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RESEARCH ARTICLE

SHOC2 mediates the drug-resistance of triple-negative breast cancer cells to everolimus

Wenwen Geng[a,b], Meiling Cao[c], Ke Dong[a], Junhua An[a], and Haidong Gao[a,b]

[a]Department of Breast Surgery, Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University, Qingdao, China; [b]Oncology Laboratory, Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University, Qingdao, China; [c]Department of Rheumatology, Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University, Qingdao, China

ABSTRACT

Aberrant activation of the mTOR pathway is a characteristic alteration in triple-negative breast cancer, but the mTOR pathway inhibitor everolimus is not effective for the triple-negative breast cancer (TNBC) patients. Presently, we showed that the activation of ERK pathway was an important mechanism of resistance to everolimus in TNBC cells in this study. SHOC2, a key protein mediating the Ras-Raf-ERK pathway, could act as a scaffolding protein to facilitate the activation of the pathway by mediating the interaction of key components of the pathway. Our results showed that everolimus activated the Raf-ERK pathway by promoting the interaction between SHOC2 and c-Raf and that knockdown of SHOC2 significantly inhibited the Raf-ERK pathway induced by everolimus. We further demonstrated that SHOC2 expression levels were closely related to the sensitivity of TNBC cells to everolimus and that interference with SHOC2 expression in combination with everolimus had significant effects on the cell cycle progression and apoptosis in vitro experiments. Western blotting analysis showed that cell cycle regulators and apoptosis-related proteins were significantly altered by the combination treatment. Xenograft model also demonstrated that knockdown of SHOC2 significantly increased the sensitivity of tumor to everolimus in nude mice. In conclusion, our study showed that SHOC2 is a key factor in regulating the sensitivity of TNBC cells to everolimus and that combined therapy may be a more effective therapeutic approach for TNBC patients.

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SHOC2; Raf-ERK pathway; TNBC; everolimus; drug resistance

Introduction

Breast cancer has become the highest incidence malignant tumor for women in the world, triple-negative breast cancer (TNBC) is the most aggressive phenotype compared to other subtypes, accounting for about 20% of all cases. However, there is still no effective targeting therapy for these patients currently, and chemotherapy and radiotherapy may be the only treatment options. However, chemotherapy is only effective for a small number of early TNBC patients who are sensitive to it, and advanced breast patients usually respond poorly, leading to metastasis, recurrence and high mortality.[1] So looking for new effective molecular targets is an urgent need for those patients.

Mutation activation of PI3KCA gene and/or PTEN mutations of loss of PTEN expression are most common in the TNBC subtype, which can lead to a constitutive activation of downstream effector mTOR (mammalian target of rapamycin, mTOR).[2] The mTOR activation has been reported to promote tumor growth, increase protein synthesis in tumor cells, inhibit tumor cells autophagy and apoptosis, and affect the invasion and metastasis ability of tumor cells.[3] So the mTOR inhibitors have been applied to various malignant tumors. Everolimus (RAD001, Afinitor), a rapamycin analog, is an oral mTOR inhibitor. For breast cancer, everolimus has been used for the treatment of postmenopausal advanced female breast cancer patients with hormone receptor-positive, epidermal growth factor receptor-2 negative, while it is not effective for TNBC patients.[4] The mTOR pathway activation is generally changed in TNBC patients, but no substantial therapeutic effect was observed with mTOR inhibitors alone.[2,5] Another study also proved that the addition of everolimus to chemotherapy was associated with more adverse events in the treatment of TNBC patients, while the clinical effect had not been improved.[6] Therefore, the primary objectives of this study are to explore the drug-resistance mechanism of everolimus and provide new biomarkers and propose novel therapeutic strategies in the treatment of TNBC patients.

One of the drug-resistance mechanisms for pathway inhibitors may be that inhibition of a single pathway increases negative feedback activation of upstream and bypass pathways.[7,8] Interestingly, our study also showed that inhibition of mTOR by everolimus could promote the feedback activation of the Raf-ERK signaling pathway in TNBC cells. Blocking the ERK pathway could promote the sensitivity to everolimus, which suggested that the Raf-ERK signaling activation was a key event of resistance to everolimus in TNBC cells. It is well known that SHOC2 is required for Ras-Raf-ERK

CONTACT Haidong Gao haidonggao@sdu.edu.cn Department of Breast Surgery, Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University, Hefei Road No.758, Qingdao 266000, China

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kinase cascade, cooperating with c-Raf to mediate the Raf-ERK pathway activation.[9,10] Previous results of our research had also shown that SHOC2 knockdown could suppress the ERK pathway in MDA-MB-231 breast cancer cells. In the present study, we showed that everolimus suppressed mTOR pathway by inhibiting mTOR phosphorylation, and SHOC2 was critical for the activation of Raf-ERK pathway. Furthermore, we found that SHOC2 binding to c-Raf was increased after everolimus treatment, which lead to the phosphorylation downstream of ERK signaling cascades. Our results also showed that SHOC2 knockdown could dramatically promote the sensitivity to everolimus for TNBC cells in vitro and in vivo. Therefore, we propose that the activated Raf-ERK pathway mediated by SHOC2 might account for the resistance to everolimus in TNBC cells.

Results

ERK pathway is activated in everolimus-resistance TNBC cells

To evaluate the response to everolimus in different breast cancer cell lines, we performed the CCK-8 assay with increasing concentrations of everolimus (0 nM to 100 μM) for 24 h. The dosing was determined based on pharmacokinetic studies that indicated the plasma concentration of everolimus in clinical trials. The results showed that IC50 of everolimus in TNBC cell lines MDA-MB-231(27.40 uM) and Hs578T (31.23 uM) was significantly higher than that in other cells. The IC50 of everolimus in ER receptor-positive T47D (6.52 uM), MCF-7 (9.95 uM) and ZR-75-1 (13.67 uM) cell lines was lower than that in TNBC cells, but higher than that in HER2 receptor-positive BT474 (0.21 uM） cells [Figure 1(a)]. Therefore, we chose the everolimus-resistant TNBC cell lines MDA-MB-231 and Hs578T for this study. Subsequently, we examined the effect of everolimus on the cell cycle distribution of TNBC cells by flow cytometry. Cells were treated with 100 nM everolimus for 48 h, and the results showed that everolimus had no significant effect on the cell cycle progression of MDA-MB -231 and Hs578T cells, compared with the control group [Figure 1(b)].

Previous studies showed that inhibition of mTOR by everolimus could promote the activation of the MAPK pathway through a PI3K-dependent feedback loop in metastatic prostate cancer patients[7]. Another study also showed that inhibition of the EGFR pathway could increase the sensitivity of TNBC cells to everolimus, but the specific mechanism is still unclear. Therefore, we decide to examine the changes of ERK pathway under different everolimus-treated conditions in two different TNBC cell lines. Interestingly, we found that the phosphorylation levels of mTOR were gradually down-regulated with the increase of drug concentration, when MDA-MB-231 and Hs578T cells were treated with 0 nM (0.2% DMSO), 1 nM, 10 nM, 100 nM, 1uM, and 10 uM everolimus. Meanwhile, the phosphorylation level of ERK was significantly enhanced, and this activation effect was positively correlated with the drug concentration [Figure 2(a)]. Next, the cancer cells were treated with 100 nM everolimus to detect the effects of everolimus on ERK pathway for different periods of time (0 h, 1 h, 2 h, 4 h, 8 h, and 24 h). The results showed that the phosphorylation level of ERK gradually increased with the extension of time, and the effect of pathway activation could even last up to 24 h [Figure 2(b)].

