

CRISPR/Cas9-mediated knockout of AKAP12 gene increases susceptibility to doxorubicin in MCF-7/ADR breast cancer cell line

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Abstract

Chemotherapy resistance remains a major obstacle to effective cancer treatment. Our previous gene co-expression network analysis identified AKAP12 as a potential regulator of Adriamycin/doxorubicin resistance in the MCF-7/ADR breast cancer cell line. To investigate the role of AKAP12 in chemoresistance, we generated AKAP12-knockout MCF-7/ADR cell lines using the CRISPR/Cas9 genome editing tool. Functional analyses revealed that AKAP12 knockout increased sensitivity to doxorubicin and disrupted key processes involved in cancer progression, including proliferation, migration, survival, and apoptosis. Specifically, AKAP12-deficient cells exhibited reduced colony formation, impaired cell migration, and diminished tumorigenic potential compared to wild-type cells. Furthermore, AKAP12 knockout led to increased apoptotic cell death. Given the role of AKAP12 in actin cytoskeleton reorganization, cellular senescence, and oncogenic signaling pathways, our findings support its involvement in chemoresistance. Although AKAP12 is largely recognized as a tumor suppressor protein, its role appears to shift in the context of chemoresistance. Collectively, these results suggest that AKAP12 could serve as a marker for chemoresistance and a potential target to enhance chemotherapy efficacy in breast cancer.

Introduction

Breast cancer is one of the leading causes of cancer-related death among women and represents a public health challenge globally. Approximately one in eight women will develop breast cancer during their lifetime, with the risk increasing with factors such as age, family history of the disease, history of female hormone therapy, and dense breast tissue [1, 2]. However, mortality rates are gradually declining thanks to the successful implementation of screening programs and the availability of effective treatment options [3]. Early detection through routine mammography screening and clinical examinations has been shown to significantly affect breast cancer prognosis. Breast cancer can be treated with a range of therapeutic options, including surgery, radiotherapy, chemotherapy, hormone therapy, immunotherapy, and targeted therapy, which may be administered individually or in combination [4]. The choice of treatment strategy varies depending on clinicopathological characteristics, tumor stage, and hormonal or growth factor receptor status.

Breast cancer is primarily classified into molecular subtypes—luminal A, luminal B, HER2-enriched, and basal-like—based on histopathological features and the expression of steroid hormone and growth factor receptors, with each subtype exhibiting distinct clinical and prognostic characteristics [5]. These subtypes are typically identified in formalin-fixed paraffin-embedded tumor tissue sections using immunohistochemistry staining for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/neu or ERBB2), and the tumor proliferation marker Ki67 [6]. The luminal A subtype is characterized by the presence of ER (ER⁺), PR (PR⁺), or both, the absence of HER2 (HER2⁻), and low proliferation (Ki67 < 20%) [5, 6]. A higher Ki67 expression ($\geq 20\%$) distinguishes the luminal B subtype from luminal A. HER2-enriched subtypes can either be luminal, where ER and/or

PR are expressed along with HER2 positivity, or non-luminal, where ER and PR are absent but HER2 is positive. Tumors that are negative for ER, PR, and HER2 are classified as the basal-like subtype [7].

Surgery (breast-conserving or total mastectomy) remains a primary treatment option for non-metastatic breast cancers, often involving the removal of the tumor and surrounding tissue [8]. However, surgery may not be the optimal choice for certain tumor subtypes. For example, both luminal A and luminal B subtypes benefit more from systemic endocrine therapies, such as selective estrogen receptor modulators (e.g., tamoxifen), aromatase inhibitors (e.g., letrozole, anastrozole, exemestane), and selective estrogen receptor down-regulators (e.g., fulvestrant) [9]. Patients with HER2-positive breast cancer, characterized by amplification or overexpression of the ERBB2 gene, benefit from targeted anti-Her2 therapies, including monoclonal antibodies like trastuzumab and pertuzumab, or small-molecule tyrosine kinase inhibitors such as lapatinib and neratinib [5, 9]. In contrast, patients with triple-negative breast cancer (TNBC) do not respond to endocrine therapies and have limited treatment options, with chemotherapy being the standard approach [9, 10]. Chemotherapy is also utilized as a preoperative systemic therapy to reduce tumor volume (downstaging), facilitate breast-conserving surgery, and avoid auxiliary lymph node dissection [4]. Standard breast cancer chemotherapy regimens, both neoadjuvant and adjuvant, typically include anthracyclines and taxanes (paclitaxel and docetaxel), either in combination or sequentially [5]. A major challenge in chemotherapy is the development of resistance to chemotherapeutic agents, which is thought to contribute to treatment failure in 90% of metastatic breast cancer cases [11].

Chemotherapy resistance in tumor cells can arise either after exposure to a chemotherapeutic agent (acquired resistance) or without prior drug exposure (intrinsic resistance). In some cases, resistance to one type of chemotherapeutic agent can extend to structurally unrelated drugs or those with different mechanisms of action, a phenomenon known as multidrug resistance [12]. Several mechanisms for chemoresistance have been identified including the overexpression of membrane efflux pumps, alterations in drug metabolism, drug sequestration in cellular organelles, modifications of drug targets, alterations in DNA repair mechanisms, changes in apoptosis signaling pathways, and changes in the tumor microenvironment [13, 14]. Upregulation of membrane proteins such as multidrug resistance protein 1 (MDR1), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP) that enhances drug efflux across cell membranes are the primary routes for chemoresistance [15]. Additionally, increased drug metabolism that is driven by the activation or overexpression of xenobiotic-detoxifying enzymes, such as cytochrome P450s, glutathione S-transferase, and uridine diphosphate-glucuronosyltransferase also important in chemoresistance in tumors [16, 17]. Certain chemotherapeutic agents, such as vinca alkaloids and taxanes (e.g., paclitaxel), impair cell division by binding to microtubules and promoting their abnormal polymerization or depolymerization. Mutations in β -tubulin and the altered expression of its isoforms have been linked to chemotherapy resistance in various cancers [18–20]. Modulation of DNA repair mechanisms including mutation in breast cancer gene 1/2 (BRCA1/2), epigenetic silencing of MLH1/2 genes involved in mismatch repair, and upregulation of cyclin-dependent kinase 12 (CDK12) and WEE1 kinase, has been linked to chemoresistance in breast cancer [21–23].

