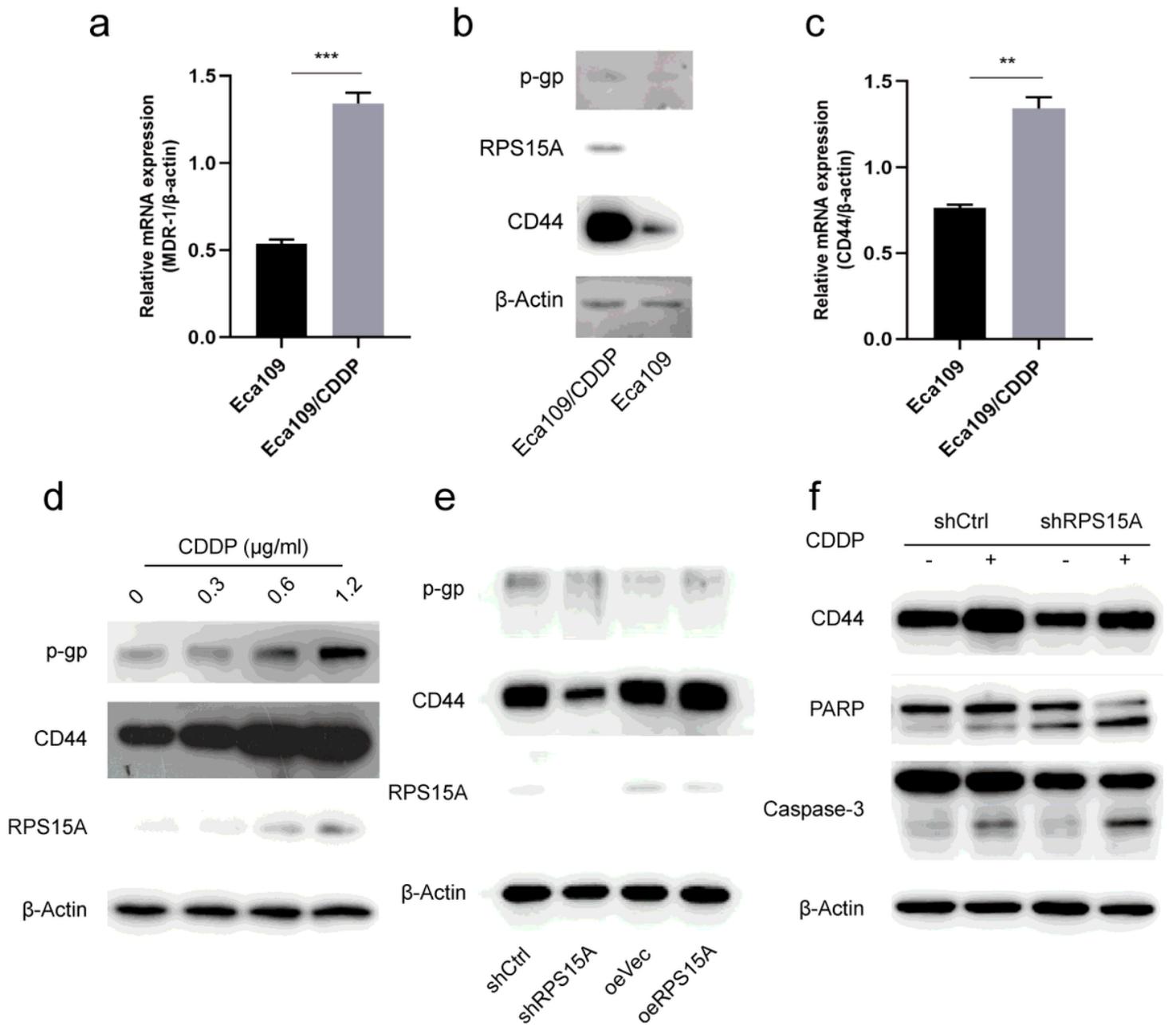
Increased expression of RPS15A in human ESCC cells A and B, RT-qPCR and WB analysis of relative RPS15A mRNA (A) and protein levels (B) in ESCC cell lines, human immortalized esophageal epithelial cell line SHEE, and normal esophageal epithelial cells HEEC. C and D, RT-qPCR and WB analysis of

RPS15A mRNA and protein levels after lentiviral transfection (MOI=20) in Eca109 cells. \*\*\*P < 0.001 vs Control.