Feedback activation of the ERK pathway is associated with drug resistance to everolimus in TNBC cells

In order to confirm whether everolimus-resistance in TNBC was associated with the feedback activation of ERK pathway, we then conducted inhibitor experiments. The results showed that the expression of p-ERK promoted by everolimus could be significantly suppressed with MEK-1-specific inhibitor PD98059, but the phosphorylation levels of mTOR and mTOR pathway were not changed [Figure 3(a)]. The CCK-8 assay showed that the proliferation of MDA-MB-231 and Hs578T cells were significantly suppressed by everolimus after inhibition of ERK pathway with PD98059, while these two inhibitors alone had no significant inhibitory effect on cell proliferation [Figure 3(b)]. Flow cytometry results also showed that everolimus combined with PD98059 could significantly affect cell cycle distribution in MDA-MB-231 and Hs578T cells. Compared with everolimus or PD98059 alone, the combination treatment induced a significant increase in the proportion of cells in the G1 phase, preventing the cell cycle transition from G0/G1 to S phase. Western blotting showed the expression of cycle-associated protein Cyclin D1 was significantly inhibited in MDA-MB-231 and Hs578T cells [Figure 3(c)]. These results indicated that the everolimus resistance in TNBC cells might not due to the limited response of the mTOR pathway but was closely related to the feedback activated ERK pathway. Inhibition of the feedback activated ERK pathway could significantly increase the sensitivity of TNBC cells to everolimus.

SHOC2 regulates the feedback activation of the Raf-ERK pathway after everolimus treatment by the interaction with c-Raf in in TNBC cells

In order to explore the mechanism of ERK pathway activation by everolimus, it is necessary to understand the process of Ras-Raf-ERK pathway activation. The activation of this pathway depends on the aggregation of substrates at specific sites, and requires scaffold proteins to regulate this process. SHOC2 is a scaffold protein and can mediate different functions with multiple interaction partners.[9] Studies have shown that SHOC2 was capable of enhancing ERK1/2 activity by forming a ternary complex with Ras and c-Raf kinase in response to stimuli from growth factor.[10] The scaffold protein SHOC2 can bind to c-Raf and recruit it to specific locations, thereby enhancing its activation.[11] Our previous studies have shown that knockdown of SHOC2 could inhibit the activity of the ERK pathway in breast cancer cells, and the high expression of SHOC2 in breast cancer tissues was associated with poor prognosis of breast cancer patients.[12] In this study, we first identified whether SHOC2 was necessary for the activation of Raf-ERK pathway in TNBC cells, and we found that SHOC2 overexpression could significantly increase the phosphorylation level of ERK. However, after SHOC2 knockdown in

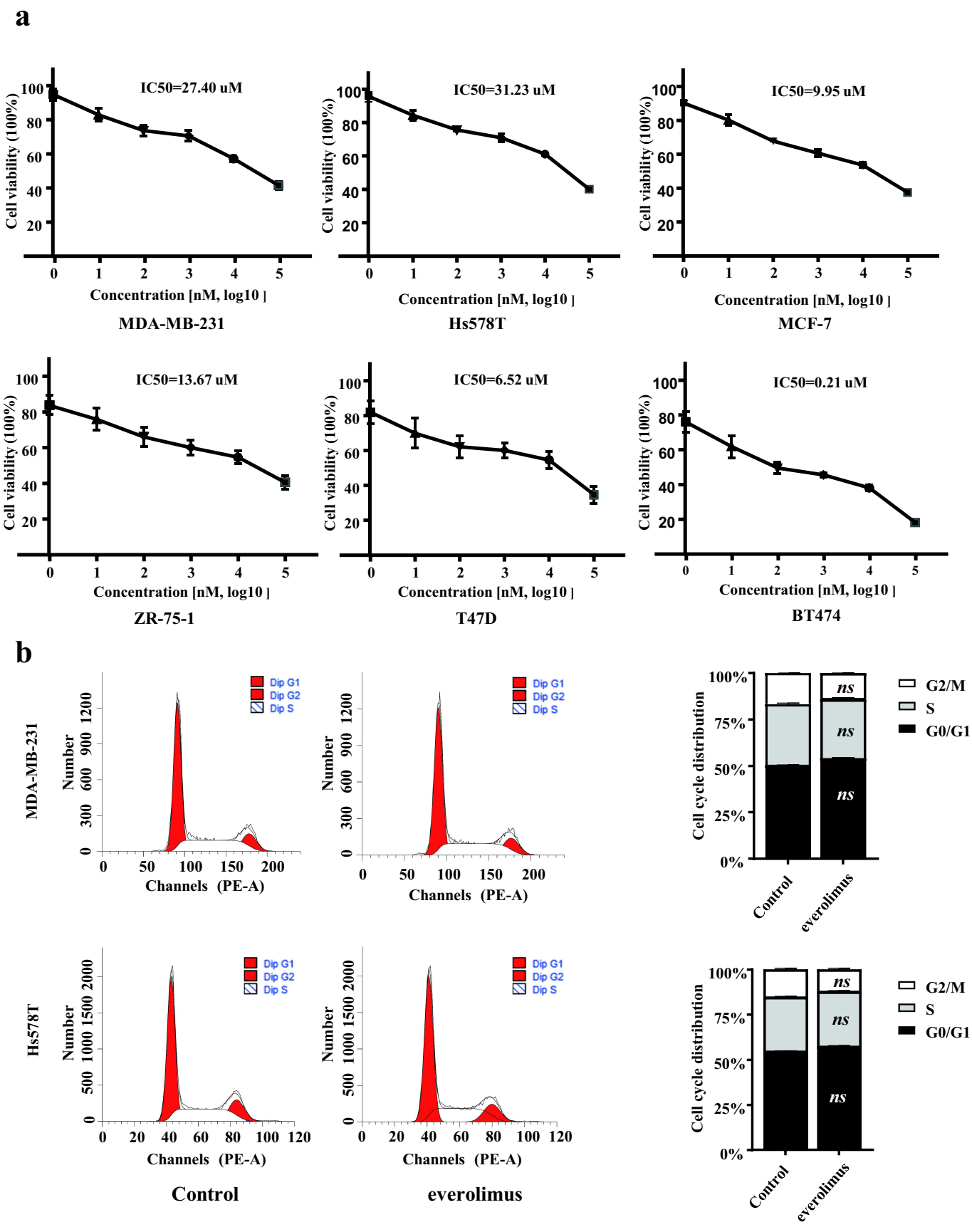

Figure 1. Cytotoxic effect and cell cycle change of everolimus on breast cancer cell lines. A. A CCK8 assay was used to detect the drug sensitivity of breast cancer cells after treated for 24 h with increasing concentrations of everolimus. Survival rate = (mean absorbance of experimental group/mean absorbance of 0.2% DMSO group) × 100%. B. Cell cycle analysis by flow cytometry in MDA-MB-231 and Hs578T cells after treated with 100 nM everolimus. Data represent the mean value ± SD of triplicate experiments. *ns*: not significant for comparison between treated group and control group (0.2% DMSO) cells using Student's t-test.

