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Research paper

Fatty acid receptor GPR120 promotes breast cancer chemoresistance by upregulating ABC transporters expression and fatty acid synthesis

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ABSTRACT

Background: Chemoresistance is the major cause of neoadjuvant treatment failure in breast cancer patients. Despite recent progress, the mechanism underlying chemoresistance remains to be further defined.
Methods: Expression of G protein-coupled receptor 120 (GPR120) was analyzed by immunohistochemistry in the biopsies of primary breast cancer who subsequently underwent preoperative neoadjuvant chemotherapy. In vitro and in vivo loss- and gain-of -function studies were performed to reveal the effects and related mechanism of GPR120 signaling pathway in the chemoresistance of breast cancer cells.
Findings: We identified that GPR120, a receptor for long-chain fatty acids, was important for the acquisition of chemoresistance in breast cancer cells. We showed that GPR120 expression was positively associated with clinical response to neoadjuvant chemotherapy in patients. In breast cancer cells, GPR120 enhanced the de novo synthesis of fatty acids that served as GPR120 ligands to activate GPR120 signaling via a feedback mechanism. Upregulated GPR120 signaling rendered cells resistant to epirubicin-induced cell death by upregulating ABC transporters expression and thus decreasing the intracellular accumulation of epirubicin. Akt/NF-κB pathway was responsible for the GPR120-mediated expression of ABC transporters leading to modulation of the concentration of chemotherapeutic drugs in cells. The functional importance of GPR120 in chemoresistance was further validated using epirubicin-treated tumor xenografts, in which we showed that blockade of GPR120 signaling with AH7614 or GPR120-siRNA significantly compromised chemoresistance.
Interpretation: Our results highlight that GPR120 might be a promising therapeutic target for breast cancer chemoresistance.
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1. Introduction

Chemotherapeutic intervention is one of the prevailing ways of treating breast cancer patients, but the development of chemoresistance limits the effectiveness of chemotherapy [1]. Therefore, identifying the molecular mechanisms contributing to chemoresistance is important for disease interventions. The mechanisms of chemoresistance, either intrinsic or acquired, are complex and multifactorial, including but not limited to reduced intracellular drug accumulation, modification of drug targets, increased repair of drug-induced DNA damage, and interactions between cancer cells and the tumor microenvironment [2,3]. ATP-binding cassette (ABC) transporters, which pump chemotherapeutic drugs outside cells, contribute to

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Research in context

Evidence before this study

GPR120, a receptor for long-chain free fatty acids, is involved in potent anti-inflammatory and insulin-sensitizing effects. However, our previous studies have reported that the expression of GPR120 was significantly associated with tumor progression and that the activation of GPR120 signaling could induce angiogenesis and epithelial-mesenchymal transition in colorectal carcinoma cells.

Added value of this study

We showed that GPR120 functioned as a chemoresistance-promoting factor in breast cancer. GPR120 expression was positively associated with clinical response to neoadjuvant chemotherapy in patients. Mechanistically, GPR120 promoted the de novo synthesis of fatty acids that served as potential GPR120 ligands to activate GPR120 signaling via a feedback mechanism. Upregulated GPR120 signaling rendered breast cancer cells resistant to epirubicin-induced cell death by upregulating the expression of ABC transporters and thus decreasing the intracellular accumulation of epirubicin.

Implications of all the available evidence

Our results suggested that targeting GPR120 in combination with chemotherapy may overcome breast cancer chemoresistance.

multidrug resistance (MDR) [4,5]. Among the 49 members of the ABC transporter family, multidrug resistance protein 1 (MDR1, also known as P-glycoprotein and ABCB1), MDR-associated protein 1 (MRP1, also known as ABCC1) and breast cancer resistance protein (BCRP, also known as ABCG2) are involved in multidrug resistance [6]. All of these ABC transporters exhibit overlapping substrate specificity and promote the removal of chemotherapeutic compounds, such as taxanes, topoisomerase inhibitors and antimetabolites, from cells [6]. Therefore, directly targeting ABC transporters or their relevant signaling pathways might represent potential MDR-reversing strategies.