Heterogeneity in tumor cell populations, driven by genomic or nongenomic alterations, contributes to the rapid development of drug resistance [15]. The high mutation load and genomic instability enable the acquisition of new mutations and epigenetic modifications, further fueling drug resistance. This can result in the emergence of distinct resistance mechanisms across different cancer cell subpopulations, further complicating the treatment. A complete understanding of the molecular basis of chemoresistance in breast cancer is still lacking. Our previous gene co-expression network analysis suggested a role for AKAP12 in conferring resistance to doxorubicin, an anthracycline chemotherapeutic agent, in the MCF-7/ADR breast cancer cell line [24]. AKAP12 encodes for a member of A-kinase anchoring family proteins that are involved in intracellular signal transduction through binding to cAMP-dependent protein kinase (PKA), protein kinase C (PKC), protein phosphatase 2B, and calmodulin [25–27]. AKAP12 regulates the cell cycle by anchoring cyclins, PKC, and PKA during the G1-to-S transition [28]. Previous functional analyses showed that AKAP12 inhibits cell migration in breast cancer [29], further supporting its potential role in tumorigenesis and malignancy.

In this study, we utilized the CRISPR/Cas9 genome editing tool to achieve targeted knockout of the AKAP12 gene in the MCF-7/ADR breast cancer cell line. The MCF-7/ADR cell line, known for its doxorubicin resistance, is an ideal model for studying chemoresistance in breast cancer. We conducted a functional analysis of the roles of the AKAP12 gene in doxorubicin resistance in breast cancer.

Materials and methods

Cell culture

Human MCF-7/ADR breast cancer cell line was obtained from Biotechnology group of Shahid Beheshti Medical Sciences University. The cells were cultivated in a high glucose Dulbecco's Modified Eagle Medium (DMEM) (DNA biotech, Iran) supplemented with 10% fetal bovine serum (FBS) (Bioldea, Tehran, Iran) and 1% penicillin and streptomycin (100 units/mL penicillin and 100 µg/mL streptomycin). The cells were maintained at 5% CO₂ and 37°C in a humidifying incubator. Cells were passaged at 90% confluency, and passage-matched cells were used for downstream analysis.

Guide RNA (gRNA) design and construction

The gRNAs were designed using the Zhang lab's online tool (<http://crispr.mit.edu/>). The coding sequence of the AKAP12 gene was submitted to the CRISPR guide RNA prediction tool and the predicted guides with the highest score were selected. The gRNAs 5'-CACCGAAACGCCCTCCTTCGGT-3' and 5'-CACCGACATCAGAGTCATCTTCGT-3', which target exon two of the AKAP12 gene were synthesized. Complementary strands for each gRNA were hybridized and ligated into BbsI linearized pSpCas9 (BB)-2A-Puro (PX459) vector (Addgene, plasmidID 48138). The vector also expresses a human codon-optimized pSpCas9 gene along with a puromycin resistance gene (PuroR) using a 2A peptide. Plasmids containing gRNA were confirmed by Sanger sequencing (Niagene, Tehran, Iran).

Cell transfection and clonal selection

The MCF-7/ADR cells were seeded at 5×10^5 density cells/well in a 24-well plate. After 24 hours, the vector containing gRNA construct was transfected using TurboFect Transfection Reagent (ThermoFisher Scientific, Wilmington, DE) according to the manufacturer's protocol. The unmodified pX459 plasmid was used as a negative control. The cells were allowed to grow in full growth medium at 37 °C for 48 h before being treated with antibiotic. At 48 h post-transfection, the culture medium was replaced with fresh medium containing puromycin at a concentration of 2 µg/mL. A non-plasmid transfection control was also set to test for the efficacy of puromycin selection. The cells were maintained in the puromycin-containing medium for a week. Colonies of surviving cells were picked, and each was transferred to a separate well in a 96-well plate. After the screening step, the cells in two parallel 96-well plates were harvested for either DNA extraction or further tests, including subsequent freezing and storage.

PCR amplification and sequencing of the target region

PCR primers targeting ~ 300 bp region around the gRNA binding site in AKAP12 gene were designed (Sup1). The genomic region surrounding the CRISPR/Cas9 target region was amplified using Ampliqon red PCR master mix (Ampliqon, Odense, Denmark). The PCR was conducted in 50 µL reaction at 95°C for 10 min, followed by 35 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 25 s and a final 72°C for 5 min. PCR products were purified and sequenced using a 3100 genetic analyzer. Sanger sequencing chromatograms were analyzed to identify potential indel mutations in the target region.

Cell viability assay

MCF-7/ADR cells knocked out for AKAP12 (MCF-7/ADR-AKAP12^{-/-}) were seeded at a density of 2×10^4 cells per well in a 96-well plate. The culture was left overnight at 37°C in a humidified incubator with 5% CO₂. When cells were fully adhered to the plate, doxorubicin at a concentration of 13 µg/mL (IC₅₀ dose for wildtype MCF-7/ADR) was added to the culture medium. MTT assay was performed on cells treated with doxorubicin by adding 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (5 mg/mL) to each well. The incubation with the MMT reagent was terminated after four hours. The culture medium was discarded, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and the plate was shaken for 15 min to dissolve the cells. The absorbance at 492 nm was measured in a microplate reader (BioRad, USA). The experiment was conducted in triplicate. Cell growth inhibition was determined using the following formula: Inhibitory rate (percentage) = $(1 - \text{mean OD value in experimental group} / \text{mean OD value in control group}) \times 100$.

Quantitative real-time PCR

The expression of proapoptotic genes, including CASP3, CASP8, and Bax were measured using qRT-PCR. Total RNA was extracted from MCF-7/ADR and engineered MCF-7/ADR cell lines using the Roche High

Pure RNA Isolation kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were evaluated using Nanodrop Spectrophotometry (MaestroGen, Taiwan) and agarose gel electrophoresis. qRT-PCR was performed using ExcelRT™ One-step RT-qPCR Kit (SMOBIO, Taiwan). The reaction was performed in triplicate in an ABI phase One Plus™ Real-Time PCR System (Applied Biosystems) with the following cycling conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were estimated using the $2^{-\Delta\Delta C_t}$ method using GAPDH as an internal control.

