

PLAC8 promotes adriamycin resistance via blocking autophagy in breast cancer

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Abstract

Background

Adriamycin (ADM) is currently one of the most effective chemotherapeutic agents in breast cancer treatment. However, growing resistance to ADM can lead to treatment failure and poor outcome. The underlying molecular mechanisms in ADM resistance in breast cancer remains unclear. PLAC8 is reported as a novel highly-conserved protein and functions as an oncogene or tumor suppressor in various tumors.

Methods

Here, we analyzed the expression profile of PLAC8 in breast cancer tissues and breast cancer cell lines, and explored the correlation of PLAC8 expression levels with patients' outcomes and ADM response. One ADM resistant MCF-7 breast cancer cell (MCF-7/ADM) and its parental cell was used as *in vitro* models to identify the underlying mechanism of PLAC8 and ADM resistance. Breast cancer cells were transfected with PLAC8 knockdown and overexpression vectors, and MTT and colony formation assays were performed to test the cell response to ADM. Then, we tested the effect of PLAC8 on autophagy pathway by flow cytometry and immunofluorescence analysis, and the change of main autophagy-correlated factors expressions: LC3 and p62. Next, combining treatment of autophagy inhibitor/inducer and PLAC8 downregulation/upregulation revealed the participation of PLAC8 in autophagy pathway to synergistically regulate ADM resistance in breast cancer.

Results

Here, higher PLAC8 expression was correlated with poorer outcome and aggressive phenotype in breast cancer, and breast cancer patients with higher PLAC8 expression showed potential ADM resistance. PLAC8 expression level was also significantly elevated in ADM resistant MCF-7 breast cancer cells (MCF-7/ADM), compared to parental MCF-7 cells. *In vitro* experiments further confirmed that PLAC8 inhibition by siRNA or enforced overexpression by infecting pcDNA3.1(C)-PLAC8 plasmid correspondingly decreased or increased the ADM resistance. Subsequently, we demonstrated that ectopic PLAC8 expression in MCF-7/ADM cell blocked the accumulation of the autophagy-associated protein LC3II, and resulted in cellular accumulation of p62. Rapamycin-triggered autophagy significantly increased cell response to ADM, while the autophagy inhibitor 3-MA enhanced ADM resistance. Actually, 3-MA and PLAC8 could synergistically enhance ADM resistance via blocking the autophagy process. Additionally, the downregulation of p62 by siRNA attenuated the activation of autophagy and PLAC8 expression in breast cancer cells.

Conclusion

Our findings suggest that PLAC8, through participation of p62, inhibits autophagy and consequently results in ADM resistance in breast cancer. PLAC8/p62/autophagy pathway may act as novel therapeutic targets in breast cancer treatment and has potential clinical application in overcoming ADM resistance.

Background

Breast cancer is the second most common cancer and is estimated to be leading cause of cancer-related death in women aged from 20 to 59 years[1, 2]. Despite curative treatment, acquired drug resistance prevents cancer therapies from achieving complete responses, and lead to later recurrence and metastatic. Adriamycin (ADM), also known as doxorubicin, is a DNA topoisomerase II inhibitor and belongs to the family of anthracycline anticancer drugs. ADM is considered to be one of the most effective agents in breast cancer treatment, for which ADM is used clinically as frontline therapy[3]. Unfortunately, chemotherapeutic resistance to ADM has emerged as one of the essential reasons for treatment failure and death associated with breast cancer [4, 5]. Significant efforts should be undertaken to resolve the molecular mechanisms that lead to ADM resistance, and identified novel biomarkers that can predict treatment response and overcome ADM resistance

Placenta-specific 8 (PLAC8, Onzin) was firstly identified via genome-wide gene expression analysis in the placenta, and PLAC8 protein was restricted to the spongiotrophoblast layer of the mouse placenta[6, 7]. Multiple functions of PLAC8 have been demonstrated in the regulation of immunity, adipocyte differentiation, and human cancers [8– 11]. Previously research revealed that PLAC8 is involved in the progression of various cancers. For example, PLAC8 recovery inhibited PI3K/Akt/GSK3b/Wnt/ β -catenin signaling to reduce cell proliferation in liver carcinoma [8]. The overexpression of PLAC8 was also revealed to be associated with malignant progression and poor prognosis in clear-cell renal cell carcinoma [9]. In colon cancer, PLAC8-overexpressing cells exhibited increased phosphorylated ERK2, which elevated cell motility and cancer invasion [10]. From our two previously published studies, PLAC8 was relatively highly expressed in lung cancer and breast cancer tissues, and contributed to cancer progression[12, 13]. A number of studies have demonstrated a role of PLAC8 in in the regulation of autophagy during pancreatic cancer progression and prostate carcinogenesis[14, 15].

Recent studies have revealed an association between autophagy and drug resistance [16]. Autophagy is a homeostatic, catabolic degradation process, by which the damaged or unwanted organelles and protein aggregates are delivered to lysosome for degradation [17]. Autophagy is essential for sustain proper function and metabolism of the cell. The involvement of autophagy in ADM resistance has been extensively investigated [18]. Some studies indicated that autophagy has a protective role in cancer cells and results in ADM resistance [19]. Other observation, however, showed that induction of autophagy helps overcome ADM resistance and leads to cancer cell death. In breast cancer MDA-MB-231 cells, treatment with ADM induced cytoprotective autophagy, and Atg5-deficient caused decreased sensitivity toward ADM [20].

The role of PLAC8 in regulation of autophagy and ADM resistance in breast cancer has not been reported until now. In this study, we firstly reported that there may be a link among PLAC8 overexpression, autophagy, and ADM resistance in breast cancer. We highlighted the higher level of PLAC8 expression in ADM-resistant breast cancer tissues, and demonstrated a significant association of PLAC8 high expression with poorer patient survival. Furthermore, enforced overexpression or inhibition of PLAC8 could modulate autophagy through p62 participation and change ADM sensitivity in breast cancer cells. Thus, our findings suggest that PLAC8 plays a key role in ADM resistance through regulation of autophagy. In-depth understanding of the mechanisms of PLAC8/autophagy regulation in relation to ADM resistance and targeted autophagy-modulating agents will provide a promising therapeutic strategy for overcoming ADM resistance and promote breast cancer treatment.

Results

High PLAC8 expression was correlated ADM resistance and predicted poor outcomes in breast cancer

To gain initial insight into PLAC8 expression patterns in breast tumors, we analyzed the relationship between PLAC8 expression, breast cancer disease stage and patients' overall survival. Based on the Cancer Genome Atlas (TCGA) breast cancer dataset and generated Kaplan–Meier survival plots (KM plots), overall survival was prolonged in patients without PLAC8 amplifications or gains, compared with those with PLAC8 aberrant expression ($p = 0.0098$) (Figure. 1A). Depending on clinical tumor subtype, the triple-negative and HER2-positive breast cancer tissues presented relatively higher PLAC8 expression ($p < 0.00001$) (Figure. 1B). Besides, the expression of PLAC8 was significantly elevated in higher histologic grade (grade2/3 compared to grade 1) (data downloaded from <http://co.bmc.lu.se/gobo/gsa.pl>) (Figure. 1C). Compared to that in breast normal tissues, PLAC8 expression was elevated in breast cancer (Figure. 1D-F).