G protein-coupled receptor 120 (GPR120), a receptor for long-chain free fatty acids (FFAs), is involved in a variety of physiological processes, including glucagon-like peptide-1 secretion, insulin sensitivity, glucose homeostasis, macrophage inactivation, taste perception, and adipocyte differentiation [7,8]. In addition, our previous studies have reported that the expression of GPR120 was significantly associated with tumor progression and that the activation of GPR120 signaling could induce angiogenesis and epithelial-mesenchymal transition in colorectal carcinoma cells [9]. Of note, it has been shown that stearic acid (C18:0) and n-3 fatty acid DHA (C22:6n-3) [10] serve as ligands to activate GPR120 signaling with varying affinity [11]. Likewise, it has been shown that the activation of GPR120 signaling via fatty acids enhances the survival of murine enteroendocrine STC-1 cells under serum-starved conditions [12]. In breast cancer, the levels of stearic acid (C18:0) and n-3 fatty acid DHA (C22:6n-3) were found to be significantly higher in tumor tissues than in adjacent normal tissues [10]. In addition to diet, de novo fatty acid synthesis is a key source of fatty acids in the tumor microenvironment [13]. Fatty acid synthase (FASN), a key lipogenic enzyme catalyzing the terminal steps of de novo lipogenesis, is strongly associated with recurrence, metastasis, and death in breast cancer patients and might be a good target for anticancer therapy [14,15].

Thus, we proposed that elevated levels of fatty acids in breast cancer tissues might activate GPR120 signaling, which sustains cancer cell survival in response to chemotherapy and drives the development of chemoresistance. We found that GPR120 expression was positively associated with chemotherapeutic sensitivity in breast cancer patients and that activation of GPR120 signaling promoted ABC transporters expression and de novo fatty acid synthesis. Our results suggest that GPR120 might be a promising pharmaceutical target for the reversal of breast cancer chemoresistance.

2. Materials and methods

2.1. Patients and breast cancer biopsies

Seventy-eight primary breast cancer patients with pathological stage II to III disease, in the age range of 27 to 87 years (mean age 51.9), who underwent preoperative neoadjuvant chemotherapy at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine from 2011 to 2015, were enrolled in this study. Tumor specimens were obtained by core needle biopsy before neoadjuvant chemotherapy. Informed consent was obtained from all study subjects prior to their inclusion in this study, and this study was reviewed and approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All patients received four to eight cycles of systemic neoadjuvant chemotherapy with the TEC (docetaxel, epirubicin, cyclophosphamide) or TAC (docetaxel, pirarubicin, cyclophosphamide) or FEC (5-fluorouracil, epirubicin, cyclophosphamide) regimens.

2.2. Immunohistochemistry

The expression level of GPR120 in paraffin-embedded biopsy sections was detected via immunohistochemical staining. Rabbit anti-human GPR120 antibody (Abcam, Cambridge, UK; CAT# ab97272, RRID# AB_10680852) was employed as the primary antibody with a 1:500 dilution, followed by an anti-rabbit secondary antibody using DAKO ChemMate™ Envision™ Detection Kit (DAKO A/S, Denmark). Positive staining for GPR120 (in brown) was mainly localized in the cytoplasm and plasma membrane. Immunohistochemical scoring was independently performed using H-score system by two expert pathologists who were blinded to the clinical response to neoadjuvant chemotherapy [16,17]. H-scores were determined based on both the intensity and the percentage of GPR120-positive tumor cells in 10 random fields at ×400 magnification. The staining intensity was classified into the following four levels: negative (0), weak (1+), moderate (2+), and strong (3+). The H-score was calculated with the following formula: 1 × (percentage of cells staining weakly [+]) + 2 × (percentage of cells staining moderately [++]) + 3 × (percentage of cells staining intensely [+++]), and the overall score ranged from 0 to 300. H-scores >150 were defined as high expression of GPR120, while H-scores ≤150 were considered low expression.