Migration assay

Unmodified MCF-7/ADR cells and AKAP12 disrupted cells were tested for migration capacity by mimicking cell migration during the wound-healing process. Cells were seeded at a density of 250,000 cells/well in six well plate and incubated at 37°C for 24, 48 and 72 hours in a humidified incubator. When the cells reached 90%-95% confluency, the cell monolayer was scratched in a straight line using a pipette tip. Cell debris was washed with 1 mL of prewarmed fresh medium. The plate was then supplemented with fresh serum-free medium and incubated at 37°C for 72 h. The scratch width was measured by imaging the plate surface every 24 hours. Images from each interval were processed with ImageJ software to assess cell migration.

Soft agar colony formation assay

Anchorage-independent growth was measured using a CytoSelect 96-well Cell Transformation Assay Kit (Cell Biolabs) according to the manufacturer's instructions. Unmodified MCF-7/ADR cells and MCF-7/ADR cells knocked out for AKAP12 were seeded at a density of 1.5×10^3 cells/well in 96-well plate in a complete medium supplemented with 0.5% agar. The plate was incubated at 37°C in a humidified incubator with 95% air and 5% CO₂ for 10 days, with colony formation monitored weekly. After dissolving the agar, the average number of colonies per well was counted.

Sphere culture

A sphere formation assay was conducted to assess the sphere-forming capacity of unmodified and genetically modified cells. Cells from each cell line were seeded in low-attachment 6-well plates at a density of 2×10^3 cells/well and cultured in serum-free epithelial basal medium supplemented with B27, insulin (4 µg/mL), epidermal growth factor (20 ng/mL), and basic fibroblast growth factor (20 ng/mL). Sphere-forming capacity was evaluated by counting the number of colonies formed over a 2-week period.

Cell viability assay by flow cytometry

To assess apoptosis in cells treated with the chemotherapeutic agent doxorubicin, unmodified and genetically modified cells were exposed to doxorubicin at a concentration of 13 µg/mL for 48 and 72 hours. The cells were then trypsinized, collected by centrifugation, and resuspended in 1X Annexin V binding buffer. Annexin V-FITC was added, and the cells were incubated at room temperature in the dark for 5 minutes. The procedure followed the protocol provided with the Annexin V-FITC/PI detection kit (Jiangsu Kaiji Biotechnology Co., Ltd.). Cells were analyzed with a fluorescence-activated cell sorter (FACS) flow cytometer and processed using FlowJo software.

Results

Knockout of AKAP12 gene in MCF-7/ADR cell line using CRISPR-Cas9

We aimed to analyze the effect of functional knockout of the AKAP12 gene on chemotherapeutic resistance in breast cancer. Our previous analysis suggested that this gene may play a key role in doxorubicin resistance in the MCF-7/ADR breast cancer cell line [24]. To genetically knock out the target gene in the MCF7-ADR cell line, we designed two gRNAs, targeting conserved regions to minimize off-target cleavage by Cas9 endonuclease. The AKAP12 gene consists of five exons, with the coding sequence beginning in exon 2 and ending in exon 4. Exon 4 encodes over 95% of the coding region and contains sequences for all domains required for the AKAP12 function. Therefore, we targeted this exon with two gRNAs to ensure a complete loss of gene function following the CRISPR/Cas9 cleavage. The gRNAs were synthesized and ligated into the BbsI-linearized pX458 vector, and cloning was confirmed by colony PCR followed by Sanger sequencing (Fig. 1).

We conducted two parallel transient transfections using vectors carrying each gRNA. Following cell transfection, successfully transfected cells were subjected to selection with puromycin. However, we were unable to establish a single colony, as puromycin-resistant cells did not grow into isolated colonies in the presence of puromycin. Therefore, we proceeded with a bulk population of puromycin-resistant cells transfected with each gRNA. To assess CRISPR/Cas9-induced genetic disruptions, genomic sequences surrounding each gRNA-target region were amplified using specific primers. PCR products were Sanger sequenced, and the resulting trace files were analyzed to identify indel mutations that disrupt the reading frame of the gene's coding sequence (Fig. 2). Our sequencing data indicated that, among the tested gRNAs, only gRNA2 substantially disrupted AKAP12 gene function (Fig. 2c). The presence of indel mutations including AC-AG was evident in Sanger sequencing chromatograms, resulting in premature stop codons.

Chemosensitivity increased in MCF-7/ADR cells lacking AKAP12

Our previous study showed that AKAP12 gene expression level increases under doxorubicin treatment in MCF-7/ADR cells compared to parental cells [24]. Therefore, knocking out this gene may clarify its role in breast cancer chemoresistance. To assess the viability of AKAP12 knockout cell lines following exposure to doxorubicin, we performed an MTT assay. No significant differences in viability were observed among wild-type MCF-7, MCF-7/ADR, and genetically modified MCF-7/ADR-AKAP12^{-/-} after 24 h of doxorubicin treatment (Fig. 3). However, extending doxorubicin treatment to 48 h and 72 h significantly reduced the viability of cell lines lacking AKAP12.

Loss of the AKAP12 gene reduces cell migration

Cell migration was evaluated using a wound-healing assay. Migration capacity was quantified by measuring the rate at which cells closed the gap created by the scratch in a monolayer culture (Fig. 4). Cells with AKAP12 deficiency exhibited reduced migration capacity compared to unmodified parental cells (Fig. 4). The impairment in migration was more severe in AKAP12-deficient cells, as the wound remained significantly open even after 72 hours of incubation.

Analysis of Doxorubicin's Impact on Wound healing and Migration in Wild-Type and AKAP12-g2-Deficient MCF-7/ADR Cells. Wound healing test was used to quantify two kinds of invasive cells. Compared to MCF-7/ADR cells, less AKAP12-g2 cells were invasive. Using ImageJ, we were able to quantify the values. Mean standard deviation (n = 3) is used to display data. *P < 0.05 and **P < 0.01.

Sphere and colony formation was reduced in AKAP12-deficient cells

The ability of cancer cells to grow in non-adherent, serum-free conditions is a distinct characteristic that reflects their tumorigenic potential. This can be efficiently tested by a sphere formation assay. AKAP12 knockout cells exhibited a reduced sphere-forming capacity, with both the number and size of spheres significantly decreased in AKAP12-deficient cells compared to parental cells (Fig. 5a). Clonogenicity was also markedly reduced, by about 50%, in cells lacking AKAP12 (Fig. 5b), suggesting a role for AKAP12 in supporting the self-renewal potential of MCF-7/ADR breast cancer cells. Clonogenicity was assessed using a soft agar colony formation assay, which enables evaluation under anchorage-independent growth.