Next, we collected breast cancer tissues and divided into ADM-sensitive or ADM-resistant groups. The immunohistochemistry analysis of PLAC8 protein further confirmed the previous results that ADM-resistant breast cancer tended to be PLAC8 overexpression (Figure. 1G). The mRNA levels of PLAC8 in ADM-sensitive and resistant breast cancer cells were further compared using database obtained from the Gene Expression Omnibus (GEO) datasets. The results from the breast cancer dataset (GSE24460) showed that PLAC8 expression was significantly up-regulated in MCF-7/ADM compared with MCF-7 cells (Figure. 1H). Here we found that PLAC8 could be a new prognostic marker in breast cancer, and there is a potential relationship between PLAC8 expression and ADM resistance in breast cancer.

Intrinsically and acquired ADM-resistance in breast cancer correlates with increased PLAC8 expression

To examine the association of PLAC8 and ADM low-response in breast cancer, we next tested PLAC8 expression profile in a range of breast cancer cell lines. PLAC8 protein and mRNA expression was found

to be relatively higher in MDA-MB-231 and BCAP37 cells (Figure. 2A and 2B, respectively). The cell line MCF-7 with acquired ADM resistance and its parental MCF-7 served as our model system. MCF-7/ADM cells were established by culturing MCF-7 cells in medium with increasing ADM till to 1 $\mu\text{g/ml}$ for 6 months. Interestingly, we observed the abnormally elevated PLAC8 expression in MCF-7/ADM, when compared with the parental MCF-7 cell (Figure. 2D and 2E, respectively). The localization of PLAC8 in breast cancer cells (MDA-MB231, MCF-7 and MCF-7/ADM) was further detected using immunofluorescence staining, and PLAC8 protein was localized in both the nucleus and cytosol of breast cancer cells. Consistently with the western blot and RT-PCR results, PLAC8 expression was relatively higher in MDA-MB231 and MCF-7/ADM, compared with the negative MCF-7 cells (Figure. 2C).

We next tested these breast cancer cells sensitivity to ADM toxicity, and explore the potential correlation between PLAC8 expression and ADM resistance. MCF-7 and MCF-7/ADM cells were exposed to ADM at different concentrations (0.5, 1 and 2 $\mu\text{g/ml}$) for 48-hours. The amount of PLAC8 protein was found to be substantially increased after 48-hour exposures to ADM at various concentrations (1 and 2 $\mu\text{g/ml}$) in MCF-7/ADM, except for the conditions in which MCF-7 cells treated with ADM (Figure. 2F). As expected, colony formation assays indicated that ADM treatment (10 μM) decreased colony formation ability in MCF-7, compared with MCF-7/ADM cells (Figure. 2G). Furthermore, cell viability assay confirmed that MCF-7/ADM cells could tolerate much higher concentrations ($\text{IC}_{50} = 50.01 \mu\text{M}$) of ADM than the parental MCF-7 cells ($\text{IC}_{50} = 1.02 \mu\text{M}$) (Figure. 2H). Besides, the IC_{50} of ADM was much lower in T47D and MCF-7 cells than MDA-MB231 and MCF-7/ADM cells, suggesting that increased PLAC8 expression might play an essential role in both intrinsically and acquired ADM resistance in breast cancer cells (Figure. 2G). Thus, our findings suggest that ADM induced upregulation of PLAC8, and the intrinsically and acquired resistance to ADM in breast cancer may partially be caused by PLAC8 over-expression.

PLAC8 silencing facilitates breast cancer cell response to ADM

MCF-7/ADM tended to be resistant to ADM and had relatively highly expression of PLAC8. We further examined the effects of altered PLAC8 expression on ADM resistance in MCF-7 and MCF-7/ADM cells.

Knock-down experiment was performed in MCF-7/ADM by transfecting with PLAC8-siRNA (siPLAC8-1, siPLAC8-2 and siPLAC8-3) (Figure. 3A and 3B), and then we tested the cell response to ADM. The sensitivity changes were explored by using MTS and colony formation assays. We here confirmed that MCF7/ADM PLAC8 silencing group become more sensitive to ADM than the control group (Figure. 3C and 3D). Both the cell growth and colony formation ability were significantly suppressed because of PLAC8 knock-down. And reversely, PLAC8 overexpression by transfection with a pcDNA3.1(C)-PLAC8 plasmid, conferred protection from ADM for breast cancer cell. We overexpressed PLAC8 in MCF-7 cell and construct the stable PLAC8-overexpression breast cancer cell (C1 and C2) (Figure. 3E and 3F, respectively). The MTS and colony formation experiments results revealed that PLAC8 overexpression in MCF-7 conferred protection from ADM for breast cancer cell MCF-7 (Figure. 3G and 3H). Here, we found PLAC8 silencing in MCF-7/ADM may be an effective way to reverse ADM resistance.

Autophagy activators reverse ADM resistance of MCF-7/ADM cell

There is mounting preclinical evidence that targeting autophagy can enhance the efficacy of many cancer therapies. Here, we found the autophagy flux was blocked in MCF-7/ADM, compared to its parental cell line. Increased LC3-II and p62 accumulation were observed in the conditions of complete medium in MCF-7/ADM cell (Figure. 4A). The use of flow cytometry to study the autophagic process revealed that decreased autophagic cell death of MCF-7/ADM (Figure. 4B). In addition, 3-MA, an autophagy inhibitor, was used to inhibit autophagy in the MCF-7/ADM groups. The mTOR inhibitor rapamycin (RAPA) have been reported to block the autophagy process. Here, western blot analysis revealed increased p62 and PLAC8 protein accumulation in the 3-MA group. In contrast, autophagic flux in MCF-7/ADM was restored by RAPA preconditioning (Fig. 4C, 4D). Thus, 3-MA and RAPA are effective autophagy inhibitor and inducer of MCF-7/ADM, respectively. PLAC8 is an autophagy-regulated gene, consistently with previous studies [14, 15].

Importantly, pretreatment with RAPA reversed ADM resistance and sensitized MCF-7/ADM to ADM therapy. However, the autophagy inhibitor 3-MA significantly reversed the effects exerted by RAPA. 3-MA enhanced the protective role in the ADM + 3-MA group, as evidenced by significantly higher levels of large colony growth and increased cell viability (Figure. 4E and 4F). Conclusively, our findings demonstrated that inhibition of autophagy can be exploited as a novel strategy to re-sensitize the breast cancer cells to ADM chemotherapy.

PLAC8 modulates ADM resistance and autophagy through p62 participation

PLAC8 has been shown to promote autophagosome lysosome fusion by activating pro-survival function of autophagy in pancreatic cancer [14, 15]. We then assessed PLAC8 regulation to autophagy in breast cancer cells. Figure. 5A results showed a significant increase in P62 and LC3-II in PLAC8 stable overexpression cells (C1 and C2). PLAC8-induced decrease of autophagy was further confirmed, when compared with its parental cell line MCF-7 (Figure. 5B and 5C). Synchronously, after PLAC8 knockdown by siRNA, LC3-II and p62 accumulation were reduced in the conditions of complete medium (Figure. 5D). The inhibition of autophagy was significantly released upon PLAC8 degradation in MCF-7/ADM (Figure. 5E and 5F).

Moreover, we demonstrated that autophagy inhibitor 3-MA promoted accumulation of autophagic vacuoles, and enhanced the cytoprotective effect induced by PLAC8 overexpression to ADM cytotoxicity in breast cancer cell (Figure. 5G and 5H). In contrast, combined therapy of siRNA-mediated PLAC8 depletion with RAPA treatment sensitized breast cancer cells to ADM treatment (Figure. 5I and 5J). Next, we detected the expression levels of autophagy-related molecules, to test whether PLAC8 affected ADM chemo-sensitivity via specific autophagy-related factors. Indeed, silencing p62 significantly suppressed autophagy flux as well as inhibition of PLAC8 gene transcription (Figure. 5K-M). Coimmunoprecipitation (Co-IP) experiments were performed to identify direct protein interactions with PLAC8, and p62-PLAC8

was demonstrated as interactive protein complex. Here, our findings suggested that PLAC8 inhibited autophagy at least partially through participation of p62, and then affected ADM resistance in breast cancer cells.

