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ORIGINAL ARTICLE

ORP5 promotes migration and invasion of cervical cancer cells by inhibiting endoplasmic reticulum stress

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Abstract

ORP5 is a transmembrane protein anchored to the endoplasmic reticulum, which mainly functions as a lipid transporter and has reportedly been linked to cancer. However, the specific mechanism of ORP5 action in cervical cancer (CC) is unclear. In this study, we found that ORP5 promotes the migration and invasive ability of CC cells in vitro and in vivo. In addition, ORP5 expression was linked to endoplasmic reticulum stress, and ORP5 encouraged CC metastasis by inhibiting endoplasmic reticulum stress. Mechanistically, ORP5 inhibited endoplasmic reticulum stress in CC cells by stimulating ubiquitination and proteasomal degradation of SREBP1 to reduce its expression. In conclusion, ORP5 promotes the malignant progression of CC by inhibiting endoplasmic reticulum stress, providing a therapeutic target and strategy for CC treatment.

Keywords ORP5 · SREBP1 · Cervical cancer · Endoplasmic reticulum stress · Ubiquitination

Introduction

Cervical cancer (CC) is the fourth most prevalent malignancy and the fourth main cause of cancer death in women globally. Due to its earlier age of diagnosis than other major malignancies, it poses a serious threat to women's life expectancy. There are approximately 6 million cases and 3 million fatalities annually, with China having the highest number of cases (Arbyn et al. 2020). CC mortality varies greatly between nations and is the primary reason why women die from cancer in low-resource nations, creating a significant public health issue. Although CC has been effectively prevented with the popularization of HPV vaccine, more new therapeutic targets still need to be found (Brisson et al. 2020, Cohen et al. 2020).

Oxysterol-binding protein–related protein 5 (ORP5), a member of the oxysterol-binding protein (OSBP)–related protein (ORP) family, is an endoplasmic reticulum (ER) membrane protein that participates in lipid transport by sensing and binding cholesterol and oxysterols. The counter-transport of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylserine (PS) between the ER and the plasma membrane (PM) was discovered to be mediated by ORP5. The link between the ER and PM is created when ORP5 transfers PS to the PM and moves PI4P to the ER for the PI4P phosphatase Sac1 to degrade (Chung et al. 2015). Additionally, it has been reported that ORP5 may mediate cholesterol efflux from late endosomes/lysosomes by cooperating with Niemann-Pick Type C1, offering fresh insights into the study of intracellular cholesterol transport (Du et al. 2011). Regarding ORP5 and cancer, there is evidence that the epithelial-mesenchymal transition (EMT) process is promoted by ORP5, which is substantially expressed in pancreatic and lung malignancies (Ishikawa et al. 2010, Nagano et al. 2015, Olkkonen 2022).

For the production, folding, and modification of proteins, the ER is a crucial organelle. The buildup of misfolded or unfolded proteins in the ER lumen is a sign of endoplasmic reticulum stress (ER stress) due to external or intracellular

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factors that disrupt its ability to fold proteins. The cell starts the unfolded protein response (UPR) as a result of ER stress. While prolonged and severe ER stress can cause cell apoptosis, moderate ER stress can aid in reestablishing ER balance and promote cell viability (Siwecka et al. 2019, Wang and Kaufman 2014). ER stress is involved in a variety of human diseases. Tumors growing in an unfavorable microenvironment of ischemia, hypoxia, nutrient deficiency, and oxidative stress often induce ER homeostasis abnormalities, leading to ER stress. ER stress has emerged as a novel feature of cancer (Chen and Cubillos-Ruiz 2021). Activation of ER stress has been detected in many forms of human tumors, such as glioblastoma, myeloma, breast cancer, gastric cancer, esophageal cancer, and liver cancer (Oakes and Papa 2015).

UPR is initiated by three proteins, inositol-requiring kinase 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These three proteins are single-channel ER transmembrane proteins with internal lumenal structural domains that can recognize misfolded proteins. Dissociation of the inactive form of the chaperone protein glucose-regulated protein 78 (GRP78), namely, binding-immunoglobulin protein (BIP), results in ER stress (Wiseman et al. 2022). Aberrant URP signaling is associated with tumor cell survival, metastasis, apoptosis, and angiogenesis, affecting the prognosis of several cancers (Shi et al. 2021, Wang and Mi 2023, Wu et al. 2022). Additionally, somatic mutations in PERK, IRE1, and ATF6 have been found in many malignancies, supporting the idea that UPR signaling plays a role in the development of tumors (Forbes et al. 2010, Guichard et al. 2012, Parsons et al. 2008).

ORP5 functions at the ER-PM contact site, and it is considered that it may be involved in controlling ER activity. Members of the ORP family ORP8 and ORP5 are distinct because they both lack the FFAT motif and have only one C-terminal transmembrane domain (Santos et al. 2020). In gastric and hepatocellular carcinomas, ORP8 was discovered to suppress tumor development or cause apoptosis via inducing ER stress (Guo et al. 2017, Zhong et al. 2015). Based on the high sequence homology of 80%, it is suggested that ORP5 may have a potential role in ER stress.

Sterol regulatory element–binding protein 1 (SREBP1) is a transmembrane protein found in the ER and is a crucial regulator of lipid metabolism. The inactive precursor of SREBP1 is activated and undergoes two steps, protein hydrolytic cleavage and translocation to the nucleus, where it functions as a transcription factor for numerous key enzymes. Target genes mainly include those related to lipogenesis and cholesterol synthesis processes (Yi et al. 2020). PERK is one of the molecular branches of the UPR, which is also located in the ER. When the UPR is activated, PERK and its signaling pathways are involved in regulating cell survival and death. There have been claims that SREBP1/mSREBP1 can activate ER stress by promoting PERK expression and phosphorylation (Hu et al. 2020). Cholesterol accumulation triggers nucleus pulposus cell apoptosis and extracellular matrix degradation by ER stress mediated by mSREBP1 (Yan et al. 2021). Nelfinavir is an HIV protease inhibitor. It may also affect cancer growth through a variety of pathways, such as induction of apoptotic cell death, activation of ER stress and autophagy, and induction of chemo- and radiosensitization (Gills et al. 2007, Pore et al. 2006). In liposarcoma cell lines, nelfinavir induced SREBP1 upregulation and resulted in ER stress (Guan et al. 2011). These events suggest that SREBP1 is crucial to ER stress.

In this study, we looked into how ORP5 affects the invasion and movement of CC cells. The outcomes demonstrated that overexpression of OPR5 greatly aided CC cell migration, invasion, and metastasis of tumors in vivo. Further studies revealed that this process is mediated by ER stress inhibited by ORP5, which attenuates ER stress by targeting SREBP1 for ubiquitination and degradation. These findings identify crucial regulators associated with ER stress and offer potential therapeutic targets and strategies for CC intervention.