AKAP12 knockout enhanced doxorubicin-induced apoptosis

To determine if the increased sensitivity to doxorubicin following AKAP12 disruption is mediated by apoptosis induction, we conducted an Annexin-V/PI assay to distinguish dead cells from apoptotic cells. FACS analysis revealed a significant reduction in cell viability in AKAP12-disrupted cells (Fig. 6a), with most dead cells undergoing apoptosis. The apoptosis rate further increased with extended exposure to doxorubicin. To confirm this effect at the gene expression level, the expression of pro-apoptotic gene

Bax and apoptotic genes CASP3 and CASP8 was quantified by real-time PCR. A marked increase in all three apoptotic genes was noted in MCF-7/ADR cells lacking AKAP12 (Fig. 6b), with caspase-3 and caspase-9 showing the most substantial expression changes under doxorubicin treatment.

b. Expression gene of apoptosis in Wild type and AKAP12-ko as knocked out cells in Response to Chemotherapeutic Drug (Dox).

Discussion

This study aimed to investigate the role of AKAP12 in acquired resistance to the anthracycline chemotherapeutic agent doxorubicin in the MCF-7/ADR breast cancer cell line. We employed CRISPR/Cas9 genome editing to disrupt the AKAP12 gene in MCF-7/ADR cells. AKAP12-deficient cells were subsequently assessed for doxorubicin sensitivity, cell viability, migration, and tumorigenic potential. Our findings demonstrate that AKAP12 disruption significantly increased the sensitivity of MCF-7/ADR cells to doxorubicin, likely by promoting apoptosis.

MCF-7/ADR cells deficient in AKAP12 exhibited reduced colony formation, sphere formation, and cell migration, highlighting AKAP12's critical role in cancer development and progression. Consistent with these findings, previous studies have shown that AKAP12 depletion in colorectal cancer cells impairs colony and sphere formation and results in smaller in vivo tumor xenografts [30]. Furthermore, AKAP12 has been demonstrated to promote a stem cell-like phenotype in these cells through the PKC/STAT3 signaling pathway [30].

Previous studies have suggested a tumor suppressor function for AKAP12 in breast cancer. Tissue array analysis also showed a higher expression of AKAP12 in normal breast tissue and a correlation between elevated AKAP12 levels and improved recurrence-free survival in breast cancer patients [29]. Interestingly, protein expression analysis in various breast cancer cell lines showed that AKAP12 expression is lower in breast cancer cell lines with less aggressive phenotype compared to cells with aggressive malignant phenotype [29]. Here, we found that disruption of AKAP12 in MCF-7/ADR cells reduced cell proliferation. In contrast, a previous study reported that siRNA-induced knockdown of AKAP12 did not affect cell proliferation in breast cell lines MCF10A, MDA-MB-231, and Hs578T [29]. However, targeted expression of AKAP12 in human fibroblast cells has been associated with decreased expression and nuclear localization of cyclin D1 and increased expression of cyclin-dependent kinase inhibitors Cip/p21 and Kip/p27, followed by a G1 cell cycle arrest [28, 31]. Additionally, transfecting AKAP12 in ras-transformed HT1080 fibrosarcoma cells showed upregulation of proapoptotic protein Bax and anti-apoptotic protein Bcl-2, leading to an increased apoptosis [31]. These findings contrast with our results, as AKAP12 disruption in MCF-7/ADR cells led to increased expression of pro-apoptotic gene Bax and apoptotic genes caspase-3 and caspase-8, suggesting a context-dependent role for AKAP12 in regulating cell proliferation and apoptosis.

Loss of AKAP12 reduced cell migration, as demonstrated by the wound-healing assay. However, previous studies reported that downregulation of AKAP12 in breast cell lines such as MCF10A, MDA-MB-231, and

Hs578T was associated with increased cell migration [29]. In prostate cancer cells, tetracycline-inducible expression of AKAP12 suppressed anchorage-independent growth, further supporting a tumor-suppressing role for AKAP12 [32]. The same result was made in scr-transformed NIH3T3 cells, where re-expression of AKAP12 led to poor anchorage-independent growth and cell invasion [33], suggesting a key role for AKAP12 in regulating cell migration. In a previous study, upregulation of AKAP12 in esophagus squamous cell carcinoma led to decreased cell migration and reduced expression of epithelial-mesenchymal transition (EMT) markers N-cadherin and vimentin [34]. AKAP12 is known to regulate chemotaxis-driven cell migration, a process mechanistically distinct from the migration assayed by the wound-healing assay [29, 32]. Since we utilized the wound-healing assay to measure migration, the reduced migration observed here contrasts with the previously reported findings. This discrepancy may arise from the specific context of our study, which focused on the role of AKAP12 in chemotherapy resistance within a doxorubicin-resistant cell line. The unique properties of MCF-7/ADR cells and their resistance mechanisms could influence the functional role of AKAP12 in ways not observed in other cancer models.

Our previous analysis found a link between AKAP12 upregulation and doxorubicin resistance in the MCF-7/ADR breast cancer cell line [24]. Here, we observed that *AKAP12* knockout increased doxorubicin sensitivity in these cells, further confirming its potential role in chemoresistance. In ovarian cancer cells, higher AKAP12 protein and mRNA levels have been linked to paclitaxel resistance, which correlated with decreased methylation of the *AKAP12* gene during the development of the chemoresistance phenotype [35]. This data suggests that epigenetic modifications may play a crucial role in regulating AKAP12 expression and, consequently, drug resistance. Additionally, elevated levels of AKAP12 protein have been identified in the mitochondrial proteome of ovarian cancer cells resistant to cisplatin [36]. These data collectively suggest that AKAP12 may serve as a marker of chemoresistance in ovarian and breast cancer. AKAP12 has been implicated in various signaling pathways contributing to cellular survival and proliferation under chemotherapeutic stress. For instance, AKAP12 is shown to interact with critical signaling proteins PKA and PKC, which are involved in cellular responses to chemotherapy [37]. In a recent study, ESR1-AKAP12 gene fusion was identified in a subset of lung cancer patients resistant to the EGFR tyrosine kinase inhibitor Osimertinib [38]. This discovery suggests a potential role for AKAP12 in mediating cellular responses to therapy, potentially through altered signaling pathways linked to drug resistance.