PLAC8 silencing effectively abrogated breast cancer formation in vivo

Next, PLAC8 knockdown in animal models abrogated breast tumor growth supporting an oncogenic role. In animal models, MCF-7/ADM cells with PLAC8-silencing grew significantly slower and smaller than their control (Figure. 6A and 6B). Significant differences were found in tumor volume between the PLAC8-silencing groups compared with those in the vector groups four weeks post-implantation (Figure. 6C). As expected, PLAC8 expression was relatively decreased in the PLAC8-knockdown groups (Figure. 6D). Results from the IHC staining of Ki67 showed increased tumor proliferation ability in the PLAC8-knockdown groups, which verified the previous *in vitro* results of PLAC8 knockdown in MCF-7/ADM cell (Figure. 6E). Furthermore, transgenic PLAC8 knockout lead to decreased expression of LC3 and P62 protein. Here, animal models further supported that PLAC8 knockdown in MCF-7/ADM cells suppressed tumor formation ability, and the process of autophagy was promoted in PLAC8 silencing groups.

Materials And Methods

Reagents and cell culture

Adriamycin was purchased from Sigma-Aldrich (catalog. D1515) and was soluble in water to final concentration of 10 mg/ml. The catalog number of 3-MA and rapamycin M9281 and S-015 (Sigma-Aldrich). Human breast cancer cell line, MCF-7, T47D, BCAP37, MDA-MB231, MDA-MB-453, MDA-MB-436, MDA-MB-468, HBL-100 and HCL-1937 were purchased from the Cell Bank of the Chinese Academy of Sciences and stored in liquid nitrogen. ADM-resistant MCF-7 cells (MCF-7/ADM) was derived from the human breast cancer cell MCF-7, which was maintained in the presence of 1 ug/ml ADM. A series of MCF-7 cells with incremental resistance to ADM were established by ADM challenge at the starting concentration of 1 ng/ml. A double concentration of ADM was then applied when the cells became tolerable. The process was repeatedly performed to increase cell tolerance to ADM. Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBREAST CANCERO) and grew at 37 °C with 5% CO₂ and 95% humidity.

Plasmids, siRNAs, and transfection

Lentiviral PLAC8-overexpressing and negative control vectors were obtained from GENE (Shanghai China). Short interfering RNAs targeting PLAC8 or p62 (siRNA-PLAC8/siRNA-p62), and a scrambled control siRNA were designed and commercially synthesized by Ribo Bio (Guangzhou, China). MCF-7 and MCF-7/ADR cells were transfected with 1 µg of LC3-EGFP-mCherry plasmid using Lipofectamine® 3000 transfection reagent (Invitrogen, L3000001). The stable cell line was selected using G418 sulfate antibiotic (Cal- biochem, 509290). Cells were seeded 24 h in 12-well plates (1 × 10⁵/well) and transfected

with siRNA duplexes (50 nM) or 2 µg of plasmids using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's instructions. Cells were harvested for RNA and protein extraction 48 h after transfection and processed for functional assays.

RNA isolation and real-time fluorescent quantitative PCR

Total RNA was extracted after 48 h transfection using Trizol reagent (Invitrogen) following the manufacturer's instruction. The concentrations were quantified by NanoDrop 2000 (Thermo Scientific, USA), and the RNA (1 µg) was reverse-transcribed by the HiFiScript cDNA Synthesis Kit (CW BIO, CW2569M). Real-time fluorescent quantitative PCR was performed using UltraSYBR Mixture (CW BIO, CW0957H). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The following primers were used: PLAC8: 5'-GGAACAAGCGTCGCAATGAG-3' (sense) and 5'-AAAGTACGCATGGCTCTCCTT-3' (anti-sense); GAPDH: 5'-TGCACCACCAACTGCTTAG-3' (sense) and 5'-AGTAGAGGCAGGGATGATGTTC-3' (anti-sense). P62: 5'-ATCGGAGGATCCGAGTGT-3' (sense) and 5'-TGGCTGTGAGCTGCTCTT-3' (anti-sense). The results were calculated using $2^{-\Delta\Delta Ct}$ method.

Protein extraction and Western blot analysis

Total protein was extracted after 48 h transfection using RIPA reagent (Beyotime Biotechnology, China) following the manufacturer's instruction. The following antibodies were used: PLAC8 (1:1000, Cell Signaling Technology, #13885), Ki67 (1:500, Sino Biological, 100130-T32-50), GAPDH (1:500, Santa Cruz, sc-47724), β -actin HRP conjugated (1:500, Santa Cruz, sc-47778), LC3 antibody (1:1000, SIGMA, L7543), and p62 (1:1000, Medical & Biological Laboratories, PM045) antibodies. The signals were detected with an ECL Kit (Bio-Rad, Clarity™ Western ECL Substrate, 500 ml #1705061).

Immunofluorescence staining

Cells at a density of 1×10^5 were briefly plated in 6-well plates, and three glass coverslips were placed onto the wells. The plates were incubated for 24–48 h until 30–40% confluence was reached. The cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100. The slides were then washed three times with phosphate-buffered saline (PBS) and blocked with 5% bovine serum albumin in PBS for 30 min at room temperature. The sections were incubated with a primary antibody against PLAC8 overnight. After rinsing three times with PBST for 5 min, an Alexa 488-conjugated (green) goat anti-rabbit antibody was applied (1:200 dilution; Life Technologies) for 1 h at room temperature. Cell nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole), and images were acquired using a Nikon laser scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA).

Cell viability assay

Cell viability assay was performed with MTS (5 mg/ml, Promega, WI, USA). Approximately 48 h after transfection, the transfected cells were transferred into a 96-well plate for one day. Cells were then treated with serial dilutions of ADM for 48 h. The absorbance at 490 nm was measured using a multi-mode reader (LD942, Beijing, China). The IC₅₀ (50% inhibitory concentration) value, which represents the drug

concentration with 50% cell growth inhibition, was calculated by normal probability transforms based on the relationship of drug concentration and inhibition rat. Samples were prepared in triplicates, and the cell viability was determined as the mean \pm SD.

Colony formation assays

Cells were seeded in 6-well plates at 500 cells/well and incubated for 10–14 days before staining with a crystal violet staining solution (Sigma-Aldrich). The colonies were then calculated and photographed.

Patient-specimen selection and Immunohistochemical analysis

The paraffin section of breast tumor and normal samples were collected from Sir Run Shaw Hospital, Zhejiang University. Breast tumor and normal samples were collected from breast cancer patients from Sir Run Run Shaw Hospital. All these selected breast cancer patients underwent primary surgery followed by ADM-based chemotherapy. The study was approved by the ethical committee of the Sir Run Shaw Hospital, School of Medicine, Zhejiang University. Slides were stained with PLAC8, Ki67, LC 3 and p62 antibodies by using the GT Vision III IHC Assay Kit (HRP/DAB, rabbit/mouse-general, two-step, GK500710, Gene Tech, Shanghai, China) in accordance with the manufacturer's protocol. Images were acquired by polarized light microscopy.