Materials and methods

Cell culture

Human CC cells HeLa and C33A were acquired from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2 and cultured in high-sugar medium DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Beyotime, Shanghai, China)

Western blot assay

Protein was extracted using RIPA protein lysis buffer containing 1% PMSF, and total protein content was assessed using a bicinchoninic acid kit (BCA, Beyotime Biotechnology, Beijing, China). Protein was then kept separated by SDS-PAGE and converted to nitrocellulose membranes, which were then blocked with 5% skim milk for 2 h. Primary antibodies were then applied to the membranes and incubated at 4 °C overnight. The membranes were incubated with secondary antibodies for 2 h the next day after being rinsed with washing buffer. The membranes were finally cleaned once more and the protein signal was detected with ECL reagents. The antibodies we bought and concentrations used were as follows: ORP5 (Abcam, ab59016) (1:1000), BIP (ABclonal, A0241) (1:1000), CHOP (ABclonal, A0221) (1:500), ATF4 (ABclonal, A0201) (1:1000), ATF6

(ABclonal, A0202) (1:1000), SREBP1 (Proteintech, 14088-1-AP) (1:1000), E-cadherin (Proteintech, 20874-1-AP) (1:10000), Vimentin (Proteintech, 10366-1-AP) (1:2000), Snail1 (Proteintech, 13099-1-AP) (1:500), GAPDH (Proteintech, 10494-1-AP) (1:10000), N-cadherin (Servicebio, GB12135) (1:1000), and HRP-labeled Donkey Anti-Goat IgG (H+L) (Beyotime, A0181) (1:1000).

IP assay

Five hundred microgram protein per tube was extracted according to the procedure referred to in the Western blot, mixed with 2 μg antibody, and then gently shaken overnight at 4 °C. Fifty microliters of ProteinA/G Beads (Beyotime) was added to each tube to capture the antigen-antibody complex and spun for 3 h at 4 °C the next day. Finally, the precipitate was collected by centrifugation and washed five times with pre-cooled PBS, and SDS-PAGE electrophoresis was used to identify the samples.

Wound healing assay

The two transfected CC cells were spread in a 6-well plate with serum-free medium, and once they had reached 95% confluence, a wound was made by scraping through the cell layer with a sterile 200-μl pipette tip. After that, PBS was used to wash the cells three times, replaced with fresh medium, and continued to be cultured. Images were taken at 0 h and 48 h. All experiments were repeated three times.

Transwell assay

To assess the cells' capacity to migrate and invade, transwell chambers with 8.0-μm pores (Corning Incorporated) were employed. Cells to be tested were resuspended in media without serum and seeded in the upper chamber with 200 μl of the suspension, and a 600 μl medium that contains serum was injected into the lower chamber. An additional 1:9 ratio of serum-free medium diluted with Matrigel (Corning Incorporated) was added to test the invasive ability of the cells. After waiting 24 h in the incubator for migration and 48 h for invasion, the cells were fixed with methanol and stained with 1% crystalline violet. Finally, the cells that failed to pass through the bottom membrane were wiped off and the cells were photographed and counted under a microscope, and the experiment was repeated three times.

Thioflavin T staining assay

Six-well plates were used to cultivate the cells, and 24 or 48 h after transfection, the supernatant of the cells was removed and washed with PBS. For fixation, 4% paraformaldehyde was added for 20 min; then, 2 ml of ThT (Aladdin) staining solution (10 μm dissolved in PBS) was added to each well, stained for 30 min, and washed three times, and nuclei were counterstained with DAPI (Biosharp). Once more washing has been completed, mount the sample on the slide using two drops of anti-fluorescence quenching mounting solution, and finally, examine with fluorescence microscopy.

ER staining

The cell culture medium was withdrawn and the cells grown on coverslips were rinsed with PBS after transfection had taken place for 24 or 48 h. ER-Tracker Red (Beyotime) was added to ER-Tracker Red dilution at a ratio of 1:3000 and incubated with the cells for 20 min at 37 °C. After being cleaned with PBS, the cells were fixed with 4% paraformaldehyde. Shortly afterward, counterstaining was performed and the anti-fluorescence quenching mounting solution was added dropwise. Finally, the sample was observed and photographed under a fluorescence microscope.

Transfection and construction of stable cell lines

Small interference RNAs (siRNAs) were designed and synthesized by IBSBIO, and siNC, siORP5, and siSREBP1 were transfected into HeLa and C33A cells using siLentFect™ Lipid Reagent (Bio-Rad, Hercules, CA, USA). The siRNA sequences were 5′-GCGGAGACAAUGAGCUCU ATT-3′ for siORP5, 5′-AGACAUGCUUCAGCUUAU CAATT-3′ for siSREBP1, and 5′-UUCUCCGAACGU GUCACGUTT-3′ for siNC. Vector and pcDNA3.1-ORP5 plasmids were transiently transfected into HeLa and C33A utilizing Hieff Trans™ Liposomal Transfection Reagent (YEASEN, shanghai, China). For the following investigations, cells were employed 24 or 48 h after transfection. The lentiviruses knocking down ORP5 expression and relevant negative control lentiviruses were obtained from GenePharma. For the acquisition of stable cell lines, both cells were infected with lentivirus-shORP5 and lentivirus-shNC and screened with 3 μg/ml puromycin (Vicmed, Xuzhou, China) for 2 weeks, and shNC was used as a control. The shRNA sequences were 5′-GCGGAGACAATGAGCTCT A-3′ for shORP5 and 5′-TTCTCCGAACGTGTCACGT-3′ for shNC.

In vivo tumor implantation

Twenty female BALB/C-nude mice (3 weeks old) were acquired from Jiangsu Jicuiyaokang Bioscience. Four groups of mice were created: shNC group, shORP5 group, shNC + 4-PBA group, and shORP5 + 4-PBA, with five mice in each group. ORP5 stable knockout HeLa cells or negative control cells were concentrated in PBS (2×10^6/100 μl) and injected into the mice through the tail vein. Subsequently, mice were

fed without or with 100 mg/kg/day of 4-PBA (MACKLIN) in drinking water. The mice were euthanized 5 weeks later, their lungs were dissected, the number of pulmonary metastatic tumors in each mouse was tallied, and their weight was noted. The last step involved gathering lung tissues for H&E and IHC staining.

Statistical analysis

All data were statistically evaluated and plotted using GraphPad Prism 8. The Student *t*-test was used to analyze group differences. Data are expressed as means ± SD from three independent experiments. $P < 0.05$ means statistically significant.

Results

ORP5 promotes the migratory and invasive capacity of CC cells

Firstly, we examined the expression of ORP5 in human skin fibroblasts (HSF), human immortalized keratinocytes cells (HaCat), and cervical cancer cells C33A, HeLa, SiHa, and Caski. The results of Western blot showed that ORP5 was highly expressed in cervical cancer cells compared to non-tumor cells (Fig. 1a). To verify the role of ORP5 in CC, we performed the following series of experiments. The abilities of tumor migration and invasion reflect the malignancy of the tumor, and we chose the transwell assay to detect it. According to the findings, ORP5 overexpression could significantly promote the migration and invasion of CC cell lines in both HeLa and C33A, as opposed to ORP5 downregulation, which inhibited these capacities (Fig. 1b). Then, as was predicted, ORP5 facilitated cell migration in the investigation of wound healing (Fig. 1c). To further verify the above experimental findings, we looked into how ORP5 affected EMT markers at the protein level. In CC cells overexpressing ORP5, epithelial marker E-cadherin was reported to suppress, and mesenchymal markers N-cadherin, Vimentin, and Snail1 were increased (Fig. 1d). In conclusion, ORP5 facilitated the migration and invasion of HeLa and C33A in vitro.