Conclusion

CRISPR technology was used to generate AKAP12-deficient MCF-7/ADR cell lines. This study provides strong evidence that AKAP12 plays a critical role in acquiring chemoresistance in breast cancer. Targeting AKAP12 may enhance the efficacy of chemotherapeutic agents like doxorubicin, offering a potential strategy for overcoming resistance in breast cancer treatment. This approach holds promise for improving treatment outcomes in patients with resistant breast cancer by incorporating new therapeutic avenues into existing oncology protocols. These findings also contribute to understanding the molecular mechanisms underlying chemotherapy resistance in breast cancer. However, a key

limitation of this study is its *in vitro* nature, as the MCF-7/ADR cell line may not fully capture the complexity of breast cancer in clinical settings.

Abbreviations

ADR
Adriamycin resistance
Dox
Doxorubicin
ECM
Extracellular matrix

Declarations

Author Contribution

A.M. performed the experiments, analyzed the data, prepared the figures and wrote the main manuscript text. A.F. and J.G. contributed to the experimental work and data analysis. H.E.G. contributed to the flow cytometry and cell-based assays. K.S. contributed to study design and provided resources and supervision. V.J. conceived and supervised the study and revised the manuscript. All authors reviewed the manuscript.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Figures

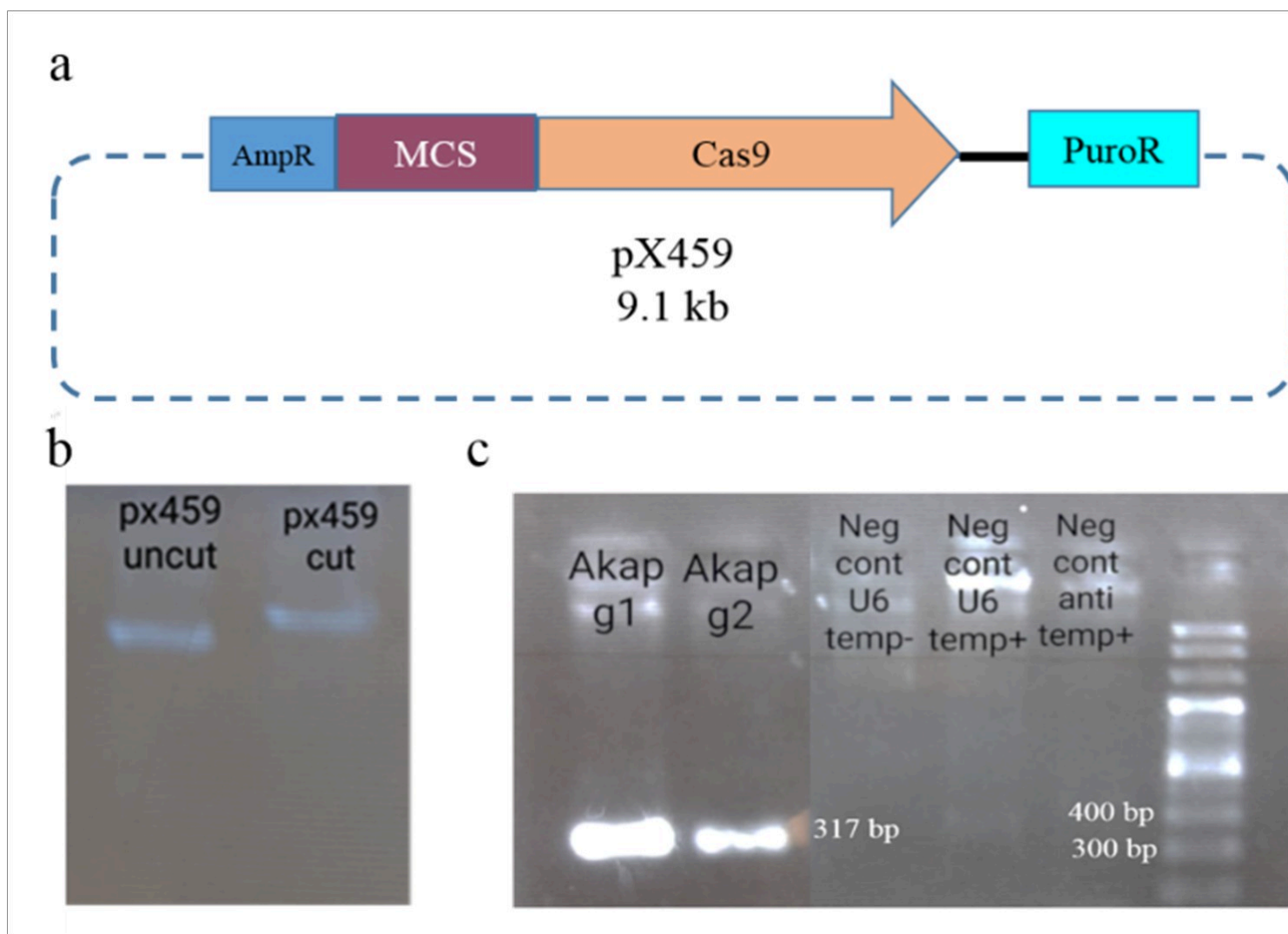
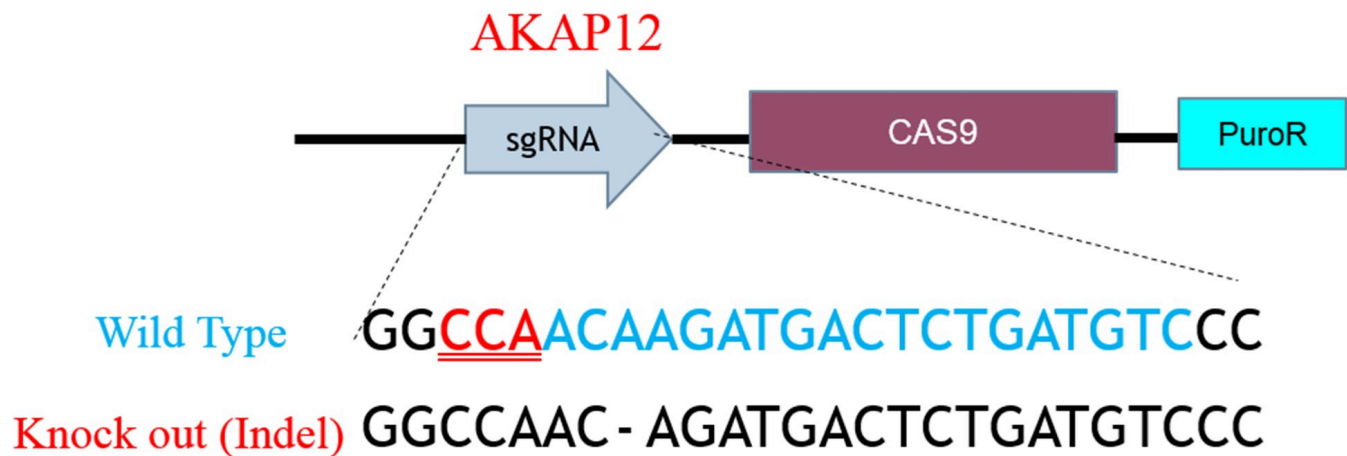