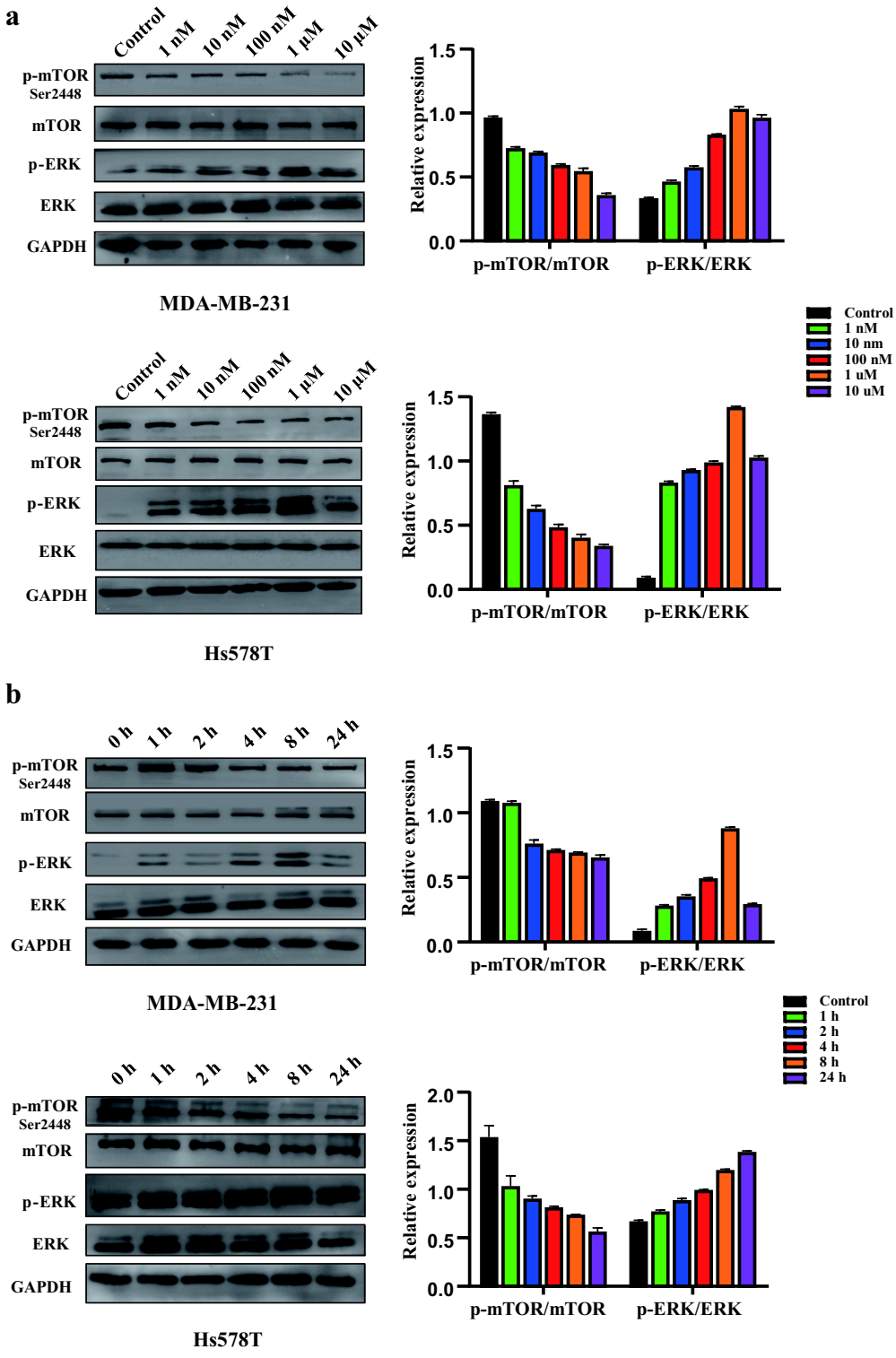

Figure 2. Treatment with everolimus promotes the activity of ERK signaling pathways. A. Western blotting analysis of ERK and mTOR signaling pathways in MDA-MB -231 and Hs578T TNBC cell lines for 4 h with increasing concentrations of everolimus. B. Western blotting analysis of ERK and mTOR signaling pathways in MDA-MB-231 and Hs578T TNBC cell lines for different times with 100 nM everolimus.

TNBC cells MDA-MB-231 and Hs578T, the phosphorylation level of ERK was dramatically down-regulated, which implied that Raf-ERK pathway was significantly inhibited [Figure 4(a)].

Based on the above results, SHOC2 and c-Raf are required for the activation of the Raf-ERK pathway. We next performed co-immunoprecipitation experiments to investigate whether the interaction between SHOC2 and c-Raf played important roles in the Raf-ERK pathway activation for the TNBC cells. The results showed that SHOC2 could interact with heterologous and endogenous c-Raf in 293T cells and TNBC cells, respectively [Figure 4(c)], suggesting that SHOC2 might mediate the Raf-ERK pathway by interacting with c-Raf. Then we used