ORP5 inhibits ER stress in CC

Since our earlier research revealed that ORP5 significantly affects the EMT process of CC, the specific mechanisms involved deserve further exploration. ORP5 is localized to the ER and may be involved in regulating ER function. ORP5 and ER stress have not yet been linked by any studies. In order to confirm the connection, we initially overexpressed ORP5 in HeLa and C33A cells and examined the changes in ER stress markers ATF6, ATF4, BIP, and CHOP protein levels. The findings demonstrated that ATF6, ATF4, BIP, and CHOP were downregulated in both cells, indicating that ORP5 could inhibit ER stress in CC cells, while knockdown of ORP5 increased ER stress marker levels, demonstrating that ER stress was produced (Fig. 2a).

Thioflavin T is a small-molecule compound that attaches to protein aggregates and fluoresces, especially β-sheets. ThT is a tool for detecting and measuring the levels of ER stress in living cells and animal tissues since it has been demonstrated to determine incorrect or unfolded proteins in ER stress (Beriault and Werstuck 2013). ThT fluorescence was dramatically diminished in ORP5 overexpressing CC cells, suggesting that ORP5 reduced the buildup of ThT-stained misfolded or unfolded proteins in CC cells. On the other hand, after ORP5 was knocked down, we noticed increased ThT fluorescence (Fig. 2b).

When stress occurs in the ER, appearance features such as swelling, expansion, and vacuolization occur (Wang et al. 2012). Here, we use ER-Tracker Red, an ER fluorescent probe with cell membrane permeability, which is highly selective for ER and can be used for ER-specific fluorescent staining of living cells. The fluorescence intensity of this dye is proportional to the degree of ER swelling (Mele et al. 2019). We up- or downregulated ORP5 in CC cells, respectively, and the results showed that ER-Tracker staining was significantly diminished after the upregulation of ORP5 and enhanced after the downregulation of ORP5, which was consistent with the previous results (Fig. 2c). In conclusion, all the above results demonstrated that ORP5 inhibited ER stress in CC cells.

ORP5 promotes migration and invasion of CC cells by inhibiting ER stress

To learn more about how ER stress affects the ORP5-mediated biological functions of CC, functional rescue experiments were carried out. We selected tunicamycin (TM) as an inducer of ER stress and then overexpressed ORP5 in HeLa and C33A cells with or without TM treatment. The outcomes showed that induction of ER stress attenuated the enhanced migration and invasion ability of CC caused by ORP5 overexpression. Detection of EMT indicators revealed that ORP5 upregulation resulted in E-cadherin downregulation; N-cadherin, Vimentin, and Snail1 upregulation was also effectively reversed by TM (Fig. 3a, c). Similarly, 4-PBA was used to inhibit ER stress. Then, we knocked down ORP5 in CC cells treated with or without 4-PBA, and the results were opposite to the above (Fig. 3b, d). In conclusion, it may be concluded that ER stress significantly influences the growth of malignancies induced by ORP5.

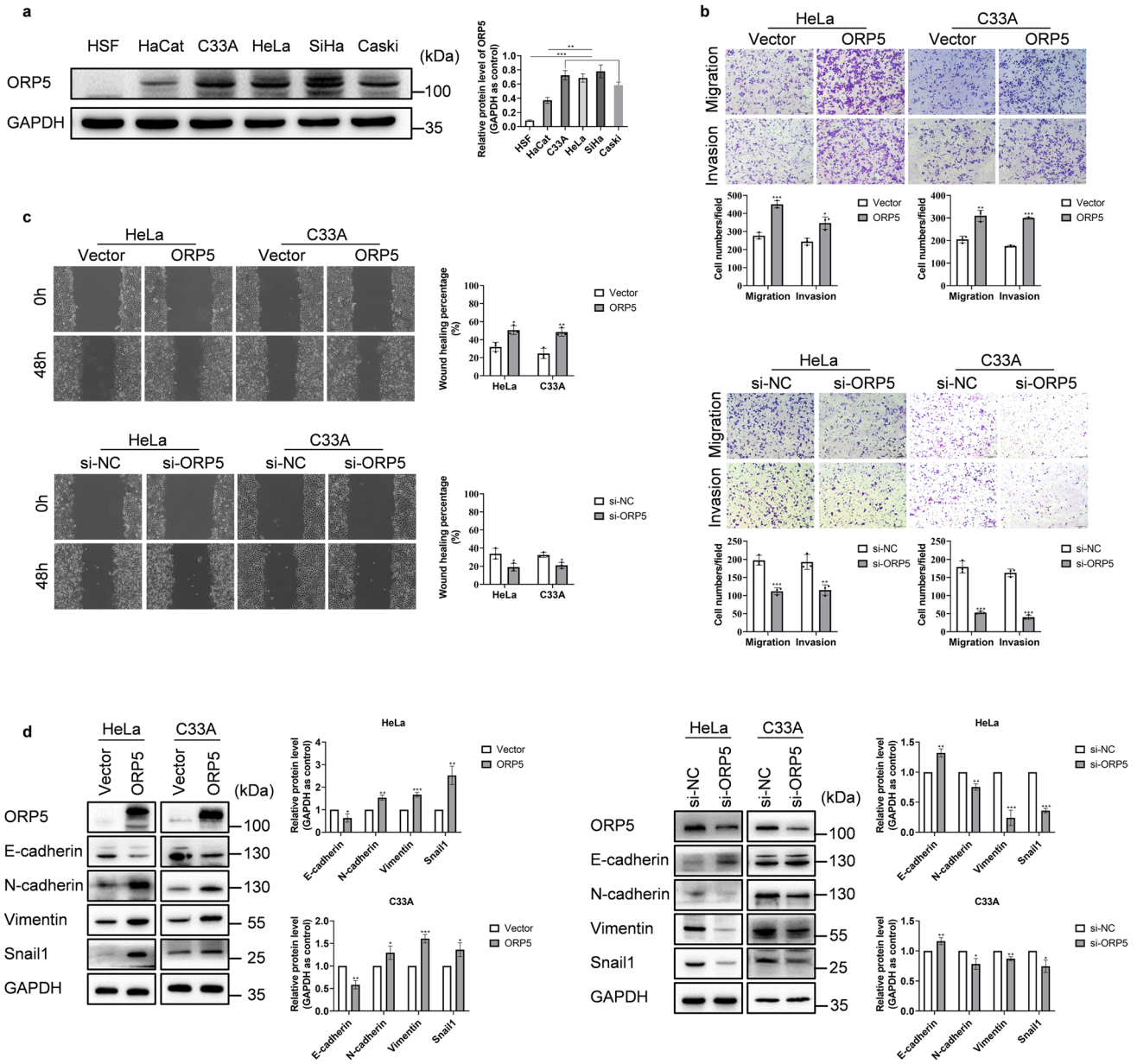


Fig. 1 ORP5 promotes the migratory and invasive capacity of CC cells. **a** The expression of ORP5 in human skin fibroblasts (HSF), human immortalized keratinocytes cells (HaCat), and cervical cancer cells C33A, HeLa, SiHa, and Caski was measured by Western blot. Statistical analysis of ORP5 expression ($n = 3$). **b** The results of the transwell assay of the migration and invasion for HeLa and C33A cell lines overexpressing or knocking down ORP5 ($n = 3$). Original magnifications, × 100 for **b**. **c** The result of wound healing assay for HeLa and C33A cell lines overexpressing or knocking down ORP5 ($n = 3$). Original magnifications, × 100 for **c**. **d** Protein levels of E-cadherin, N-cadherin, Vimentin, and Snail1 in HeLa and C33A cell lines overexpressing or knocking down ORP5. Statistical analysis of E-cadherin, N-cadherin, Vimentin, and Snail1 expression ($n = 3$). Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