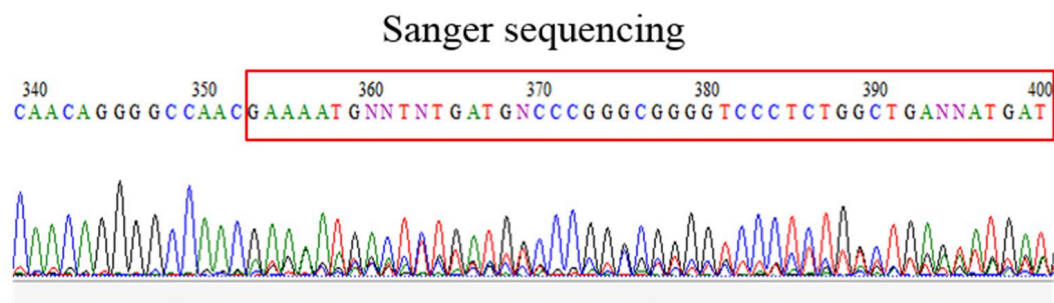
Figure 1

CRISPR design and AKAP12 knockout experimental process. **a** Genetic map of pX459 vector. **b** Confirmation of Digestion and preparation of linear CRISPR vector for clone guides with Bbs1 enzyme. **c** Colony PCR results, Clones resulting from the transformation of the ligation product of a cloned AKAP12 gene.

a



b



c

	The Sequence of Colonies
AKAP12 WT	GGCCAACAAGATGACTCTGATGTCCC
AKAP12-g2	GGCCAAC-AGATGACTCTGATGTCCC

Figure 2

Validation of indels generated by sequencing for target region in AKAP12 gene. **a** The gRNA sequences and indel after CRISPR-directed gene editing of the AKAP12 gene. **b** The result of sanger sequencing to determine the efficacy of gene Indel through CRISPR/Cas9. **c** Specific population and in established clonal cell lines(Confirmed cell line gRNA2 for AKAP12).

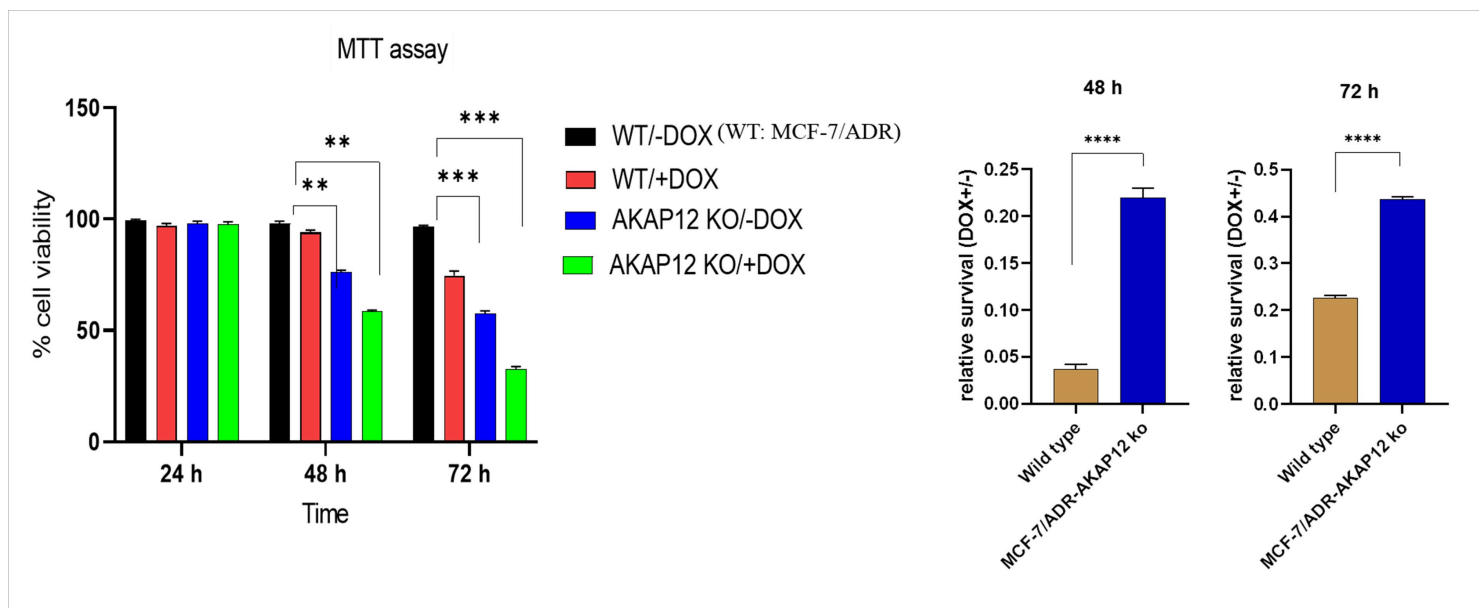


Figure 3

Decreased survival in cells containing AKAP12 knockout gene compared to the control at 48 and 72 hours in the conditions with and without Dox treatment (MCF-7/ADR = Wild type).