Direct immunofluorescence

MCF-7 and MCF-7/ADM cells were transfected with a GFPLC3 plasmid overnight and transferred to coverslips. Nuclei were stained with 40,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D9542) followed by fixing with 4% paraformaldehyde (Sigma-Aldrich, 158127) and permeabilizing with 0.5% Triton X-100 (Solarbio, T8200-100) in PBS (Corning, R21-040-CV). Images were taken with a spinning disk confocal fluorescence microscope (the system is composed of a CSU-X1 spinning disk from Yokogama. The amounts of GFP-LC3-II-positive puncta in 50 GFP-positive cells for each group were counted using the Metamorph offline 7.7.8.0 software package and the GFP-LC3-II-positive puncta per cell were calculated.

Analysis of autophagy by flow cytometry

Cells added 5ug/ml ADM and 3-MA or RAPA incubated for 48 h. The cells were washed, and the autophagic vacuoles were quantified using a cyto-ID autophagy detection kit (Enzo, ENZ-51031) according to the manufacturer's instructions. The signals of labeled autophagic vacuoles were analyzed using a flow cytometer with an FL1 (488 nm excitation, green) channel.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed using protein lysates of cells transfected with siP62 and PLAC8 plasmid and antibody following standard protocol. Normal rabbit IgG served as negative control. The immunoprecipitates was immunoblotted with anti-PLAC8 and anti-P62 antibodies that were described previously. The immunocomplexes on the Western blot were detected by chemiluminescence and photographic films and quantified with multi-Gauge soft (Fujifilm).

Tumor xenograft assay

MCF-7/ADM cells were transfected with lentiviral PLAC8-silencing and negative control vectors. An equal number (2×10^6) of cells were resuspended in 60 μ l of PBS with 40 μ l of growth factor-reduced basement membrane matrix (#356231 Corning) and then injected into four-week-old nude mice ($n = 5$). The mice were observed three times a week, and the tumor volumes were measured twice per week. Tumor volume was calculated with the formula $V = 0.5 ab^2$ (a , longest tumor axis; b , shortest tumor axis). All mice were sacrificed four weeks after tumor cell injection. Tumor tissues were collected and processed for further analyses, including the immunohistochemistry analysis of PLAC8, Ki67, LC3 and p62 expression. Animal studies were reviewed and approved by the Ethics Committee for Animal Studies of Zhejiang University.

Statistical analysis

GraphPad Prism 6.0 software was used for statistical analysis. Data were independently collected from at least three experiments. The survival curve was conducted using the Kaplan–Meier method with a log-rank test. Two-tailed unpaired Student's t-tests were used to compare mean data. The results are presented as the mean \pm SD, and $p < 0.05$ was considered statistically significant.

Discussion

ADM remains to be one of the most active and widely used chemotherapy agents in the treatment of early and advanced breast cancer. However, drug resistance has limited the effectiveness of the agent in cancer treatment [21, 22]. The exact mechanisms behind the resistance are still poorly understood. Our study highlighted that breast cancer cells with high PLAC8 expression responded weakly to ADM treatment, and PLAC8 was necessary and sufficient to induce ADM-resistant phenotypes. And, PLAC8 may alter the ADM sensitivity of breast cancer cells by modulating autophagy process. And combined treatment of autophagy inducer RAPA and PLAC8 knockdown could efficiently reverse ADM resistance in breast cancer.

The lysosomal protein PLAC8 localizes to the inner surface of the plasma membrane and interacts with other cellular components to execute its functions, which include autophagy, cell cycle arrest, and apoptosis. The function of PLAC8 differs based on specific cell type. For example, ectopic expression PLAC8 protected fibroblasts from apoptosis but induces apoptosis in epithelial cells [23, 24]. In colon cancer, PLAC8 induced epithelial–mesenchymal transition, and cell cycle was reported to be regulated by PLAC8 in pancreatic cancer cells[25]. In our studies, high PLAC8 expression is correlated with breast cancer resistant to ADM and poorer survival. Moreover, silencing PLAC8 expression enhances ADM response in MCF-7/ADM cells, whereas PLAC8 overexpression causes ADM resistance in MCF-7 cells. Genetic ablation of PLAC8 suppressed breast cancer proliferation, as well as decreased tumorigenicity *in vivo*. Our results further added evidence to the versatility of PLAC8 in cellular functions. PLAC8 may be used as a novel biomarker in the prediction of patient responsiveness to ADM and serves as a unique therapeutic target for overcoming ADM resistance in breast cancer.

Autophagy is an evolutionarily conserved catabolic process, in which cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled to sustain cellular metabolism [26]. Autophagy has dual roles in cancer, acting as a tumor suppressor by preventing the accumulation of damaged proteins and organelles and a mechanism of cell survival to promote the growth of established tumors [27, 28]. Autophagy process is also a promising target for drug development in cancer [29–31]. Autophagy can be monitored using marker proteins, namely, LC3 and p62. During autophagosome formation, cytosolic LC3 I is lipidated, recruited to the membranes, and designated as LC3 II. Lipidated LC3 II migrates faster than LC3 I on polyacrylamide gels, and the ratio of autophagosomal LC3 II to cytosolic LC3 I is used as an indicator of autophagy [32]. p62 is a key adaptor of target cargo that interacts with the autophagy machinery. p62 binds to misfolded aggregate proteins in autophagosomes and then degraded in the autolysosomes [33, 34]. Previously, PLAC8 is found to be correlated with autophagosome and promotes autophagy by facilitating autophagosome–lysosome fusion in pancreatic cancer and prostate carcinogenesis [14, 15, 35]. Our experiments suggested that PLAC8 may affect ADM sensitivity by modulating autophagy in breast cancer. Besides, PLAC8 protein expression and function was downregulated upon p62 suppression in breast cancer cells. Co-immunoprecipitation analysis revealed that PLAC8 may directly interact with p62, which mediated the function of p62 in suppressing breast cancer autophagy process. Overall, these data demonstrated that PLAC8 overexpression can collaborate with p62 to suppress autophagy, and lead to ADM resistance in breast cancer.

Specifically, autophagy activators and inhibitors are an active area of investigation in cancer therapeutics, and some inhibitors are being examined for their efficacy in clinical trials. An increased understanding of autophagy in cancer is important for its optimal utilization to enhance the effects of chemotherapy and improve clinical outcomes of treatment in cancer patients [36, 37]. In ADM-resistant breast cancer cell MCF-7/ADM, combining treatment of autophagy inducer RAPA and PLAC8 knockdown could synergistically and efficiently reverse ADM resistance. Thus, targeting PLAC8 and/or autophagy inducers combined with chemotherapy may be a new strategy for the treatment of ADM resistance in breast cancer patients. This finding might help to develop novel molecular therapeutic strategies for breast cancer metastasis treatment.

Conclusions

In this study, we examined the clinical significance and biologic effects of PLAC8 in breast cancer progression and ADM sensitivity regulation. Our findings proposed that PLAC8 overexpression contributed to ADM resistance in breast cancer via modulation of autophagy, partially by collaborating with p62 transcription. A better understanding of the novel PLAC8/p62/autophagy regulatory pathway may allow us to develop a promising therapeutic strategy to enhance the effects of ADM chemotherapy and improve clinical outcomes in the treatment of breast cancer patients.

Abbreviations

Adriamycin

ADM.
Placenta-specific 8
PLAC8.
Cancer Genome Atlas
TCGA.
Kaplan–Meier survival plots
KM plots.
Gene Expression Omnibus
GEO.
Rapamycin
RAPA.
Coimmunoprecipitation
Co-IP.

Declarations

Ethics approval and consent to participate: For the analyzed tissue specimens, all patients provided informed consent to use excess pathological specimen for research purposes. The protocols employed in this study and the use of human tissue were approved by the Ethics Committee of the Sir Run Run Shaw Hospital, affiliated with Zhejiang University, and conducted in full accordance with the ethical principles cited in the World Medical Association Declaration of Helsinki and local legislation.