ORP5 inhibits ER stress and promotes migration and invasion of CC cells through the downregulation of SREBP1

SREBP1, an ER membrane-bound protein that acts as a transcription factor for numerous essential enzymes, is crucial for lipid metabolism. It has been reported that the mature form of SREBP1 (mSREBP1) amplifies ER stress, and in addition, mSREBP1 mediates ER stress brought on by cholesterol, suggesting that the maturation of SREBP1 is closely related to ER stress. ORP5 has been shown to affect intracellular cholesterol transport, and the activation of SREBP1 is closely related to cellular cholesterol levels.

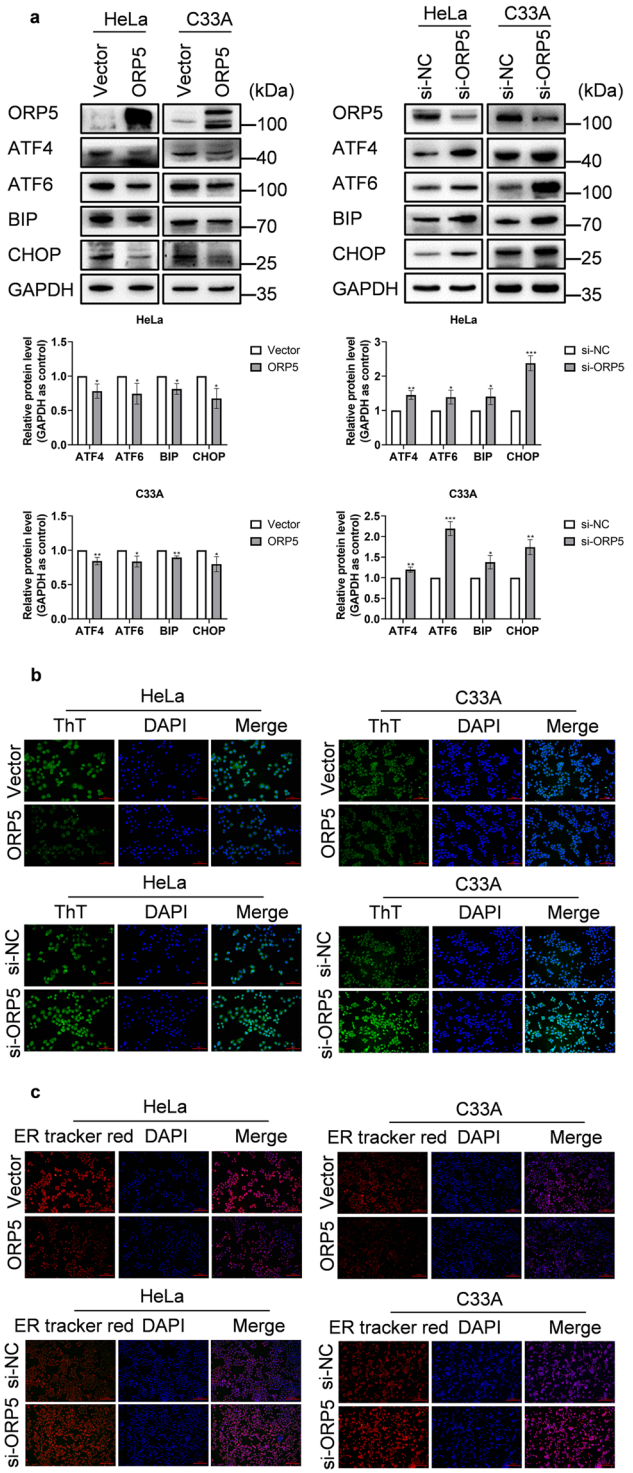


Fig. 2 ORP5 inhibits ER stress in CC. **a** Protein levels of ER stress sensors in HeLa and C33A cell lines overexpressing or knocking down ORP5. Statistical analysis of ATF6, ATF4, BIP, and CHOP expression ($n = 3$). **b** ThT staining of protein aggregates in HeLa and C33A cells overexpressing or knocking down ORP5 ($n = 3$). Scar bar, 100 µm. **c** Immunofluorescence technology traces ER in HeLa and C33A cells overexpressing or knocking down ORP5 ($n = 3$). Scar bar, 200 µm. Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

ORP5 may adversely affect SREBP1 precursor and mature form expression in HeLa and C33A cells, which is relevant to the interaction between ORP5 and SREBP1 according to a Western blot investigation (Fig. 4a). The effects of SREBP1 on ER stress and EMT in cervical cancer cells were the first thing we looked at. SREBP1 was knocked down in HeLa and C33A cells respectively, and the changes in ER stress and EMT marker protein levels were detected. The outcomes demonstrated that in cervical cancer cells, the knockdown of SREBP1 decreased ER stress and accelerated the EMT process (Fig. 4b, c). To investigate whether SREBP1 mediates the inhibition of ER stress by ORP5, we examined the changes in the levels of ER stress marker proteins. The findings revealed that the upregulation of ATF6, ATF4, BIP, and CHOP was suppressed by the knockdown of SREBP1 after the downregulation of ORP5, indicating that the involvement of SREBP1 is required for ORP5 to inhibit ER stress (Fig. 4d). We continued to look at EMT indicators to better understand if SREBP1 influences CC cell motility and invasion capability through ER stress. Western blot showed that knockdown of ORP5 significantly suppressed N-cadherin, Vimentin, and Snail1 and promoted E-cadherin expression. Following the simultaneous knockdown of SREBP1, these modifications were undone. This was also demonstrated by the transwell experiment (Fig. 4e, f). It was hypothesized that ORP5 facilitated the migration and invasion of CC cells HeLa and C33A via inhibiting ER stress by decreasing SREBP1 expression.