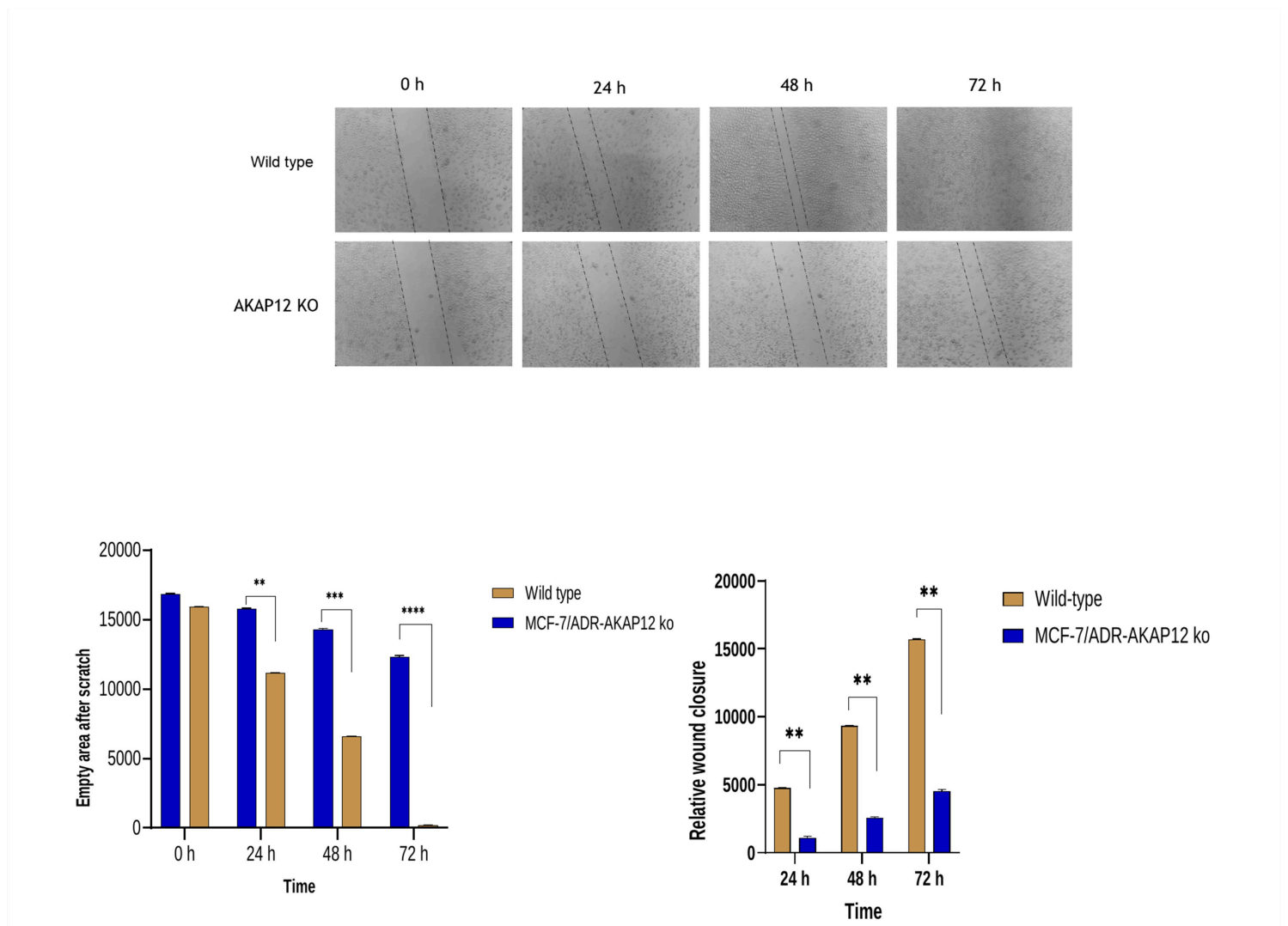


Figure 4

Effect of AKAP12-knockout on cell proliferation and migration of MCF-7/ADR cells.

Analysis of Doxorubicin's Impact on Wound healing and Migration in Wild-Type and AKAP12-g2-Deficient MCF-7/ADR Cells. Wound healing test was used to quantify two kinds of invasive cells. Compared to MCF-7/ADR cells, less AKAP12-g2 cells were invasive. Using ImageJ, we were able to quantify the values. Mean standard deviation (n=3) is used to display data. *P<0.05 and **P<0.01.