2.3. Cell culture and drug treatments

MCF-7, T47-D, SK-BR-3 and ZR-75-1 cell lines were purchased from American Type Culture Collection. MCF-7, T47-D, and ZR-75-1 cells were cultured in RPMI-1640 medium plus 10% fetal bovine serum, and SK-BR-3 in McCoy's 5a medium plus 10% fetal bovine serum. The MCF-7/ADM cell line was a gift from Dr. Jian Jin, Jiangnan University (Wuxi, Jiangsu, China), and it was cultured in RPMI-1640 medium plus 10% fetal bovine serum and 1 μg/ml epirubicin. All cell lines were mycoplasma free and cultured no longer than 2 months after recovering. Cells were seeded, allowed to adhere, and subsequently treated with 10 μM GW9508 (Sigma-Aldrich, St. Louis, MO, USA), 10 μM TUG891 (Tocris, Minneapolis, MN, USA), 50 μM AH7614 (Tocris), 5 μM PGP-4008 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 25 μM

MK-571 (Sigma-Aldrich), 5 μM fumitremorgin C (Sigma-Aldrich), or 10 μM BAY11–7082 (Selleck Chemicals, Houston, TX, USA).

2.4. WST-1 assay

Cell viability was determined using a WST-1 assay kit (Roche Applied Science, Indianapolis, IN, USA). Scramble and GPR120 KD MCF-7 or T47-D cells were respectively seeded in 100 μl RPMI 1640 medium with 10% serum in 96-well flat bottom plates with cell density 8000 cells/well. After cell adhesion overnight, the medium was changed with 100 μl RPMI 1640 medium without serum, but containing 10 μM GW9508 or vehicle control. After 24 h of culture, the medium was changed with 100 μl new RPMI 1640 medium containing 10 μM GW9508 or vehicle control and different concentrations of epirubicin (0, 0.03, 0.125, 0.5, 2, and 8 μg/ml). After 48 h of epirubicin treatment, 10 μl of WST-1 reagent was added and plates were further incubated up to 2 h at 37 °C in 5% CO_2. Then, the absorbance was read at 450 nm with a reference wavelength set at 690 nm using a plate reader (TECAN safire 2, TECAN, Männedorf, Switzerland).

2.5. Quantitative real-time PCR and chromatin immunoprecipitation (ChIP)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse transcribed to cDNA with a PrimeScript™ RT reagent kit (Takara Bio, Tsu, Japan). Quantitative real-time PCR were performed using the FastStart SYBR Green Master kit (Roche Applied Science) and Applied Biosystems ViiA™ 7 system. Primer pairs are shown in Supplementary Table 1.

ChIP analysis was performed by using a kit from EMD Millipore according to the manufacturer's instructions, and the primers are shown in Supplementary Table 2.

2.6. Western blot assay

Western blot assay was conducted as previously described [18]. Primary anti-human antibodies against GPR120, ABCB1 (CAT# ab170903), ABCC1 (CAT# ab180960) and ABCG2 (CAT# ab108312, RRID: AB_10861951) were purchased from Abcam, and primary antibodies against GAPDH (CAT# 2118S), β-actin (CAT# 4970S, RRID: AB_2223172), FASN (CAT# 3180S, RRID: AB_2100796), Phospho-IκBα (CAT# 2859S, RRID: AB_561111), Phospho-Akt (Thr308) (CAT# 13038S, RRID: AB_2629447), Phospho-Akt (Ser473) (CAT# 4060), Phospho-p65 (Ser536) (CAT# 3033S, RRID: AB_331284), IκBα (CAT# 4812S, RRID: AB_10694416), Akt (CAT# 9272S, RRID: AB_329827), p65 (CAT# 8242S, RRID: AB_10859369) and an apoptosis antibody sampler kit were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.7. Establishment of GPR120 knockdown cell lines