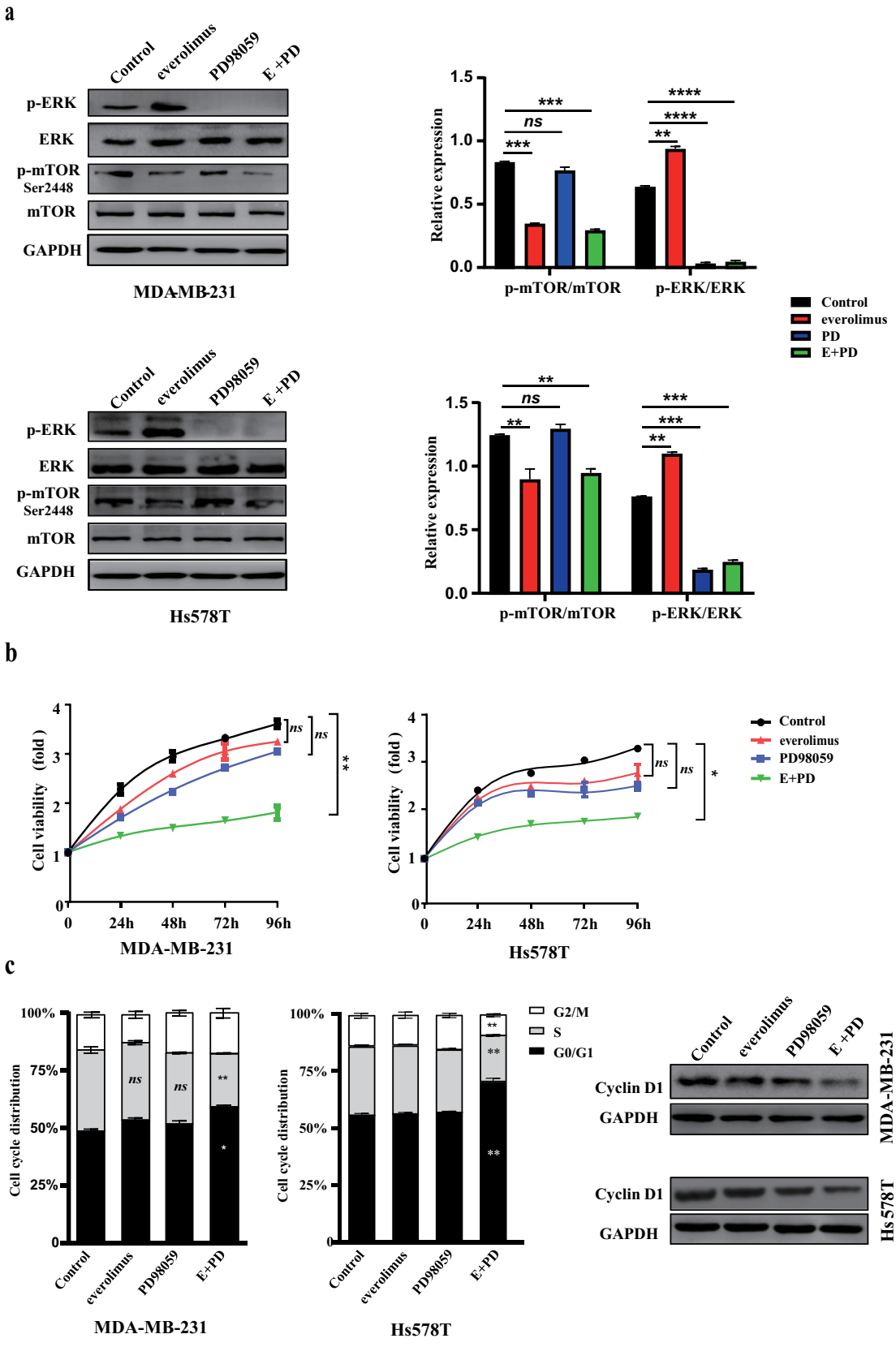

Figure 3. Effect of everolimus combined with the ERK signaling pathway inhibition on cell proliferation and cycle progression in TNBC cell lines. The cells were treated with 100 nM everolimus (E) for 4 h and then followed with or without 5 μM PD98059 (PD) as single agents (everolimus, PD98059) or in combination (E+PD). A. Western blotting analysis of ERK and mTOR signaling pathways in TNBC cell lines. B. Cell proliferation was determined using a CCK-8 assay in MDA-MB-231 and Hs578T cells. C. Cell cycle distribution and changes in the protein expression of cell cycle-related gene Cyclin D1 in MDA-MB-231 and Hs578T cells. The cell cycle distribution and quantification of positive cells were evaluated by flow cytometry. Data represent the mean value ± SD of triplicate experiments. *ns*: not significant, *$p < 0.05$,**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ for comparison between treated group and control group (0.2% DMSO) cells using Student's t-test.

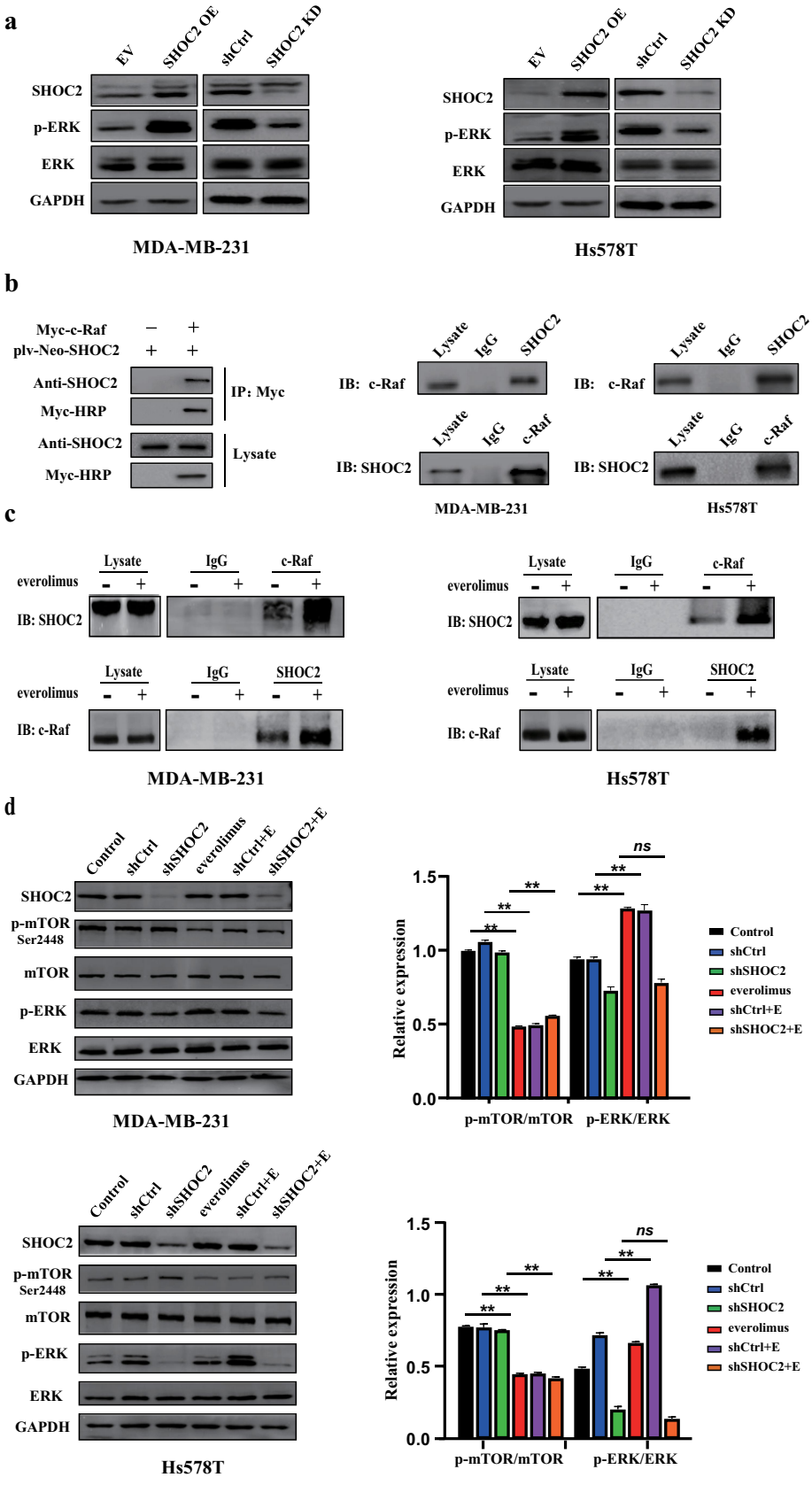

Figure 4. SHOC2 mediates the Raf-ERK pathway activated by everolimus in TNBC cell lines. A. Western blotting showing the activity of ERK pathway in SHOC2-overexpressing (OE) or SHOC2 knockdown (KD) MDA-MB-231 and Hs578T cells. B. Co-immunoprecipitation with anti-Myc antibody in whole-cell extracts of HEK293T cells over-expressing Myc-c-Raf and Flag-SHOC2 (left). Co-immunoprecipitation of endogenous c-Raf with anti SHOC2 antibodies (upper) and endogenous SHOC2 with anti c-Raf antibodies (lower) in MDA-MB-231 and Hs578T cells (right). C. Co-immunoprecipitation of endogenous SHOC2 with anti c-Raf antibodies (upper) and endogenous c-Raf with anti SHOC2 antibodies (lower) in MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E) for 4 h. D. Western blotting showing the activity of mTOR and ERK pathway in control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E) for 4 h. Data represent the mean value ± SD of triplicate experiments. *ns*: not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, for comparison between groups treated with or without everolimus using Student's t-test using Student's t-test.

co-immunoprecipitation assay to explore the effect of everolimus on the interaction of SHOC2 with c-Raf. The results showed that the binding of SHOC2 to c-Raf was significantly increased after treated with everolimus in TNBC cells [Figure 4(d)]. So our results indicated that everolimus might activate the Raf-ERK pathway by enhancing the interaction between SHOC2 and c-Raf in TNBC cells.