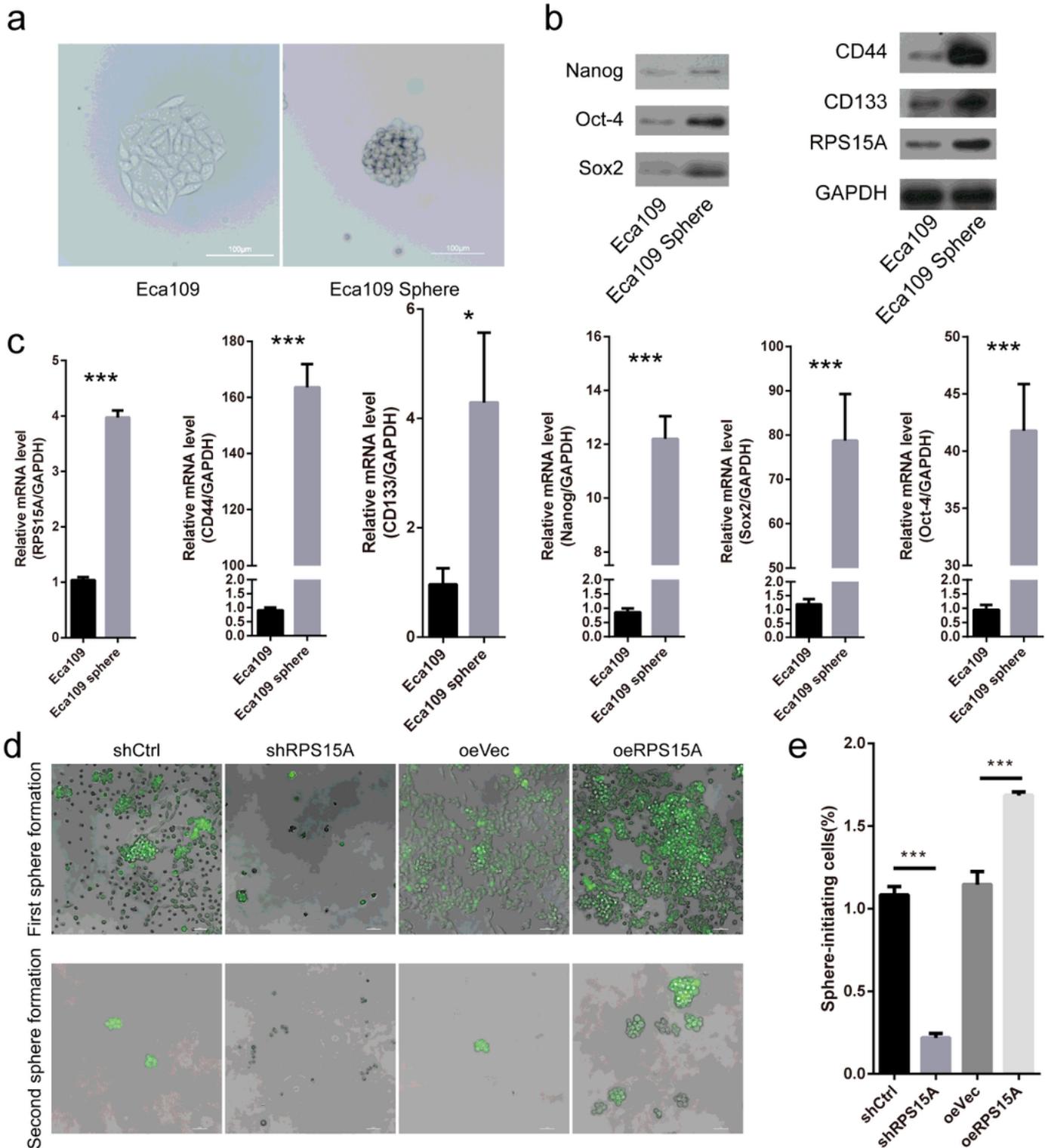
**Figure 2**

RPS15A expression modulates ESCC cells' sensitivity to chemotherapeutic agents A and B, The effects of RPS15A on the sensitivity of Eca109-shRPS15A cells to CDDP (A) and 5-Fu (B) examined by CCK-8 assay. C and D, The effects of RPS15A on the sensitivity of Eca109-oeRPS15A cells to CDDP (C) and 5-Fu (D) examined by CCK-8 assay. E, The effects of RPS15A knockdown or overexpression on the p-gp and MRP-1 proteins detected by WB. β-Actin were used as a loading control. \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 3**

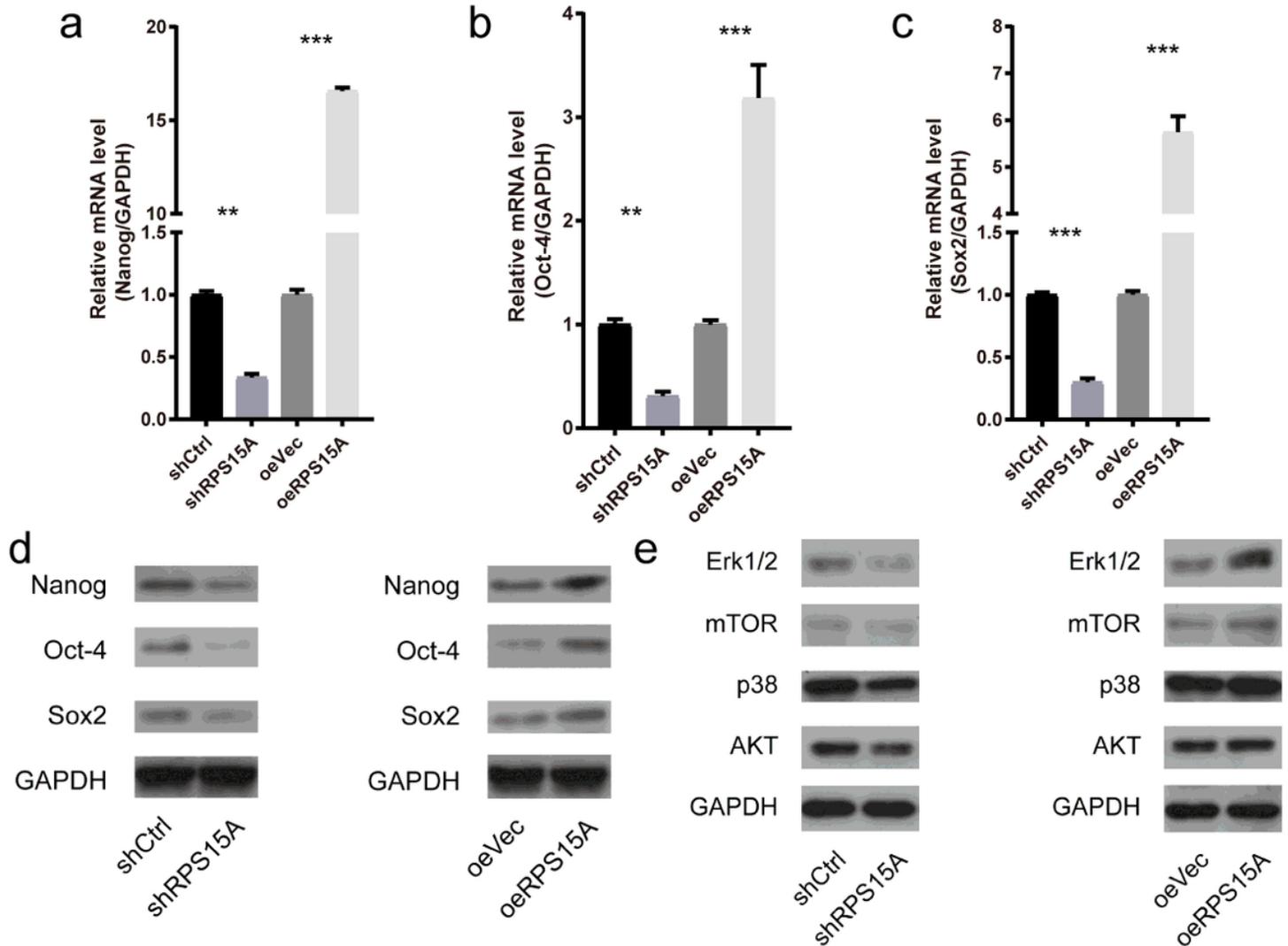
RPS15A regulates CD44 expression A, Relative expression of MDR1 mRNA in Eca109 and Eca109/CDDP cells. B, WB analysis of RPS15A, CD44, and p-gp in Eca109 and Eca109/CDDP cells. C, Relative expression of CD44 mRNA in Eca109 and Eca109/CDDP cells. D, WB analysis of RPS15A, CD44, and p-gp in Eca109 cells incubated with varying concentrations of CDDP for 48 hours. E, WB analysis of CD44 and p-gp in Eca109 cells following changes in RPS15A expression. F, WB analysis of CD44, PARP, and Caspase-3 in Eca109 cells with or without RPS15A knockdown after 48 hours of 0.4 $\mu$ g/ml CDDP treatment.  $\beta$ -Actin was used as a reference in RT-qPCR and  $\beta$ -Actin used as a loading control in WB. \*\*P < 0.01, \*\*\*P < 0.001.