To establish GPR120 knockdown MCF-7 and T47-D cell lines, lentivectors expressing GPR120-specific shRNA (5'-GAGTAACTGATCAC AATGACCAGTCCTGG-3′) or negative control shRNA (Scramble) were purchased from Applied Biological Materials Inc. (ABM, Richmond, BC, Canada). Lentivirus production and infection were conducted according to the manufacturer's protocol. The transfected cells were expanded for follow-up experiments.

2.8. Measurement of intracellular epirubicin accumulation

Flow cytometry was used to measure intracellular epirubicin accumulation. Cells treated with or without epirubicin were analyzed by FACSCalibur flow cytometer with CellQuest Pro Software (BD Biosciences, Franklin Lakes, NJ, USA), and geometric mean fluorescence intensity (MFI) of epirubicin was calculated using FlowJo software (Treestar, Ashland, OR, USA). Relative epirubicin fluorescence intensity (relative EPI fluo) was calculated as follows: [(MFI of cells with epirubicin treatment - MFI of cells without epirubicin treatment) / MFI of cells without epirubicin treatment] × 100%.

2.9. Construction of luciferase reporter vectors and dual-luciferase assays of promoter activity

Fragments of the ABCC1 promoter (−660 to +103) and the ABCG2 promoter (−243 to +362) were individually subcloned into pGL3-basic reporter plasmids. MCF-7 cells were seeded in 6-well plates and transiently co-transfected with 2.5 μg of reporter construct and 100 ng of the Renilla luciferase vector pRL-TK using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and analyzed on the Lumat [3] LB 9508 Tube Luminometer (Berthold Technologies, Germany).

2.10. Cell fatty acid extraction and analysis

Fatty acid extraction: 1 ml 5% H_2SO_4/methyl alcohol was added to tubes containing cell pellets (2×10^6) and supplemented with 100 μg nonadecanoic acid methyl ester as an internal standard. N2 was used to exclude air from tubes, and sealed tubes was heated at 80 °C for 90 min. After cooling the tubes at 4 °C for 10 min, 1.5 ml double distilled H_2O and 1 ml hexane were added into the tubes. Vortexed mixtures were centrifuged at 2000 rpm for 2 min, and the upper phase was used for fatty acids analysis.

Fatty acid analysis: Gas chromatography-mass spectrometry (GC–MS, 7890A-5975C, Agilent Technologies, Santa Clara, CA, USA) was used to measure fatty acid types and contents. Fatty acid concentrations were calculated according to an internal standard.

2.11. In vivo tumorigenicity assay

Four-week-old female BALB/c nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were housed in specific pathogen-free conditions and humanely cared for according to the criteria outlined in the National Guidelines for the Care and Use of Laboratory Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The estrogen hormone is necessary for the growth of MCF-7 cells, and thus all mice received subcutaneous implantation of estrogen pellets (0.72 mg of 17β–estradiol 60-day release, Innovative Research of America, Sarasota, FL, USA) three days prior to cell injection. Approximately 5×10^6 MCF-7/ADM cells suspended in 100 μl PBS were subcutaneously injected into mouse flanks. Tumor volume was estimated by using the following formula: length × $width^2/2$. When the tumors reached 150 to 200 mm^3, mice carrying xenograft tumors were assigned to different treatments: 1. vehicle, 2. epirubicin (4 mg/kg, intraperitoneal injection, once every 4 days), 3. GPR120-siRNA (10 nmol, intratumoral injection, once every 4 days), 4. AH7614 (50 μg, intratumoral injection, once every 4 days), 5. GPR120-siRNA in combination with epirubicin (10 nmol of GPR120-siRNA and 4 mg/kg of epirubicin, administration of GPR120-siRNA one day prior to epirubicin injection), and 6. AH7614 in combination with epirubicin (50 μg of AH7614 and 4 mg/kg of epirubicin, administration of AH7614 one day prior to epirubicin injection). At the end of 20 days of treatment, the mice were euthanized, and tumors were removed for further experiments. The GPR120-siRNA sequence was as follows: 5'-AAAGAAATGACTTGTCGAT-3′.