Consent for publication: All authors read and approved the manuscript.

Availability of data and materials: All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests: No conflict of interest exists in the submission of this manuscript.

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Authors' contributions: LBW, WQ and JCZ conceived and designed the study. YFG, CPX, JJY, WXH and JS performed the experiments. YXC, YLJ and MSM wrote the paper. DDH, CC, ZQL, LNC JR and PS reviewed and edited the manuscript.

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Figures

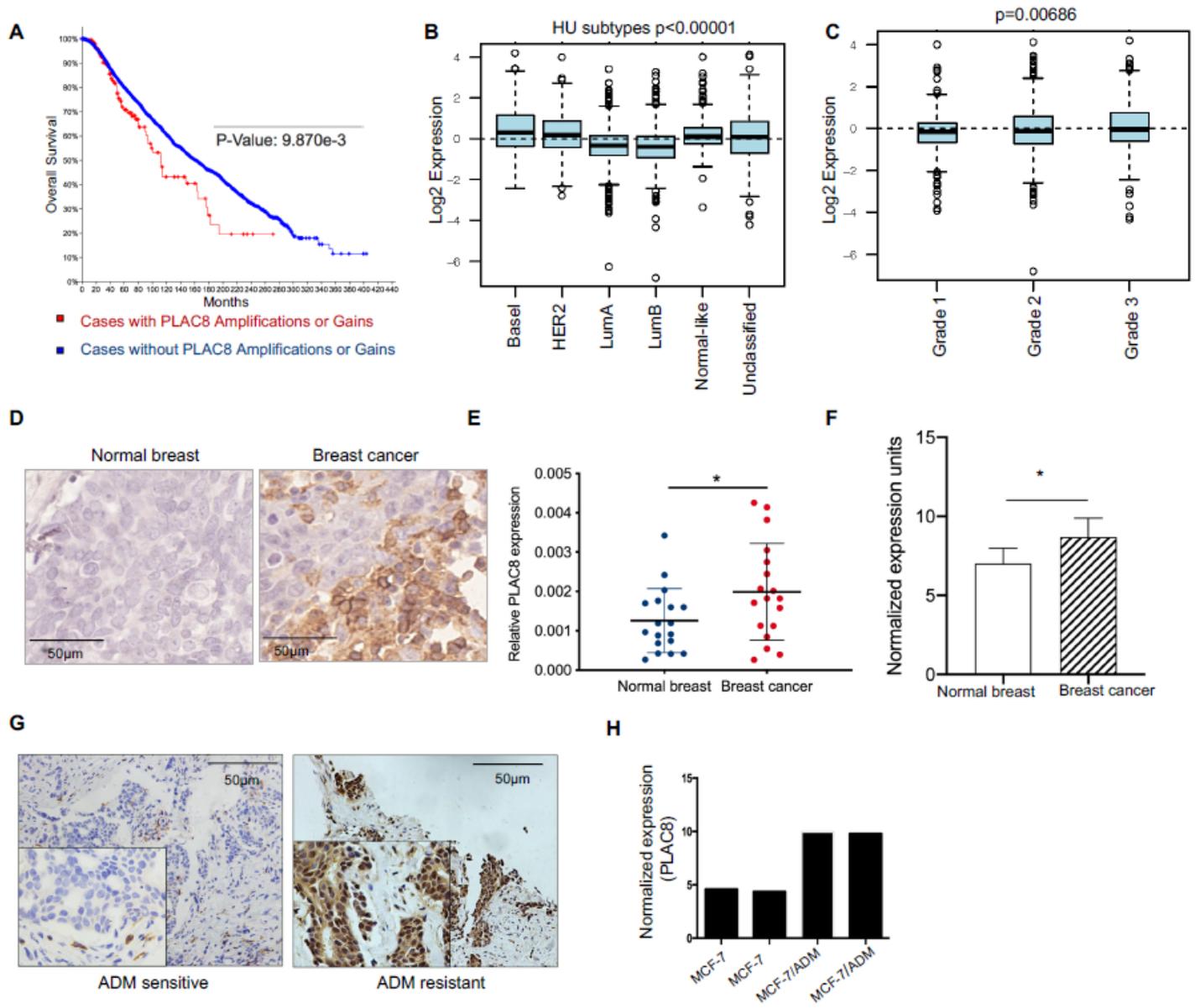


Figure 1

PLAC8 expression was associated with breast cancer outcome and ADM response. A. The survival curve was compared using the Kaplan–Meier method according to the PLAC8 expression level. Cases with PLAC8 amplifications or gains significantly predicted poor outcomes in patients with breast cancer. P-value=0.00987; B, C. The correlation between PLAC8 expression levels with breast cancer subtype (B) and histological grade (C); D. Immunohistochemistry analysis of PLAC8 expression in normal breast and correspondent breast cancer tissues; E. mRNA expression of PLAC8 between normal breast and breast cancer; F. Analysis results based on GEO dataset (GSE6883) showed that the expression of PLAC8 is relatively higher in breast cancer; G. From immunohistochemistry staining, PLAC8 expression was increased in ADM resistance breast cancer patients. H. The results from GEO dataset (GSE24460) showed that PLAC8 expression was significantly up-regulated in ADM-resistant MCF-7/ADM compared

with that in parental MCF-7. Each bar represents the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001