**Figure 4**

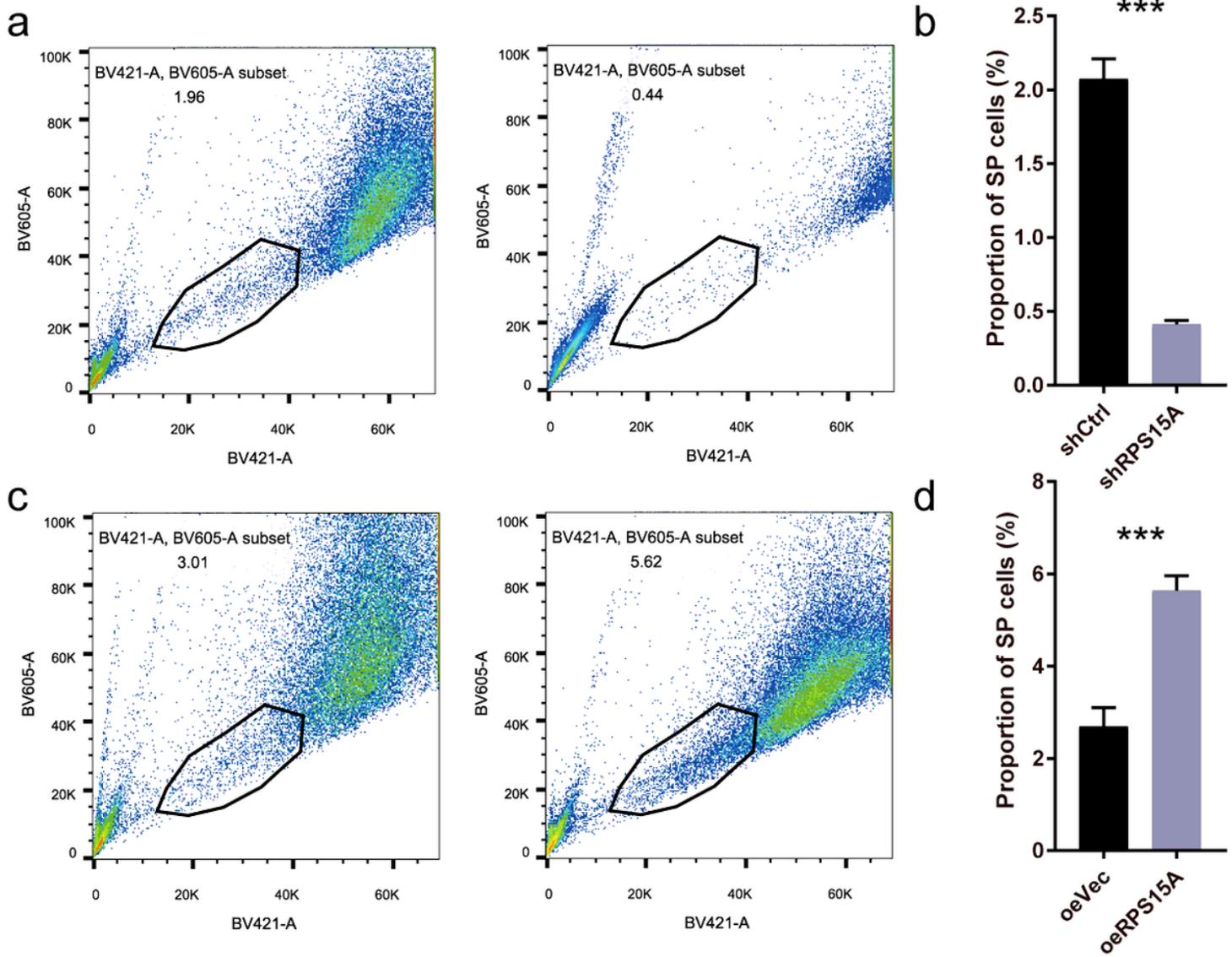
RPS15A regulates the spherogenesis ability of Eca109 cells A, Comparison of cell morphology between Eca109 parental cells (left) and tumorspheres (right). B, WB analysis of RPS15A, Nanog, OCT-4, Sox2, CD44, and CD133 proteins in Eca109 parental cells and tumorspheres. C, Relative expressions of RPS15A, Nanog, OCT-4, Sox2, CD44, and CD133 in Eca109 parental cells and tumorspheres. D and E, Representative images of tumorsphere formation capacity of primary (top panel) and secondary (lower

panel) and a histogram showing the number of sphere-formed (E). GAPDH was used as a reference in RT-qPCR and  $\beta$ -Actin was used as a loading control in WB analysis. \* $P < 0.05$ , \*\*\* $P < 0.001$ .



**Figure 5**

RPS15A regulates the expression of stem cell markers and critical signaling molecules that maintain Eca109 cell stemness A-C, The relative expression of Nanog (A), OCT-4 (B), Sox2 (C) mRNA in Eca109 cells after RPS15A knockdown and overexpression, analyzed by RT-qPCR. D and E, WB analysis of the levels of protein expression of Nanog, OCT-4, and Sox2 (D) and Erk1/2, mTOR, p38, and AKT in Eca109 cells with RPS15A silenced and overexpressed. GAPDH was used as a reference in RT-qPCR and as a loading control in WB analysis. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 6**

RPS15A regulates SP cells in ESCC A and B, Hoechst 33342 dye staining profiles of Eca109-shRPS15A and SP cells analyzed using flow cytometry and quantification of the percentage of SP cells (B). C and D Hoechst 33342 dye staining profiles of Eca109-oeRPS15A cells and SP cells analyzed using flow cytometry and quantification of the percentage of SP cells (D). \*\*\* $P < 0.001$ .

## Supplementary Files

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