In the next steps, to further confirm the important role of SHOC2 in the feedback activation of Raf-ERK pathway induced by everolimus, we used TNBC cells with stable knockdown of SHOC2 to conduct related experiments. Western blotting showed that the phosphorylation levels of ERK induced by everolimus were significantly down-regulated after SHOC2 knockdown, compared with the control and empty vector groups in the TNBC cells MDA-MB-231 and Hs578T [Figure 4(b)]. These results confirmed that SHOC2 was indispensable for the feedback activation of the Raf-ERK pathway after everolimus treatment in TNBC cells.

Knockdown of SHOC2 increases the sensitivity of TNBC cells to everolimus in vitro and in vivo

Based on the above results, we further studied whether SHOC2 expression affected the sensitivity of TNBC cells to everolimus. The CCK-8 and clonogenic assays showed that SHOC2 knockdown dramatically enhanced the inhibitory effect of everolimus on proliferation and clone forming ability in MDA-MB-231 and Hs578T [Figure 5(a,b)]. Next, we conducted cell cycle and apoptosis analysis using a flow cytometry. The results of cell cycle experiments showed that the cell cycle of cells was significantly suppressed by everolimus after SHOC2 knockdown, preventing the cell cycle transition from G0/G1 to S phase. The proportion of S phase was significantly reduced compared with other groups. Meanwhile, western blotting showed that the expression of cycle-related protein Cyclin D1 was also significantly inhibited in the TNBC cells [Figure 6(a)]. The cell apoptosis analysis showed that the proportion of apoptotic cells increased significantly in the combination-treated group [Figure 6(b)], which meant the apoptotic effect of everolimus on TNBC cells was enhanced by SHOC2 knockdown. Furthermore, western blotting results showed that

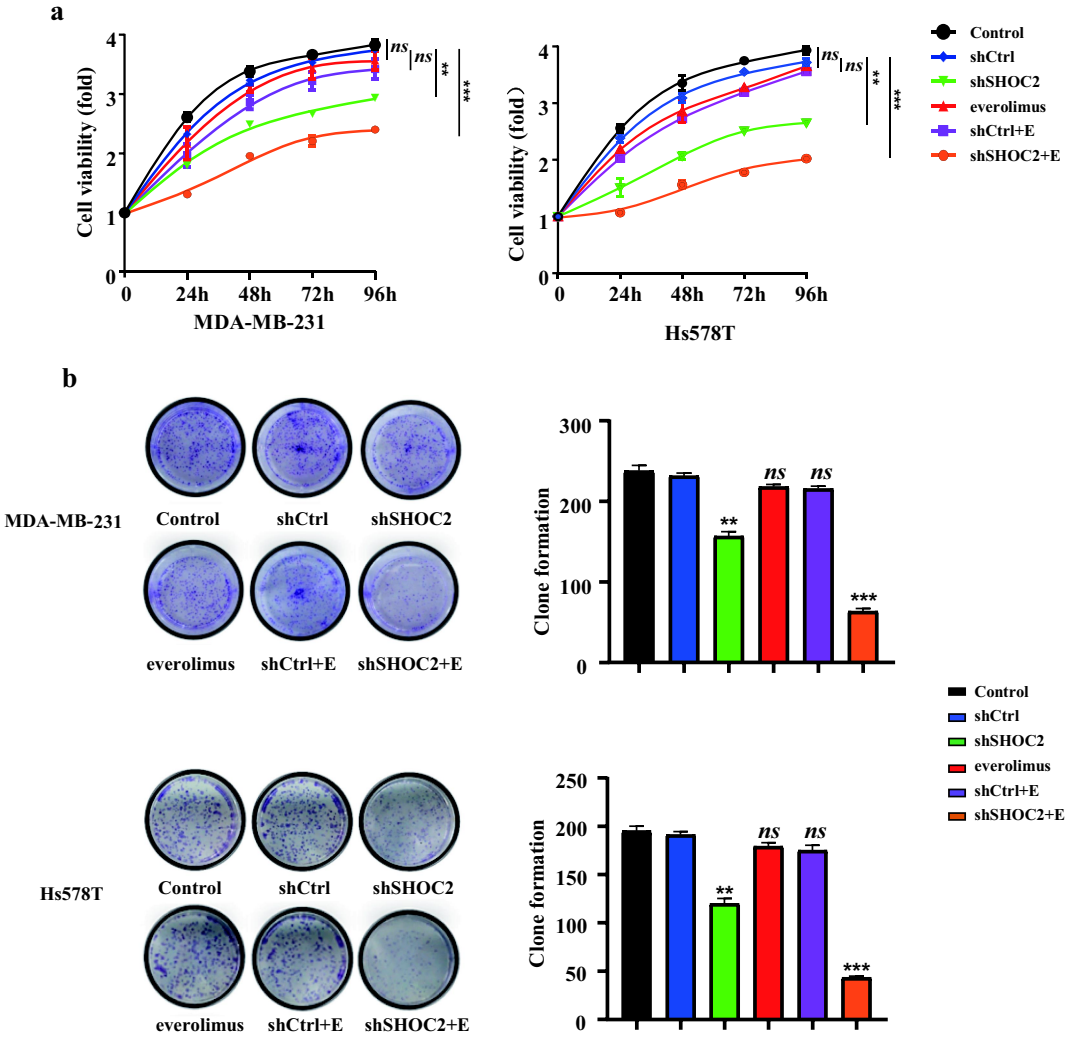

Figure 5. SHOC2 knockdown can potentiate the inhibition of cell proliferation and clone formation by everolimus in TNBC breast cancer cells. A. Cell proliferation was determined using a CCK-8 assay in Control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E). B. Clone formation assay for Control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E). Data represent the mean value ± SD of triplicate experiments. *ns*: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$ for comparison between the treated group and the control group (0.2% DMSO) cells using Student's t-test.

the expression of pro-apoptotic proteins Cleaved-Caspase 3 and Bax protein were significantly up-regulated, and the expression of anti-apoptosis protein Bcl-2 was down-regulated [Figure 6(c)]. These results showed that SHOC2 knockdown could significantly promote the sensitivity of TNBC cells to everolimus by inhibiting cell proliferation and clone formation and increasing the cycle arrest and apoptosis of breast cancer cells.