ORP5 reduces SREBP1 expression by promoting ubiquitination

As our previous experiments proved, SREBP1 precursor and mature forms were downregulated in HeLa and C33A cells after overexpression of ORP5. We first proposed that this might be caused by ORP5 encouraging the degradation of SREBP1 to further investigate the mechanism. CC cells in the vector and ORP5 groups, respectively, were treated with CHX, an inhibitor of protein synthesis, and proteins were collected at 0 h, 6 h, 12 h, and 24 h for western blot. The findings demonstrated that the SREBP1 protein stability was decreased in the cells of the ORP5 overexpression group, proving that our conjecture may be valid (Fig. 5a). Regarding the potential of ORP5 to regulate SREBP1 degradation, the fact that the ORP5-induced drop in SREBP1 expression was reversed following exposure to the proteasome inhibitor MG132 raises the possibility that ORP5 might encourage SREBP1 breakdown via the proteasome (Fig. 5b). The results of ubiquitination experiments indicate that in the presence of

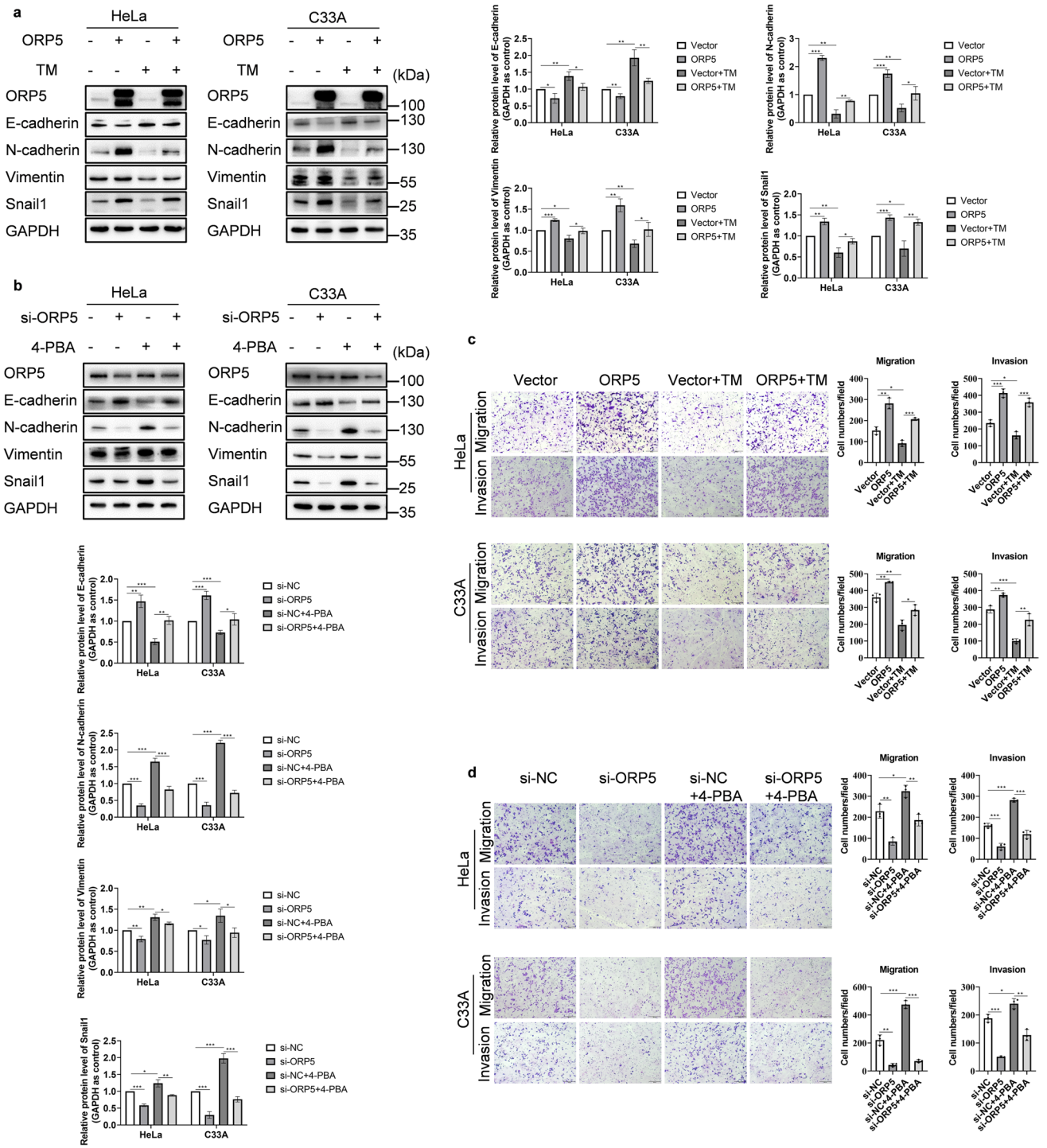
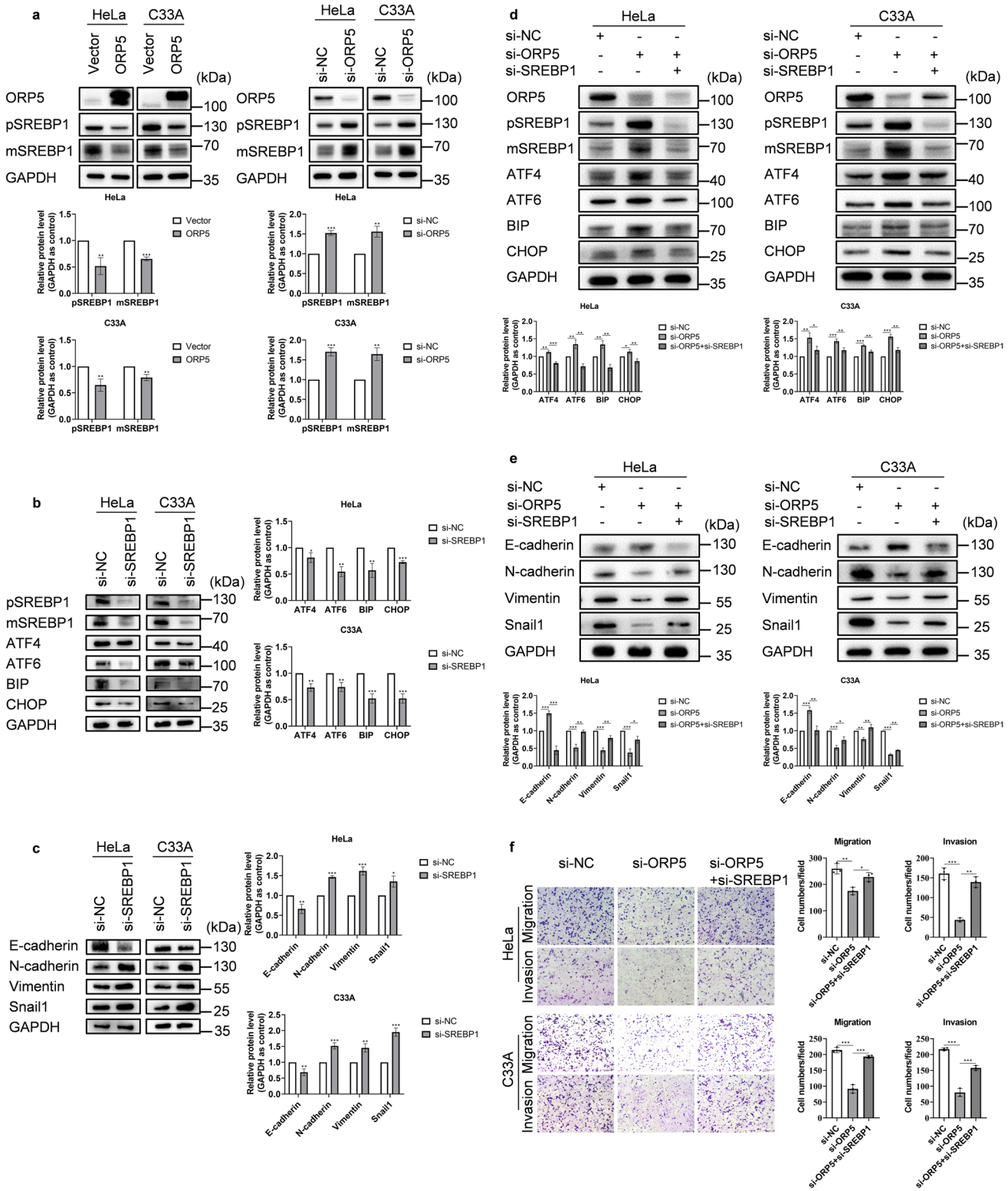