2.12. Statistical analysis

All data were expressed as the mean ± SEM, and statistical significance was evaluated by one-way ANOVA. Correlations between GPR120 expression and clinicopathologic patterns were assessed by the χ^2 test or Fisher's exact test. All statistical tests were carried out using Statistical Package for Social Science software (version 20.0). $P < .05$ was considered statistically significant.

3. Results

3.1. GPR120 expression is positively associated with clinical response to neoadjuvant chemotherapy

To explore the potential importance of GPR120 in breast cancer chemoresistance, we firstly examined GPR120 expression in breast tumor tissues from patients who underwent preoperative neoadjuvant chemotherapy. Clinicopathologic characteristics of the 78 patients are summarized in Supplementary Table 3. According to the Response Evaluation Criteria in Solid Tumors (RECIST), 51 out of 78 patients achieved complete response (CR, disappearance of tumor) or partial response (PR, more than a 30% decrease) after chemotherapy and therefore were defined as clinical responders, while 27 out of 78 patients had stable disease (SD, less than a 30% decrease or less than a 20% increase) or progressive disease (PD, more than a 20% increase) and were defined as non-responders. We detected GPR120 expression in breast tumor tissues by immunohistochemistry (Fig. 1a) and found that patients with high GPR120 expression displayed a lower response rate to neoadjuvant chemotherapy than did patients with low GPR120 expression (51.43% vs. 76.74%, χ^2 test, $P = .019$, Fig. 1b). To validate our findings, we employed a publicly available breast cancer dataset GSE16446 with 109 patients undergoing epirubicin monotherapy as neoadjuvant chemotherapy [19] (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse16446). In this dataset, pathologic complete response (pCR) was the primary end point, and GPR120 mRNA expression in pre-epirubicin biopsies ranged from 2.90 to 5.29. The median value of 3.40 was selected as the cut-off. pCR was obtained in 20% of patients with relatively lower GPR120 expression and in 7.4% of patients with higher GPR120 expression (Fisher's exact test, $P = .093$, Supplementary Table 4 and Supplementary Fig. 1). Taken together, these results suggested that GPR120 expression was positively associated with poor response to neoadjuvant chemotherapy.

3.2. GPR120 promotes the development of chemoresistance

The above results prompted us to investigate the potential importance of GPR120 in breast cancer chemoresistance. To this end, we first examined GPR120 expression in a panel of human breast cancer cell lines including SK-BR-3, ZR-75-1, MCF-7 and T47-D. The results showed that GPR120 was expressed in all of these cancer cell lines. However, MCF-7 and T47-D cells displayed a relatively higher level of GPR120 (Fig. 2a) and were subsequently used for further investigations. First, we treated the cells with GW9508, an agonist of GPR120, to determine the roles of GPR120 in chemoresistance. As shown in Fig. 2c, GW9508-treated MCF-7 cells were relatively more resistant to different concentrations of epirubicin. Of note, we showed that the effect of GW9508 in promoting cell survival was significantly compromised in GPR120 knockdown MCF-7 cells, indicating that the chemoresistance effects exerted by agonists were dependent on GPR120 (Fig. 2b-c and Supplementary Fig. 2a). Since GW9508 could