To further confirm the effect of SHOC2 on the sensitivity of TNBC cells to everolimus, we performed animal experiments using a xenograft tumor mouse model. The volume of subcutaneous tumors in nude mice was compared to detect the influence of SHOC2 expression on everolimus sensitivity in vivo. The results showed that SHOC2 knockdown could reduce the size of the tumor, but the tumor size of the everolimus treatment group was not significantly reduced compared with the control group. Most importantly, the tumor size of the combination-treated group with everolimus and SHOC2 knockdown was significantly smaller than the other three groups. These results show that the knockdown of SHOC2 could significantly enhance the tumor suppressor effect of everolimus on breast cancer cells in vivo [Figure 7(a)]. In addition, we also take tumor tissues of nude mice for western blotting assay, which showed that the ERK pathway in tumor cells was indeed activated as the mTOR pathway inhibited after everolimus treatment in nude mice [Figure 7(b)]. This evidence indicated that this feedback pathway also existed in vivo, and the feedback effect of everolimus on the ERK pathway could be significantly weakened by SHOC2 knockdown.

Discussion

The activation of PI3K/AKT/mTOR signaling pathways is a key event in TNBC,[13] and there are far fewer mutations and alterations in the core component of the mTOR pathway, compared with PTEN loss-of-function mutation or PIK3CA mutation.[14] Oncogenic activation of the mTOR signaling pathway is considered to be associated with the promoting of proliferation, invasion and metastasis ability for cancer cells. Clinical studies have shown that higher expression levels of mTOR indicated worse prognosis for breast cancer patients,[15] and increased mTOR phosphorylation levels were more commonly found in TNBC patients. So the activation of the mTOR signaling pathway is an important mechanism for tumorigenesis and metastasis in TNBC. Unfortunately, the overall treatment effect of everolimus for TNBC patients is not satisfactory.[16] One of the considerable reasons for resistance to everolimus could be the activation of bypass pathway, and inhibition of mTORC1 leads to MAPK pathway activation in human cancers.[7,17] However, the specific mechanism remains unclear.

SHOC2 is associated with the Ras-Raf-ERK signaling pathway activation as a scaffold protein. It can promote the c-Raf activation, resulting in the activation of c-Raf-MEK-ERK signaling pathway.[10] Recently, many studies have reported the important role of SHOC2 in regulating drug resistance. One study showed SHOC2 was a key regulator for the sensitivity to EGFR-TKI therapy in patients with non-small cell lung cancer.[18] Additionally, Jones et al. showed that SHOC2 deletion sensitized MDA-MB-231 breast cancer cells to MEK inhibitors.[11] However, there is still no study on the modulatory role of SHOC2 in the regulation of cellular sensitivities toward mTOR inhibitor everolimus. Therefore, the primary purpose of this study was to address the mechanism by which SHOC2 regulated the drug resistance to everolimus in TNBC. We have previously demonstrated that SHOC2 knockdown could suppress the activation of the ERK signaling pathway in breast cancer cells. In this study, our results verified that SHOC2 could interact with c-Raf, and the activity of ERK signaling could be regulated by SHOC2 expression in TNBC cell lines MDA-MB-231 and Hs578T. We also found that the binding of SHOC2 to c-Raf was enhanced after everolimus treatment, so these results suggested that SHOC2 could mediate the activation of Raf – ERK pathway by interaction with c-Raf in TNBC.

Furthermore, our study reveals that SHOC2 was essential for feedback activation of ERK pathway, and knockdown of SHOC2 significantly increased the sensitivity to everolimus for TNBC in vitro and in vivo. Therefore, the interaction of SHOC2 with c-Raf is required for the downstream activation of ERK pathway, which was the major mechanism of drug resistance to everolimus in TNBC. Therefore, we can assert that SHOC2 is an important regulator of the sensitivity of TNBC to everolimus. Combinatory treatment using everolimus and SHOC2 knockdown may be more effective for treating TNBC patients.

Nowadays, combined inhibition of signaling pathways may be a reasonable therapeutic strategy for cancer patients, which has already been proven as a promising treatment in preclinical models.[19,20] Researchers have shown that the efficacy of gefitinib in gefitinib-resistant NSCLC cells could be improved by everolimus, and the combination has a synergistic growth-suppressive effect by dramatically downregulating the activity of the EGFR signaling pathway in NSCLC cells[21]. The combined inhibition of mTOR and EGFR could significantly and synergistically suppress CRC cell proliferation and cycle progression.[22] Guerrab et al. showed that the combination of everolimus and gefitinib also could synergistically inhibit the breast cancer cells by activating mutations in PI3K or loss of PTEN.[23] Taken together, these studies indicated that dual inhibition of pathways might be an efficient treatment for TNBC. In this study, we examined the changes of bypass pathway after everolimus treatment in everolimus-resistance TNBC. Our results showed that everolimus promoted the phosphorylation ERK, resulting in enhanced activity of the Raf-MEK-ERK signaling pathway. Furthermore, the inhibition effects of everolimus could significantly be enhanced with suppress of feedback activated ERK pathway by specific MEK inhibitor PD98059. Therefore, we proposed that the feedback activation of the Raf-MEK-ERK signaling pathway by everolimus was an important mechanism of drug resistant for TNBC, and inhibition of this pathway may be of great value in improving the sensitivity to everolimus for TNBC.

Although preclinical studies suggest enhanced antitumor effects with combined targeted therapy, the toxicity of combined pathway inhibitors has limited its widespread application, given that mTOR and Raf-MEK-ERK signaling pathways mediate important signaling transduction in cell biological progression. Results have shown that inhibiting EGFR and mTOR signaling by lapatinib and rapamycin combination fostered more cytotoxic effects than that with a single inhibitor.[24] Recent studies