Fig. 3 ORP5 promotes migration and invasion of CC cells by inhibiting ER stress. **a** Protein levels of EMT sensors in ORP5 overexpressing CC cells with or without TM treatment. Statistical analysis of E-cadherin, N-cadherin, Vimentin, and Snail1 expression ($n = 3$). **b** Protein levels of EMT sensors in ORP5 knockdown CC cells with or without 4-PBA treatment. Statistical analysis of E-cadherin, N-cadherin, Vimentin, and Snail1 expression ($n = 3$). **c** Migration and invasion assay of ORP5 overexpressing CC cells with or without TM treatment ($n = 3$). Original magnifications, × 100 for (**c**). **d** Migration and invasion assay of ORP5 knockdown CC cells with or without 4-PBA treatment ($n = 3$). Original magnifications, × 100 for **d**. Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$



MG132, a significant increase of ubiquitinated SREBP1 can be observed in HeLa and C33A cells overexpressed with ORP5 (Fig. 5c). These results suggest that ORP5 overexpression induces the ubiquitin-dependent proteasome degradation of SREBP1.

4-PBA restores the inhibition of CC tumor metastasis in vivo by knockdown of ORP5

Finally, we established a mouse tumor metastasis model by tail vein injection of HeLa cells with stable knockdown

◂**Fig. 4** ORP5 inhibits ER stress and promotes migration and invasion of CC cells through downregulation of SREBP1. **a** Protein levels of SREBP1 in ORP5-upregulated or ORP5-downregulated CC cells. Statistical analysis of SREBP1 expression ($n = 3$). **b** Protein levels of ER stress sensors in SREBP1 knockdown CC cells. Statistical analysis of ATF6, ATF4, BIP, and CHOP expression ($n = 3$). **c** Protein levels of EMT sensors in SREBP1 knockdown CC cells. Statistical analysis of E-cadherin, N-cadherin, Vimentin, and Snail1 expression ($n = 3$). **d** Protein levels of ER stress sensors in ORP5 knockdown CC cells with or without si-SREBP1. Statistical analysis of ATF6, ATF4, BIP, and CHOP expression ($n = 3$). **e** Protein levels of EMT sensors in ORP5 knockdown CC cells with or without si-SREBP1. Statistical analysis of E-cadherin, N-cadherin, Vimentin, and Snail1 expression ($n = 3$). **f** Migration and invasion assay of ORP5 knockdown CC cells with or without si-SREBP1 ($n = 3$). Original magnifications, × 100 for **f**. Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

ORP5. The animals were slaughtered 5 weeks later, and the lungs were taken out for recording. The number of metastases and the weight of the lungs were reduced in the shORP5 group in comparison to the negative control shNC group, decreasing the lung metastasis of CC. However, 4-PBA treatment increased the number of metastases and the weight of the lungs in mice compared to the untreated group, indicating that treatment with 4-PBA rescued the downregulation of lung metastasis by shORP5 (Fig. 6a–c). The nodules in the mice's lungs were metastatic tumors, as revealed by H&E staining (Fig. 6d). Finally, these nodules were excised for IHC staining, and we found that ORP5 expression was diminished in the shORP5 group and SREBP1 expression was upregulated compared to the control group, demonstrating a negative correlation between ORP5 and SREBP1 levels (Fig. 6e).

Discussion

As a transmembrane protein that is anchored to the ER, ORP5 senses, binds, and transports lipids in the membrane contact sites (MCS). In addition, since MCS is an important