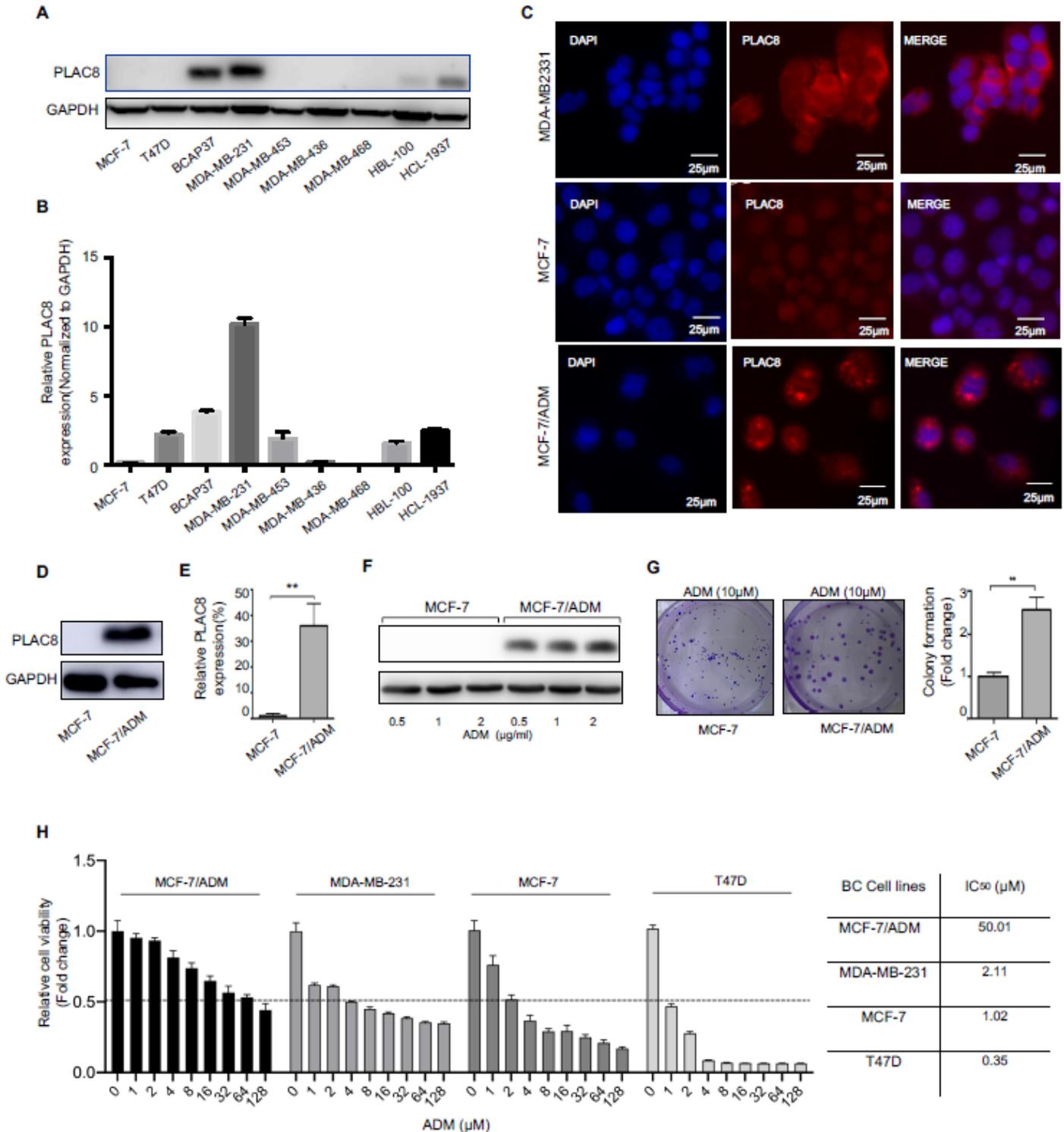


Figure 2

PLAC8 expression was increased in ADM-resistance breast cancer cells A, B. The protein and mRNA expression level of PLAC8 protein (A) and mRNA (B) in various breast cancer cell lines. C. Immunofluorescence analysis presented the location of PLAC8 protein in MDA-MB231, MCF-7 and MCF-

7/ADM cell lines. D, E. PLAC8 protein (D) and mRNA (E) expression is increased in MCF-7/ADM, when compared with its parental cell line. F. MCF-7 and MCF-7/ADM cells were treated with increasing ADM (0.5, 1, 2 ADM $\mu\text{g/ml}$) for 48 hours, and the expression of PLAC8 was significantly elevated in MCF-7/ADM cell after ADM treatment. G. Colony formation indicated the proliferative potential of MCF-7 and MCF-7/ADM cells with ADM treatment (10 μM , 48 hours). H. The sensitivity of MCF-7, MDA-MB231, T47D and MCF-7/ADM cells to ADM (left). The calculated IC50 was also presented (right). Each bar represents the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

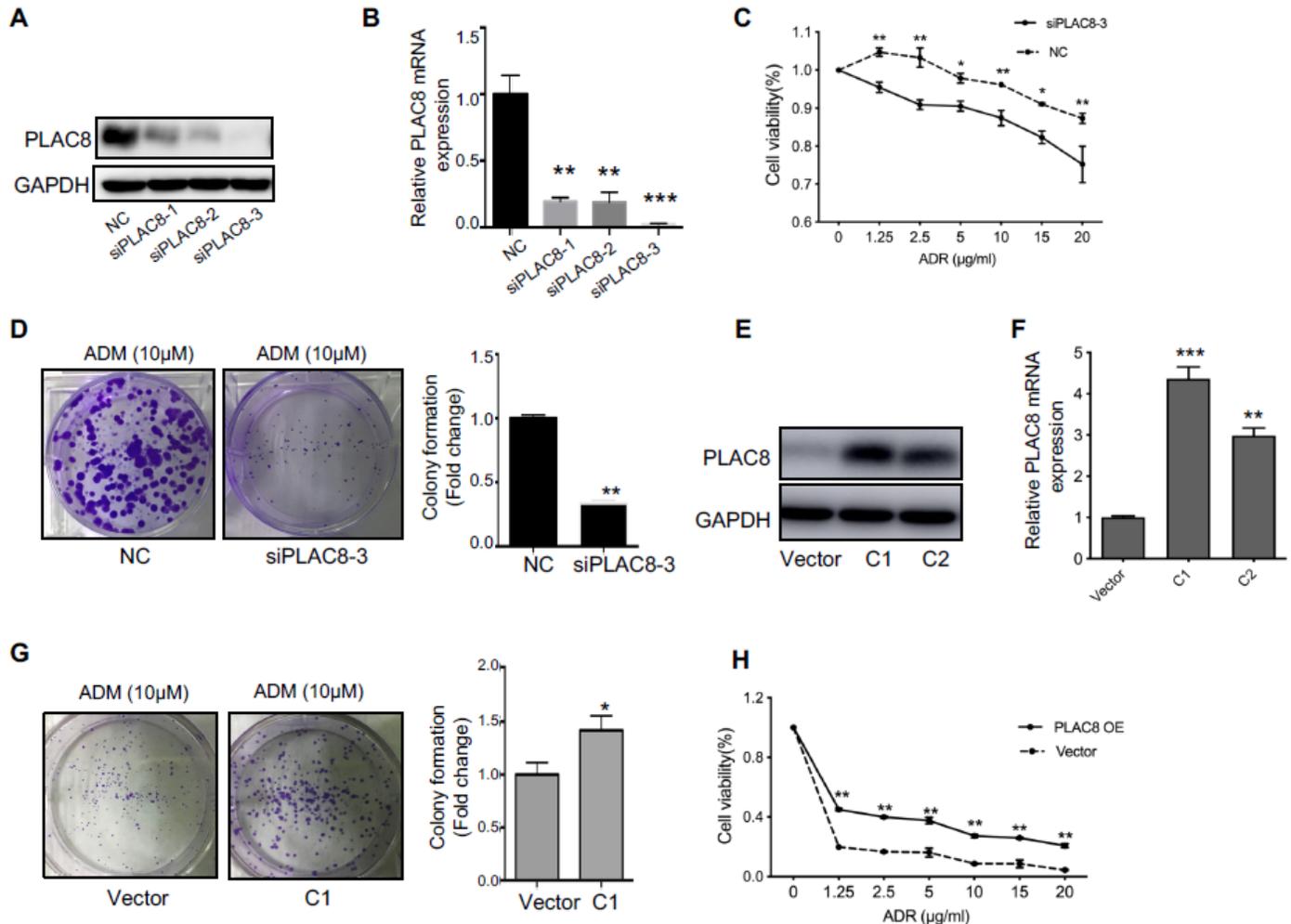


Figure 3

PLAC8 knock-down promoted breast cancer cell sensitivity to ADM. A, B. MCF-7/ADM was transfected with control siRNA, PLAC8 siRNA (siPLAC8-1, siPLAC8-2, siPLAC8-3). The western blot and RT-PCR were used to confirm the knockdown efficiency upon PLAC8 expression. C, D. PLAC8 knockdown in MCF-7/ADM increased cell response to ADM. Cell viability and colony formation ability was significantly suppressed in MCF-7/ADM PLAC8 silencing cells. E, F. MCF-7 cells were transfected with PLAC8 overexpression vector, and C1 and C2 were constructed-PLAC8 stable expression cell clones. G, H. PLAC8 overexpression in MCF-7 caused acquired ADM resistance. Colony formation capability and cell viability

were increased in PLAC8-overexpression stable cells. Each bar represents the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