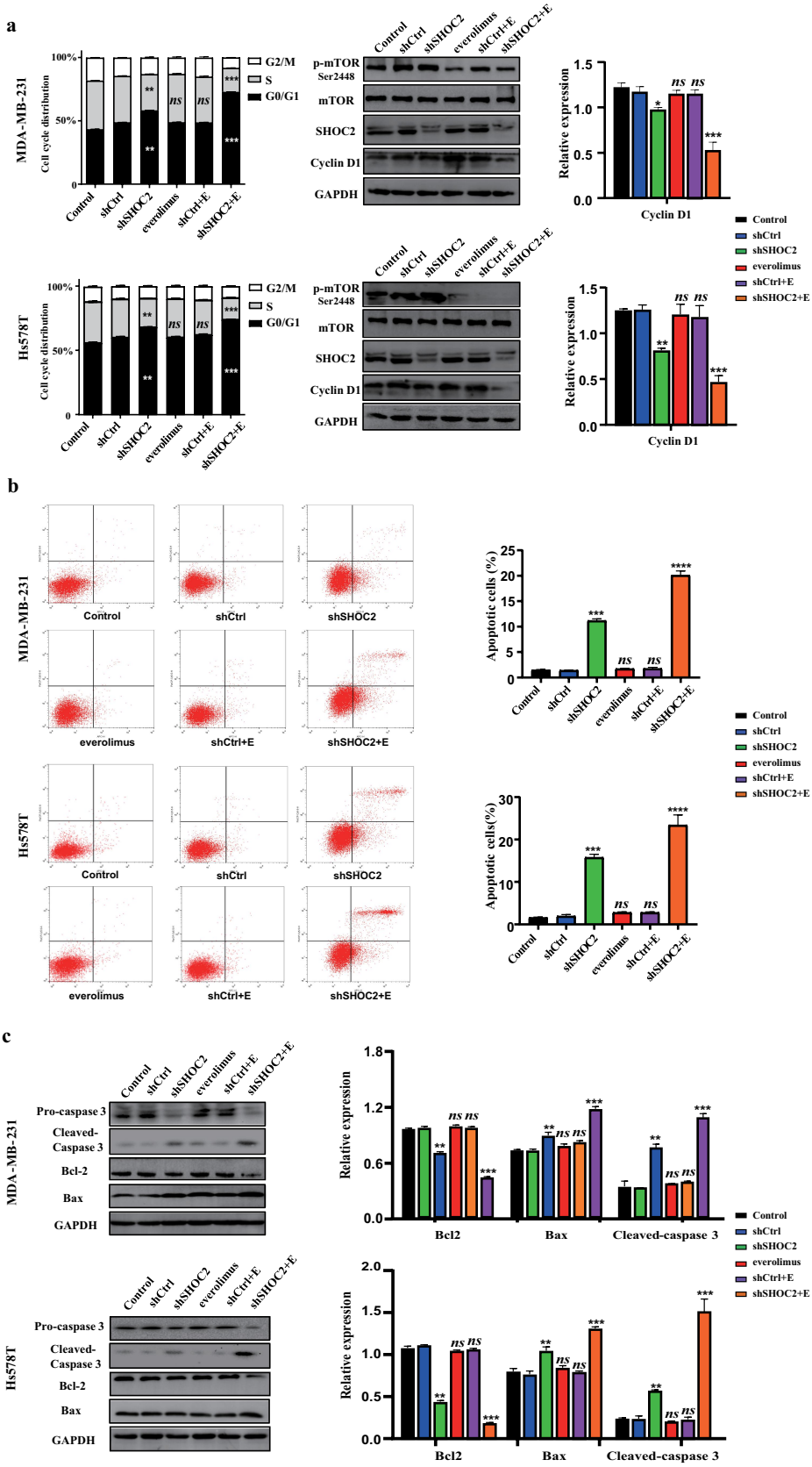

Figure 6. SHOC2 knockdown can potentiate the cell cycle arrest and apoptosis induced by everolimus in TNBC breast cancer cells. A. Cell cycle distribution and changes in the protein expression of cell cycle-related gene Cyclin D1 in Control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E). B. Cell apoptosis analysis in Control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E). C. Western blotting showing the changes in the protein expression of cell apoptosis-related genes in Control, shControl or SHOC2 knockdown MDA-MB-231 and Hs578T cells treated with 100 nM everolimus (E). The cell cycle distribution and quantification of positive cells were evaluated by flow cytometry. Data represent the mean value ± SD of triplicate experiments. *ns*: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ for comparison between the treated group and the control group (0.2% DMSO) cells using Student's t-test. B. Western blotting analysis of tumor tissues showed that the ERK pathway was feedback activated in vivo and could be significantly weakened by SHOC2 knockdown.

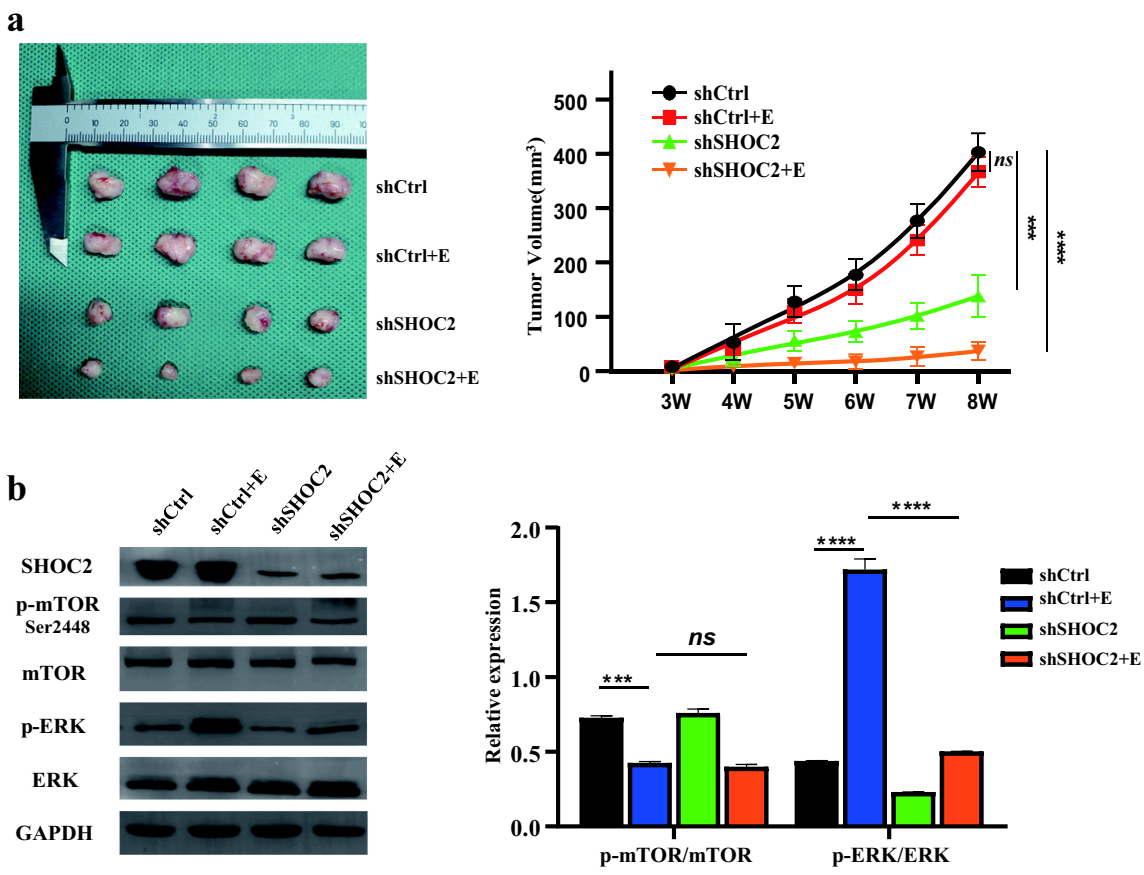

Figure 7. Knockdown of SHOC2 increases the sensitivity of TNBC breast cancer cells to everolimus in vivo. A. Effect of everolimus on nude mice burdened with SHOC2 knockdown MDA-MB-231 cell xenografts. The Nude mice inoculated subcutaneously with shControl or SHOC2 knockdown MDA-MB-231 for 8 weeks. Randomly chosen mice were receive either (3.5mg/kg) everolimus (n = 4) or normal saline (n = 4) by gastric lavage daily starting the day. At the end of the treatment period, mice were sacrificed and tumor specimens harvested. The tumor volume (V) was determined by measuring the length (a) and width (b) of calipers every week and calculated using the formula: V (mm^3) =π/6×ab^2. The mean tumor volume for each treatment group is indicated. The results are represented as the mean ± SD of 4 mice per group (n = 4, *ns*: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). B. Western blotting analysis of tumor tissues showed that the ERK pathway was feedback activated in vivo and could be significantly weakened by SHOC2 knockdown.

have shown that compared with targeting the core components of the Raf-MEK-ERK pathway that can affect the whole ERK signaling pathway, targeting SHCO2 would only inhibit the activation of the ERK pathway driven by oncogene, while not affecting the ERK pathway regulated by the SHOC2-independent mechanism under normal conditions in multiple cancer cells. Furthermore, the conditional knockout mouse model showed that SHOC2 knockout had little impact on the mice normal physiological functions, which suggesting that compared with MEK inhibition, targeting SHOC2 might have less interference with the normal cell function.[11] Together, our results suggested that targeting SHOC2 combined with everolimus might be a potentially novel therapeutic approach against TNBC. However, there are still some limitations for this study. Since everolimus has not been applied to triple-negative breast cancer currently, it is difficult to further verify the effect of SHOC2 expression on the sensitivity of everolimus therapy in patients with triple-negative breast cancer by histological experiments. This problem may be preliminarily verified by constructing a PDX model for tumor tissues of TNBC patients with different SHOC2 expressions.