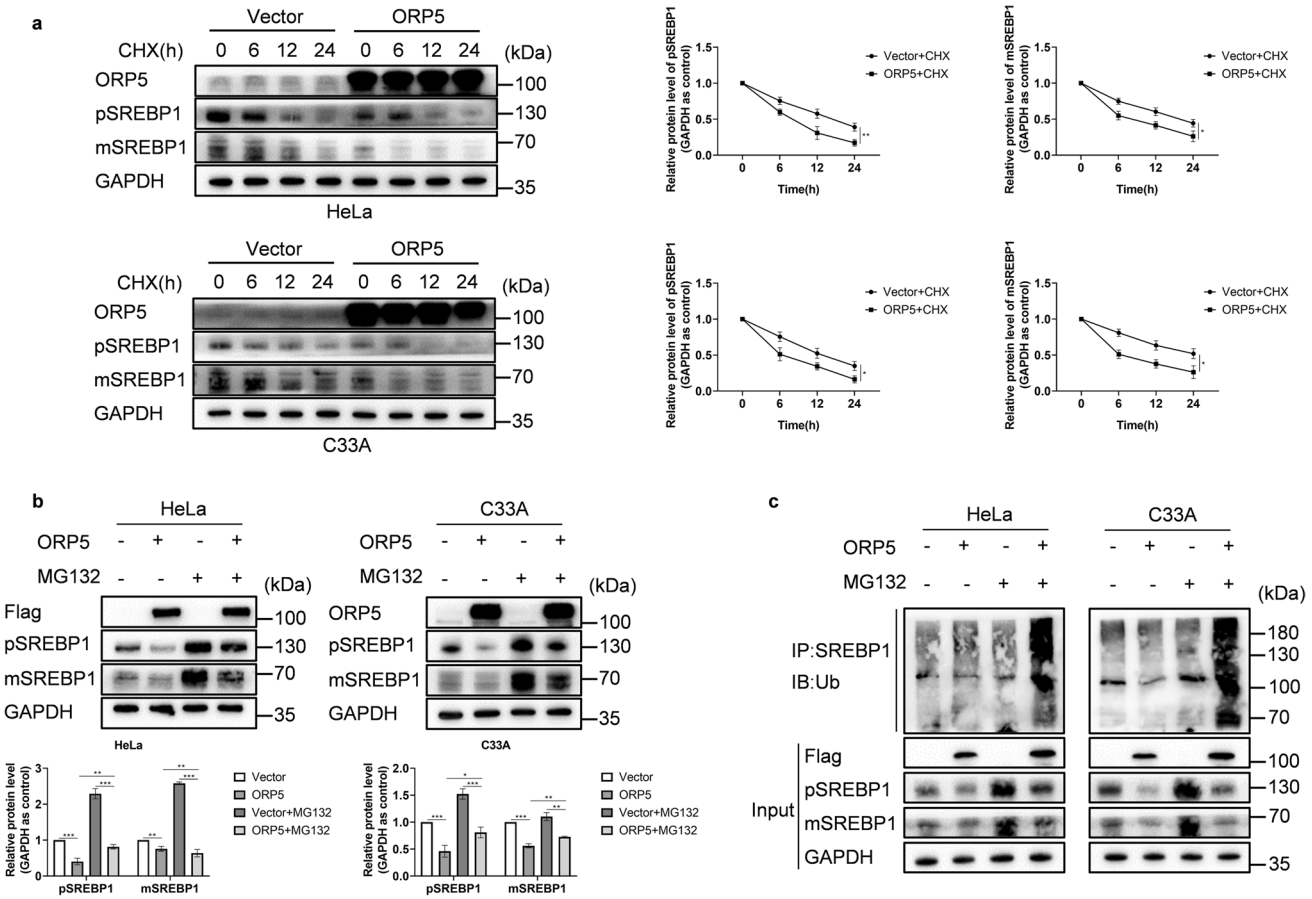


Fig. 5 ORP5 reduces SREBP1 expression by promoting ubiquitination. **a** Protein levels of SREBP1 after 0 h, 6 h, 12 h, and 24 h in CC cells of vector and ORP5 groups treated with CHX. Statistical analysis of SREBP1 expression ($n = 3$). **b** Protein levels of SREBP1 in ORP5-upregulated CC cells treated with or without MG132. Statistical analysis of SREBP1 expression ($n = 3$). **c** Immunoprecipitation and immunoblotting were performed to analyze the ubiquitination of SREBP1 in HeLa and C33A cells overexpressing ORP5 ($n = 3$). Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

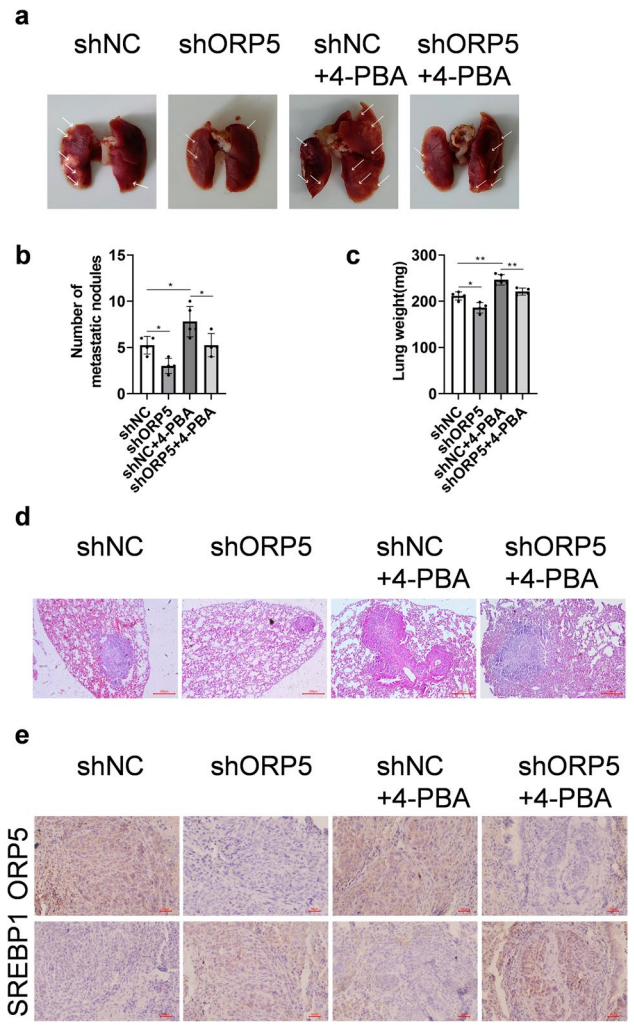


Fig. 6 4-PBA restores the inhibition of CC tumor metastasis in vivo by knockdown of ORP5. **a** HeLa cells transfected with lentivirus negative control shNC or shORP5 were injected into nude mice through the tail vein, and the lungs were finally removed and photographed ($n = 5$). **b** Quantitative analysis of the number of metastatic tumors in the lung. **c** The lungs of nude mice were weighed and recorded. **d** H&E staining results of metastatic tumors in the lung. Scar bar, 200 μm. **e** IHC staining of tumor sections with ORP5 and SREBP1 antibodies. Scar bar, 50 μm. Error bars represent SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

regulator of cellular signaling events, ORP5 has also been found to regulate Ca^{2+} signaling, and in the presence of histamine stimulation, upregulation of ORP5 can increase Ca^{2+} concentrations in mitochondria and PM niche sub-structural domains, which may affect various cellular functions such as migration, respiration, and proliferation (Pulli et al. 2018). Although ORP5 has been linked to the growth of some malignancies, the underlying processes are rarely discussed (Ishikawa et al. 2010, Nagano et al. 2015, Olkkonen 2022).

In this study, we successfully demonstrated that ORP5 is a metastasis-associated protein of CC that facilitates tumor migration and invasion. Previously, studies on human pancreatic cancer cells and analysis of clinical samples revealed that high expression of ORP5 not only enhanced tumor invasion but was also linked to a bad prognosis in human pancreatic cancer (Ishikawa et al. 2010); in human non-small cell lung cancer, ORP5 was found to have a similar role (Nagano et al. 2015). As a result, we speculated that ORP5 would have an oncogenic function in CC, and our prediction came true. In CC cells, ORP5 encouraged HeLa and C33A to migrate and invade, and among EMT indicators, ORP5 elevated mesenchymal marker protein levels while downregulating epithelial marker protein levels.

It is well known that ER plays crucial roles in folding, secretion, modification of proteins, lipid metabolism, and calcium storage. When the ER's homeostasis is disturbed, all these functions will be affected to varying degrees, leading to ER stress and inducing UPR (Wang and Kaufman 2014). In studies on cancer, ER stress has frequently been mentioned. Cancer tends to spread and metastasize to other tissues, and the adverse milieu in these tissues, as well as the properties of the tumor itself, increase the stress on protein secretion, folding, etc. and cause ER stress. Numerous cancers have been shown to have high levels of UPR activation (Chen and Cubillos-Ruiz 2021, Oakes and Papa 2015). ER stress has also been reported in CC. A traditional Chinese herb called *Sanghuangprous vaninii* has been noted as an effective treatment for several tumor- and gynecological-related illnesses. According to studies, *Sanghuangprous vaninii* inhibits the proliferation of CC cells and promotes apoptosis by inducing ER stress and mitochondrial apoptotic, ultimately exerting anti-tumor effects (He et al. 2021). Ginsenoside CK is a natural product of saponins and it has a number of advantages, such as anti-inflammatory, antidiabetic, anti-cancer, and neuroprotective properties. Treatment with ginsenoside CK increases reactive oxygen species levels, promotes Ca^{2+} leak, further induces autophagy and ER stress, and promotes apoptosis in CC cells (Yin et al. 2021). Long noncoding RNAs are often used as indicators in the detection and prediction of different forms of cancer, among which upregulation of the tumor suppressor maternally expressed gene 3 in CC triggered ER stress-related apoptosis, giving CC therapy a new theoretical foundation (Pan et al. 2021). The aforementioned results show how ER stress may act as a cancer suppressor in CC.