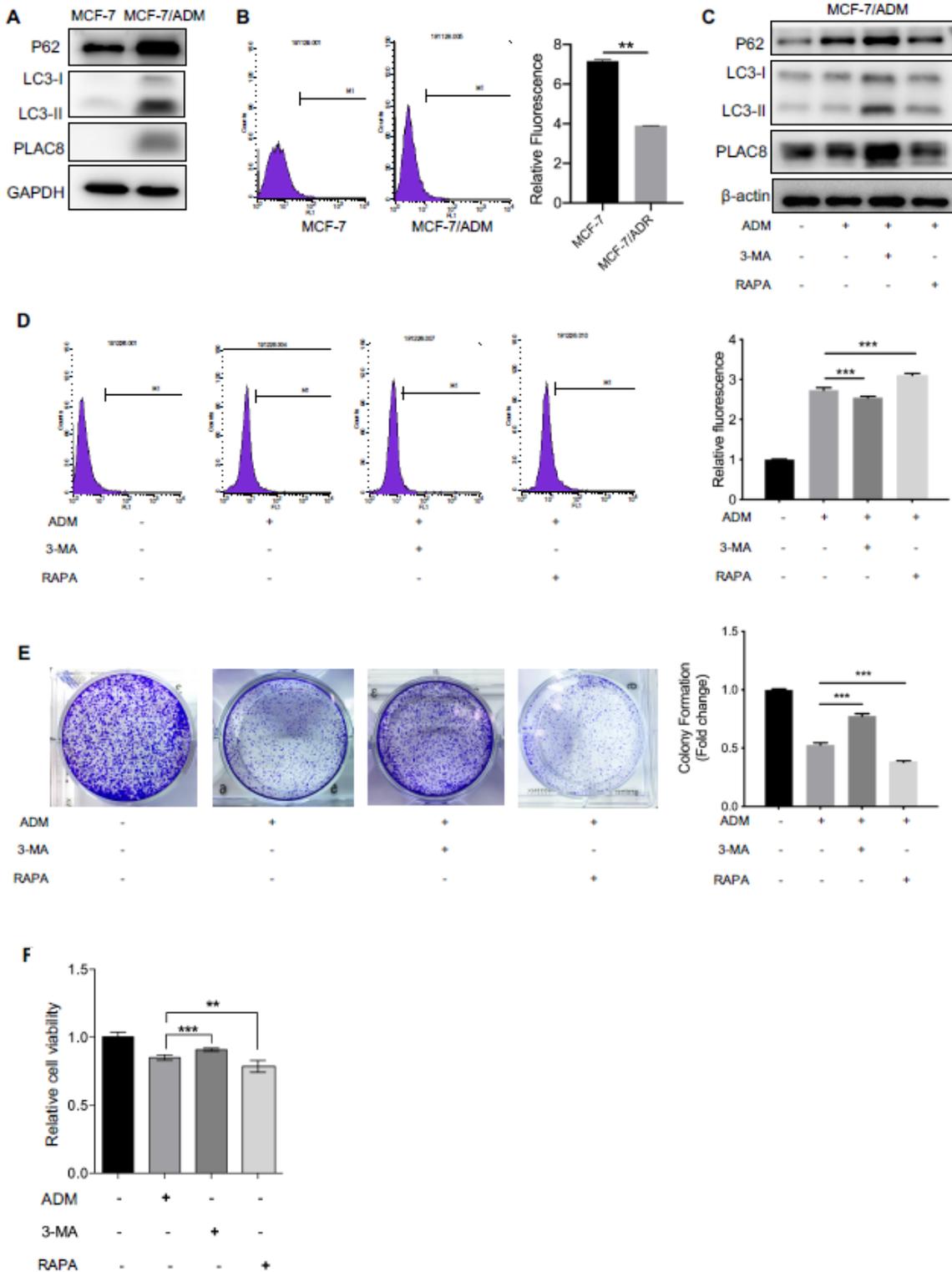


Figure 4

Autophagy activators re-sensitize MCF-7/ADM cell response to ADM A. Protein abundance of p62, PLAC8 and LC3 was analyzed by western blot in MCF-7 and MCF-7/ADM. B. Autophagy process was aberrant blocked in MCF-7/ADM, compared with that in MCF-7. C. p62, PLAC8 and LC3 protein expression was

analyzed by western blot in MCF-7/ADM cells treated with ADM (), the autophagy inhibitor 3-MA (10mM) or autophagy inducer rapamycin (RAPA) (500nM). D. Autophagy process was detected in MCF-7/ADM treated with ADM, the autophagy inhibitor 3-MA or autophagy inducer RAPA. E, F. Autophagy inducer RAPA increased ADM response in MCF-7/ADM, and treatment with 3-MA blocked this function. Each bar represents the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