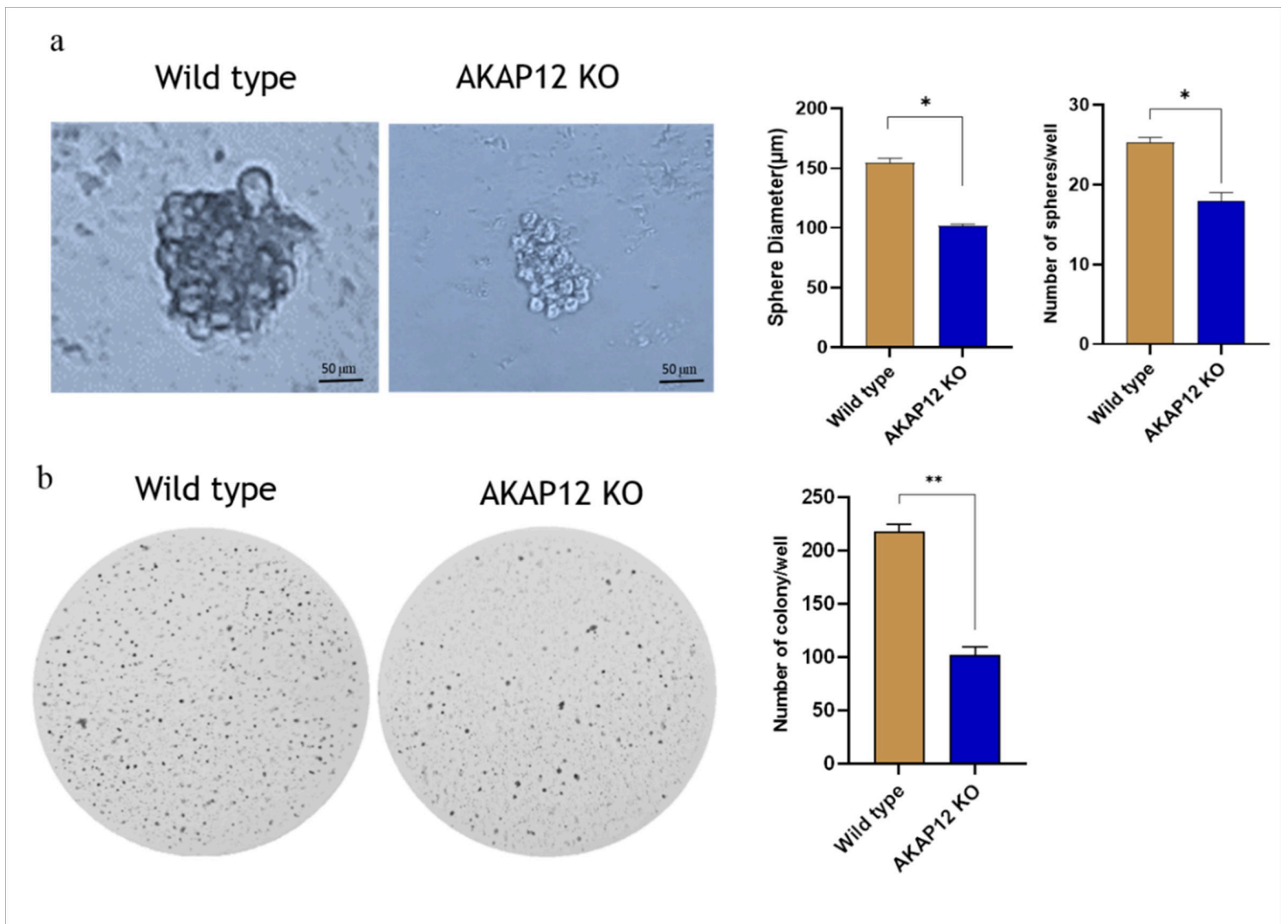


Figure 5

AKAP12 knockout decrease cell proliferation of MCF-7/ADR cells. The sphere-forming assay in MCF-7/ADR as Wild type and AKAP12-ko as knock outed cell. **a** Representative photographs of spheres formed after 7 days(Scale bar 50 μ m). **b** Representative photographs of colony formation in MCF-7/ADR as Wild type and AKAP12-ko as knock outed cell.

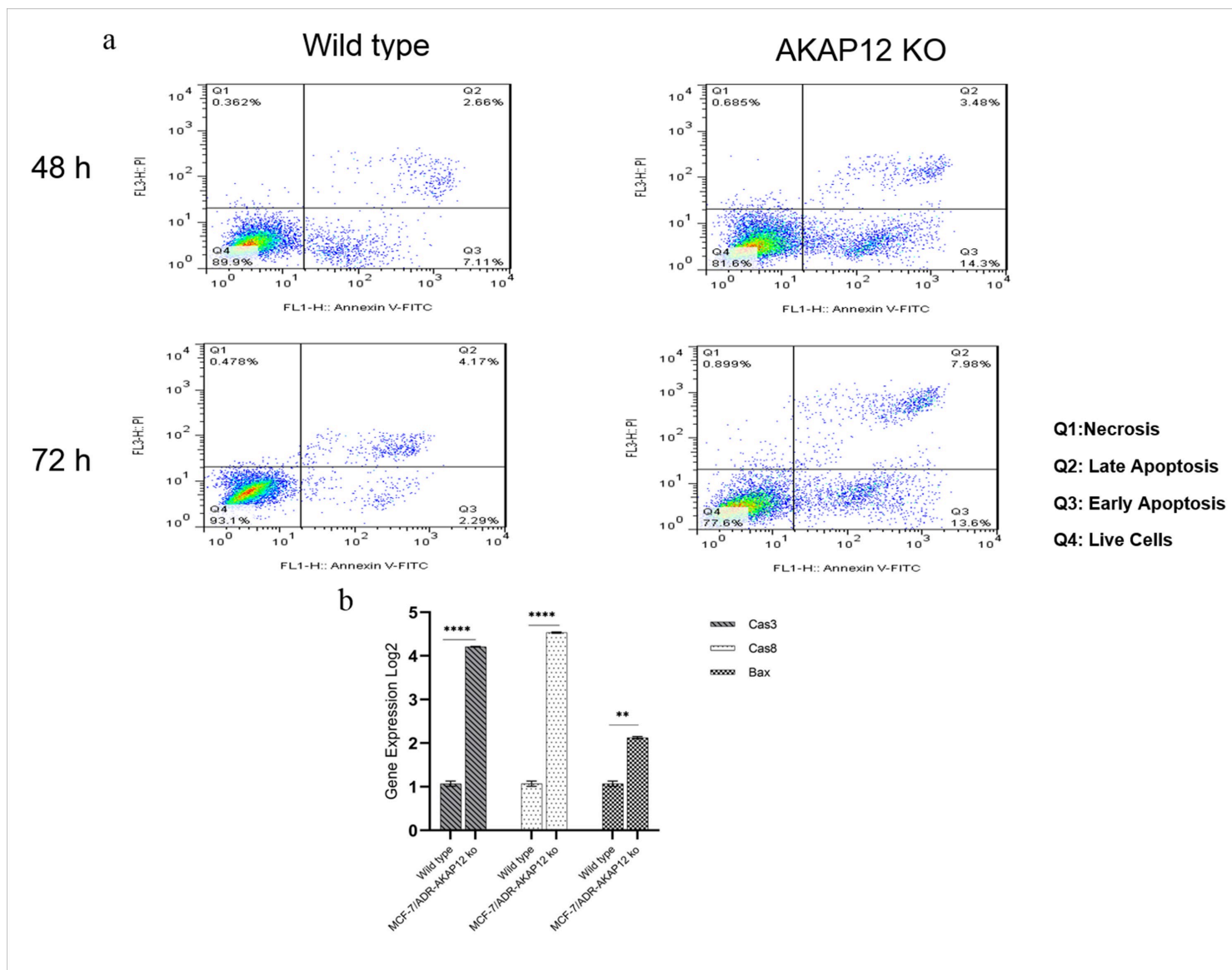


Figure 6

a. Annexin V/PI assay of manipulated MCF-7/ADR and MCF-7/ADR cells. Apoptosis in modified MCF-7/ADR as Wild type and AKAP12-ko as knock outed cells at 48 and 72 hours to determine by flow cytometric analysis. A bar chart displaying the frequency distribution of annexin V-FITC/PI staining. Three separate experiments' worth of data were averaged to get at the findings shown here. Means standard error of the mean are shown. Annexin V + /PI cells and Annexin V + /PI + cells were both apoptotic. * $P < 0.05$ and ** $P < 0.01$

b. Expression gene of apoptosis in Wild type and AKAP12-ko as knocked out cells in Response to Chemotherapeutic Drug (Dox).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUP1.xls