The function of ER is affected by many factors, such as the accumulation of reactive oxygen species, glucose starvation, lipid metabolism disorder, calcium loss, and redox state change; the ability of lipid components to regulate ER folding and transporter may also be related to the disturbance of ER calcium homeostasis (Chen and Cubillos-Ruiz 2021). Cholesterol is essential for normal cell membrane function. The ER contains extremely little cholesterol and is sensitive to abnormal distribution of free cholesterol. Cell membrane

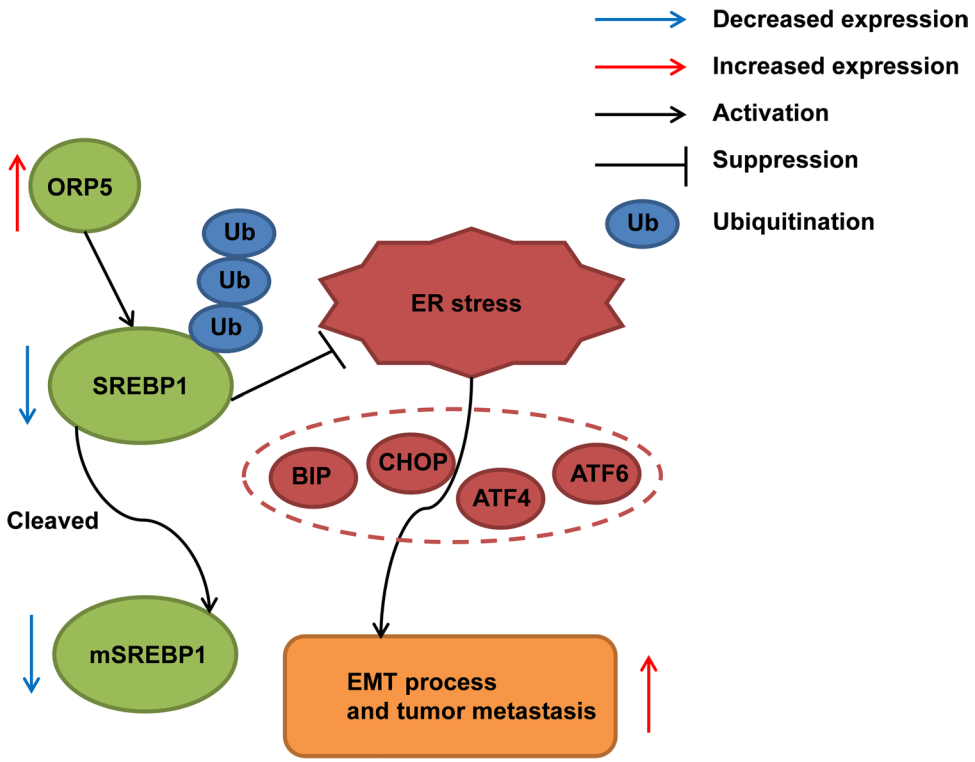


Fig. 7 A model of ORP5 regulation of ER stress and CC migration and invasion. ORP5 reduces SREBP1 expression by promoting ubiquitination, then inhibits ER stress and promotes the malignant progression of CC

composition can change a cell's biophysical characteristics. According to studies, cholesterol accumulation in ER membranes stimulates ER stress and induces apoptosis in macrophages, and cholesterol enrichment is accompanied by loss of calcium reserves in the ER (Devries-Seimon et al. 2005, Feng et al. 2003). Previous studies have rarely addressed the relationship between ORP5 and ER stress, and only two papers have reported that ORP8 plays an anti-tumor role by inducing ER stress in gastric and hepatocellular carcinoma (Guo et al. 2017, Zhong et al. 2015). But there has not been any information about ORP5 controlling ER stress. We investigated the relationship between ORP5 and ER stress in CC in light of the fact that ORP5 and ORP8 play similar roles in transporting lipids and regulating calcium signaling (Chung et al. 2015, Pulli et al. 2018), as well as the fact that ORP5 mediates cholesterol transport from the lysosome to the ER (Du et al. 2011). The results demonstrated that ORP5 could inhibit ER stress to promote CC progression, and the overexpression of ORP5 reduced the protein level expression of key molecules in the UPR branch, like ATF6, ATF4, BIP, and CHOP, and attenuated the fluorescence intensity of ER staining in living cells, all demonstrating that ER stress was alleviated. Further experiments revealed that all changes brought about by upregulation of ORP5 could be restored by ER stress inducer TM, which means that our study confirms for the first time that ORP5 can promote tumor metastasis by inhibiting ER stress levels in tumors and, therefore, can be a target for CC therapy.

SREBP1 is a crucial nuclear transcription factor. Processed SREBP1 enters the nucleus to stimulate target genes to promote transcription, including those implicated in adipogenesis (FASN, SCD, and ACLY), as well as important enzymes in the gluconeogenic and pentose phosphate pathways (PCK1, PKLR, and G6PC) (Yi et al. 2020). SREBP1 has also been shown to be a key regulator of the mTOR pathway (Yi et al. 2020, Yin et al. 2017). Cholesterol accumulation has been linked to ER stress activation and apoptotic promotion by maturing SREBP1 (Yan et al. 2021), while if ER function is impaired inducing UPR, it can increase cholesterol and triglyceride synthesis by further activating SREBP1, ultimately leading to lipid dysregulation (Colgan et al. 2011). All of the aforementioned data point to an association between ER stress and SREBP1. Given that ORP5 transports cholesterol, PS, and PI4P and that SREBP1 controls the transcription of fatty acid and cholesterol homeostasis, it stands to reason that ORP5 might influence ER stress via SREBP1. Through ubiquitination and other experiments, we verified this hypothesis, and ORP5 enhanced the proteasomal degradation of SREBP1, which in turn downregulated the protein level expression of major UPR downstream effectors and suppressed ER stress.

Notably, the depletion of Ca^{2+} leads to the buildup of unfolded proteins in the ER lumen and is a potent stimulus for ER stress. A study of Mannan-binding lectin deficiency found that MBL deficiency initiated inositol 1, 4, 5-trisphosphate receptor-mediated Ca^{2+} release into the cytoplasm,

and calcium imbalance in the ER further generated ER stress in the liver (Chen et al. 2021). The degeneration of the intervertebral disc may be due to the accumulation of advanced glycation end products. Luo et al. found that AGE treatment of nucleus pulposus cells resulted in a sustained increase in cytoplasmic Ca^{2+} concentration, while ER lumen Ca^{2+} was almost depleted, which then induced ER stress and subsequent apoptosis, and these processes could be significantly alleviated by Ca^{2+} antagonists, above illustrating the impact of impaired Ca^{2+} homeostasis on ER stress (Luo et al. 2019). The ability of ORP5 to modulate intracellular Ca^{2+} signaling suggests that it may contribute to the suppression of ER stress by ORP5. Since this is not ORP5's primary role, we neglected to address it in this experiment and will need to conduct additional research in the future.

In conclusion, our results demonstrate the pro-cancer role of ORP5 in CC progression, providing evidence for a link between ORP5, ER stress, and EMT markers. ORP5 inhibits ER stress by encouraging SREBP1 ubiquitination and lowering its protein levels, which induces EMT and ultimately promotes CC metastasis. Therefore, this might offer a fresh, promising approach to treating CC (Fig. 7).

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Declarations

Conflict of interest The authors declare no competing interests.

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