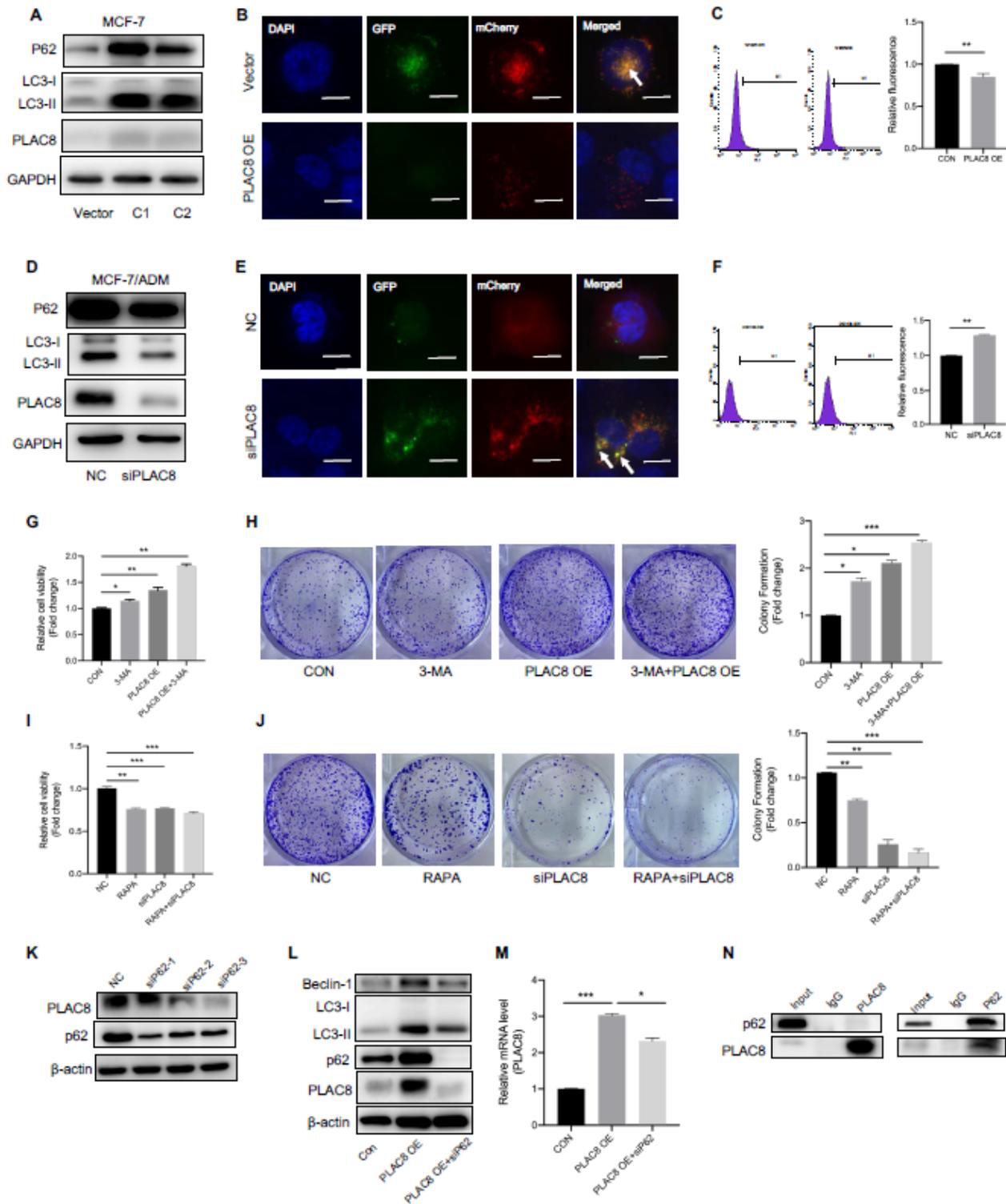


Figure 5

PLAC8-mediated suppressed autophagy and contributed to ADM resistance A. Protein abundance of p62, PLAC8 and LC3 was analyzed by western blot in MCF-7 and the stable PLAC8-overexpression cells (C1 and C2). B. Immunofluorescence staining analysis of autophagy marker LC3 in MCF-7 and MCF-7 cells overexpressing PLAC8 transfected with LC3-EGFP-mCherry plasmid. Nuclei were counterstained by DAPI. Representative images of at least 3 individual experiments are shown. Arrows indicate staining of LC3. Scale bars: 5 mm. C. The change of autophagy flux in MCF-7 and MCF-7 cells overexpressing PLAC8 from flow cytometry analysis results. D. Protein abundance of p62, PLAC8 and LC3 was analyzed by western blot in MCF-7/ADM and MCF-ADM PLAC8 silencing groups. E. Immunofluorescence staining analysis of autophagy marker LC3 in MCF-7/ADM and MCF-7/ADM cells silencing PLAC8 transfected with LC3-EGFP-mCherry plasmid. Nuclei were counterstained by DAPI. Representative images of at least 3 individual experiments are shown. Arrows indicate staining of LC3. Scale bars: 5 mm. F. The change of autophagy flux in MCF-7/ADM and MCF-7/ADM cells silencing PLAC8 from flow cytometry analysis results. G, H. In MCF-7 cells, based on the MTS and colony formation results, treatment with autophagy inhibitor 3-MA facilitated ADM resistance, and PLAC8 upregulation synergistically promoted cell resistant to ADM. I, J. In MCF-7/ADM cells, based on the MTS and colony formation experimental results, treatment with autophagy inducer RAPA increased ADM response, while combining with PLAC8 silencing enhanced the inhibition function. K. The protein expression level of PLAC8 and p62 in MCF-7/ADM cells transfected with control siRNA and p62 siRNA. L, M. Autophagy-related makers Beclin-1, LC3, p62 and PLAC8 expression level in MCF-7/ADM cell line transfected with a PLAC8-overexpressing vector or/and a p62 siRNA. β -actin served as the endogenous control. N. Co-immunoprecipitation assay presented the potential interaction between PLAC8 and p62. Each bar represents the mean \pm SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

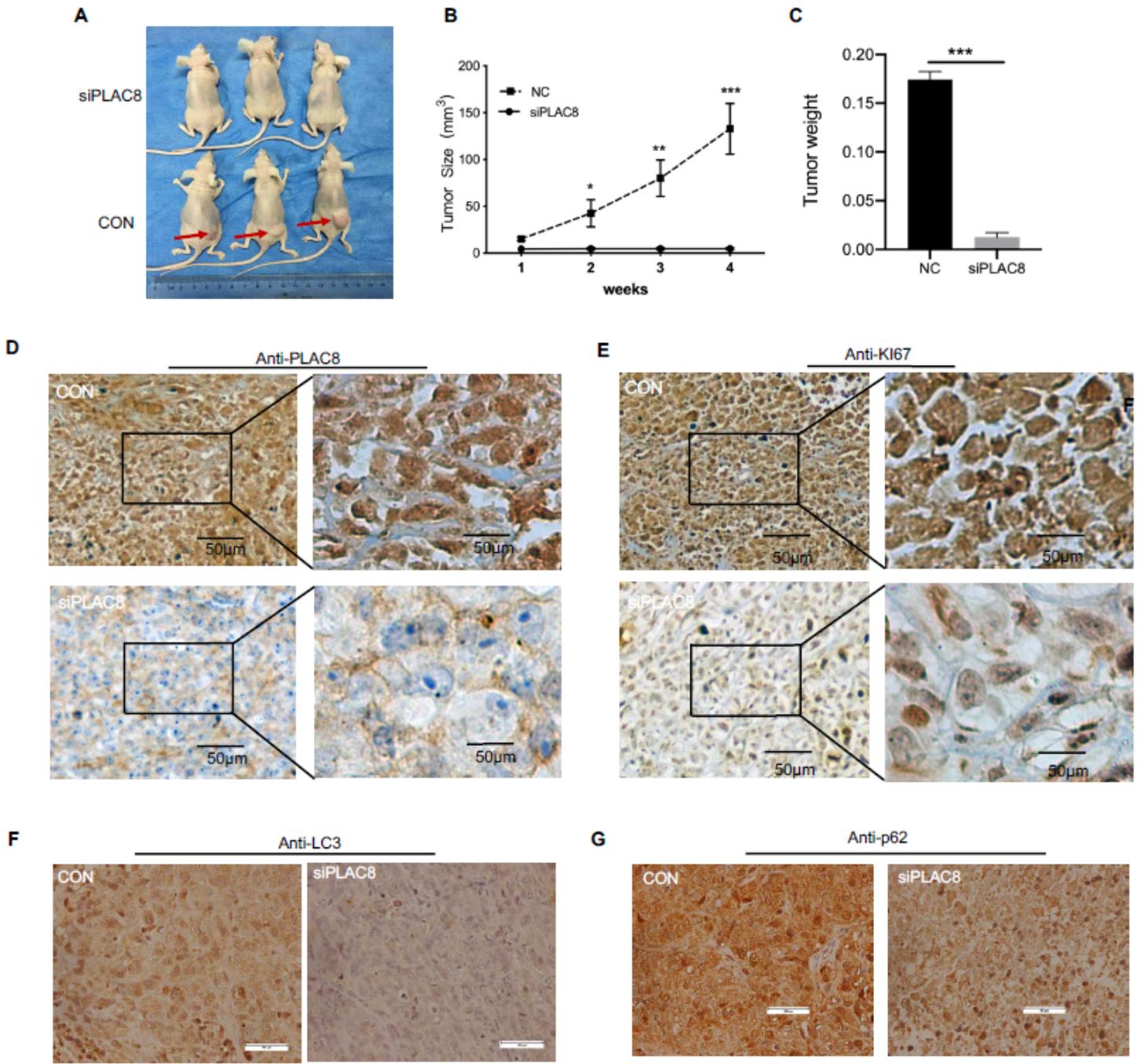


Figure 6

PLAC8 knockdown in MCF-7/ADM abrogates xenograft tumor growth. A. Arrowheads represented tumors, the down lane was mouse injected with MCF/ADM (CON), and the up lane was MCF-7/ADM (siPLAC8) group. B. The growth curve of the tumor in the two-group mice. The speed of the tumor growth was significantly increased compared with the mouse inoculated with MCF-7/ADM (siPLAC8). C. The tumor weight in MCF/ADM (CON) and MCF-7/ADM (siPLAC8) groups. D, E. PLAC8 (D) and Ki-67 (E) were expressed specifically low in the tumor from mice inoculated with MCF-7/ADM (siPLAC8). F, G. The expression of LC-3 and p62 in MCF/ADM (CON) and MCF-7/ADM (siPLAC8) groups. Each bar represents the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