Materials and methods

Cell lines, culture conditions and reagents

ER positive MCF-7, ZR-75-1 and T47D cell lines, HER2 positive BT474 cell line, triple-negative cell lines MDA-MB-231 and Hs578T, and HEK293T were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), respectively. All cell lines were conserved in the center lab of Qilu Hospital of Shandong University. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO2. T47D and ZR-75-1 cells were cultured in RPMI 1640 and MCF-7, BT474, MDA-MB-231 in DMEM medium, and the medium was supplemented with 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA). Hs578T cells were cultured in DMEM medium supplemented with 10% FBS and 5 μg/ml insulin (Soribo, Beijing, China). The MEK-1 inhibitor PD98059 and the mTOR inhibitor everolimus were purchased from Selleck Chemicals. Drugs were dissolved in DMSO (Soribo, Beijing, China) and stored at −20°C. DMSO was present in all samples, including the control samples, to a concentration of 2%. The stable SHOC2 knockdown cells of Hs578T and MDA-MB-231 were constructed as previously

described.[12] The plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Cell viability assay

CCK-8 (CCK8, Dojindo, Japan) was used to measure the viability of the cells according to the manufacturer's protocols. Two thousand cancer cells were seeded per well in 96-well microplates (Corning, Lowell, MA, USA), and 100 uL of culture medium was added to each well. During the cell viability test, 10 μL of CCK-8 reagent was added to each well and incubated for 2 h at different time points and with different treatments. The relative cell viability was expressed as a percentage of that of the control group cells, and the IC50 values were extrapolated from dose–response curves after nonlinear regression analysis. All experiments were performed in triplicate. The absorbance was analyzed at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) using wells without cells as blanks. The proliferation of cells was expressed by the absorbance.

Colony formation assay

Cells were treated with 100 nM everolimus for 24 h, then the treated cells were resuspended into the six-well plate at a density of 300 cells/well to form cell clones. Cultured in DMEM containing 10% FBS at 5% CO_2, 37°C for 15 d, cells were fixed and stained, and the colonies more than 100 cells were counted by microscope (Leica Microsystems, Germany). Each experiment was done in triplicate in this study.

Western blotting and immunoprecipitation

Confluent cells and tumor tissues were lysed in a radioimmunoprecipitation assay (RIPA) buffer, supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). 30 ug of each protein samples from cell lysates were loaded in 7–12% SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes (GE Healthcare, Westborough, MA, US). Membranes were blocked and incubated with the indicated primary antibodies overnight at 4°C, antibody information was listed in Supplementary Table 1. Western blots signals were visualized using a chemiluminescence kit (Amersham Bioscience, Piscataway, NJ, USA). For immunoprecipitation, cells with indicated treatments were lysed in 200 mM KCl, 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 10% glycerol, 0.2 mM EDTA, and 0.1% NP-40, supplemented with protease inhibitor cocktail. Clear cell lysates were then incubated with the respective antibodies or controlled IgGs at 4°C overnight. Beads-bound immunoprecipitates were washed, eluted in a Laemmli loading buffer and analyzed by western blotting.

Cell cycle analysis

Cell cycle analysis was carried out as described in our previous studies.[12] Briefly, cells were cultured in 6-well plates with 5×10^4 cells per well and treated with 5 μM PD98059 and/or 100 nM everolimus for 24 h. Cells were by trypsinization and centrifuged at 500 g for 3 min. After centrifugation, the supernatants were removed, and cells were fixed in 75% cold ethanol for 1 h. The fixed cells were resuspended with PBS solution and permeabilized with 0.1% Triton X-100 and 20 ug/ml RNase A for 30 min. Then the cells were resuspended with PBS solution and stained with 50 ug/ml propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA). Fluorescence of cells was analyzed by flow cytometry on FACSAria flow cytometer (BD, USA), and the percentage of cells at G0-G1, S and G2-M phases was determined using ModFit LT 2.0 software (Verity Software House, Topsham, ME, USA).

Apoptosis assay

The cells were prepared and treated for 72 h as in the cell cycle experiments. After centrifugation and washing with cold PBS, cell pellets were resuspended in 100 μl of binding buffer and incubated with FITC Annexin V (Thermo Fisher Scientific) and propidium iodide (PI) solution at room temperature in the dark for 15 min, then the cells were analyzed by flow cytometry.

Mouse xenograft model

All animal experiments were approved by the Laboratory Animal Center, Qilu Hospital of Shandong University. Four weeks of female BALB/c nude mice were purchased from Charles River (Beijing, China). Mice were anesthetized with pentobarbital at 50 mg/kg (i.p.) and Matrigel (Corning, Tewksbury, MA, USA) suspensions containing 1×10^7 cells of shSHOC2 or shCtrl MDA-MB-231 were subcutaneously injected. Tumor volumes were measured using calipers starting from the third week. Randomly chosen mice were receive either (3.5 mg/kg) everolimus ($n = 4$) or placebo ($n = 4$) by gastric lavage daily starting from the fourth week.

Statistical analysis

All results are shown to be the mean ± SD of multiple independent experiments. Student's *t*-test (for two-group comparisons) or one-way ANOVA (for more than two-group comparisons) was used for statistical analyses. All statistical analyses were performed with GraphPad Prism 8 software. All statistical tests were two-sided, and P values <0.05 were considered statistically significant.

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Disclosure statement

The authors declare that they have no competing interests.

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Notes on contributors

Haidong Gao is a research scientist at Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University.

Wenwen Geng received his Master degree in oncology from Tianjin Medical University in 2013, and his MD. degree in Surgery from Cheeloo College of Medicine, Shandong University in 2020. His research focuses on the mechanisms of drug resistance in targeting therapy for breast cancer.

Meiling Cao received her B.S. degree from Shandong University, and is currently working at Qilu Hospital (Qingdao). Her research interests focus on the construction of animal models for tumor experiments.

Ke Dong and Junhua An received their master's degree in surgery from Cheeloo College of Medicine, Shandong University, and they are currently working at Qilu Hospital (Qingdao).

ORCID

Haidong Gao http://orcid.org/0000-0001-5560-6323

Data availability statement

The data that support the findings of this study are available from the corresponding author Gao HD upon reasonable request.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of Qilu Hospital of Shandong University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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