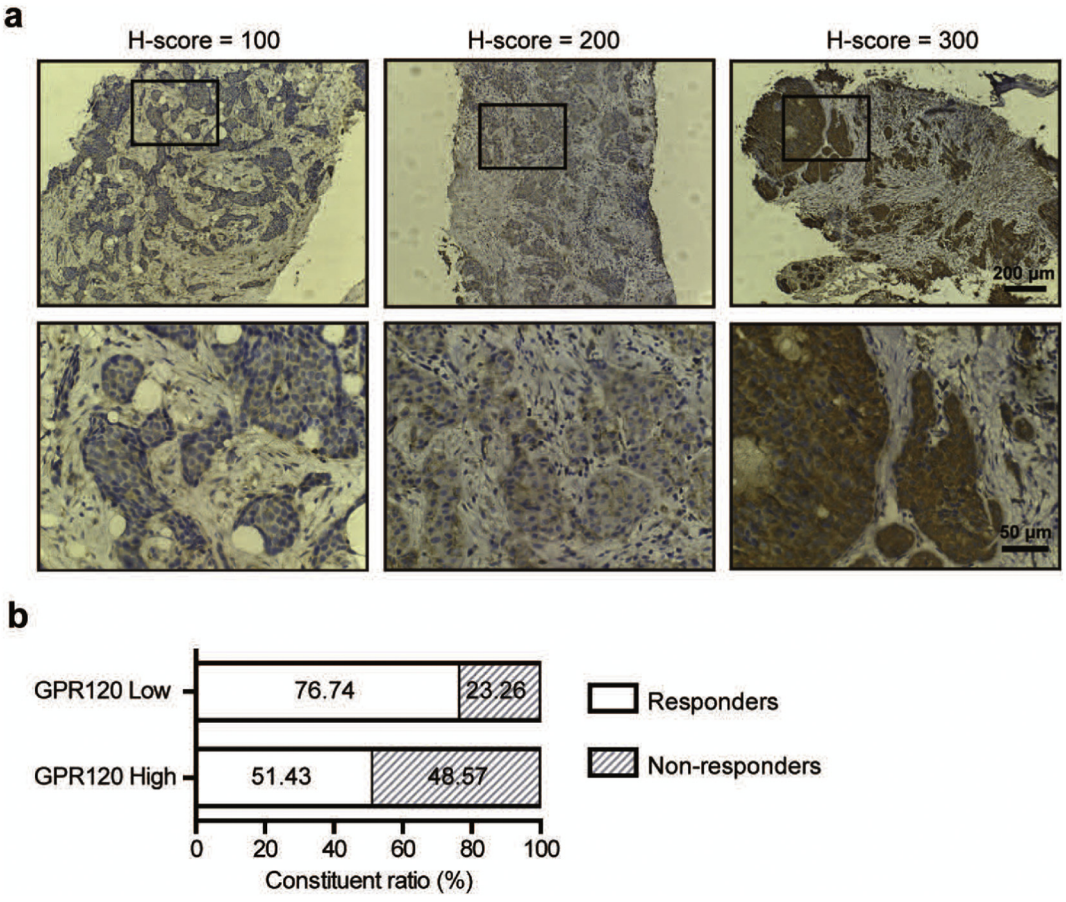

Fig. 1. GPR120 expression in tumor tissues of breast cancer patients. a, GPR120 expression in breast tumor tissues from patients was measured by immunohistochemistry. Representative images showing different expression levels were presented. b, Comparison of response in breast cancer patients with different levels of GPR120 expression.

also agonize GPR40, we utilized the more selective GPR120 agonist TUG891 to rule out the involvement of GPR40, and got the same conclusion with GW9508 (Supplementary Fig. 2b).

To further exclude the possibilities that GPR120 functions were cell type-specific, we utilized T47-D cells and showed that the activation of GPR120 made the cells resistant to epirubicin-induced apoptosis (Fig. 2d). Moreover, the effect of GW9508 was reversed when MCF-7 and T47-D cells were pretreated with the selective GPR120 antagonist AH7614 (Fig. 2e). In addition to epirubicin, GPR120 activation also promoted resistance to 5-FU-induced cell death in MCF-7 cells

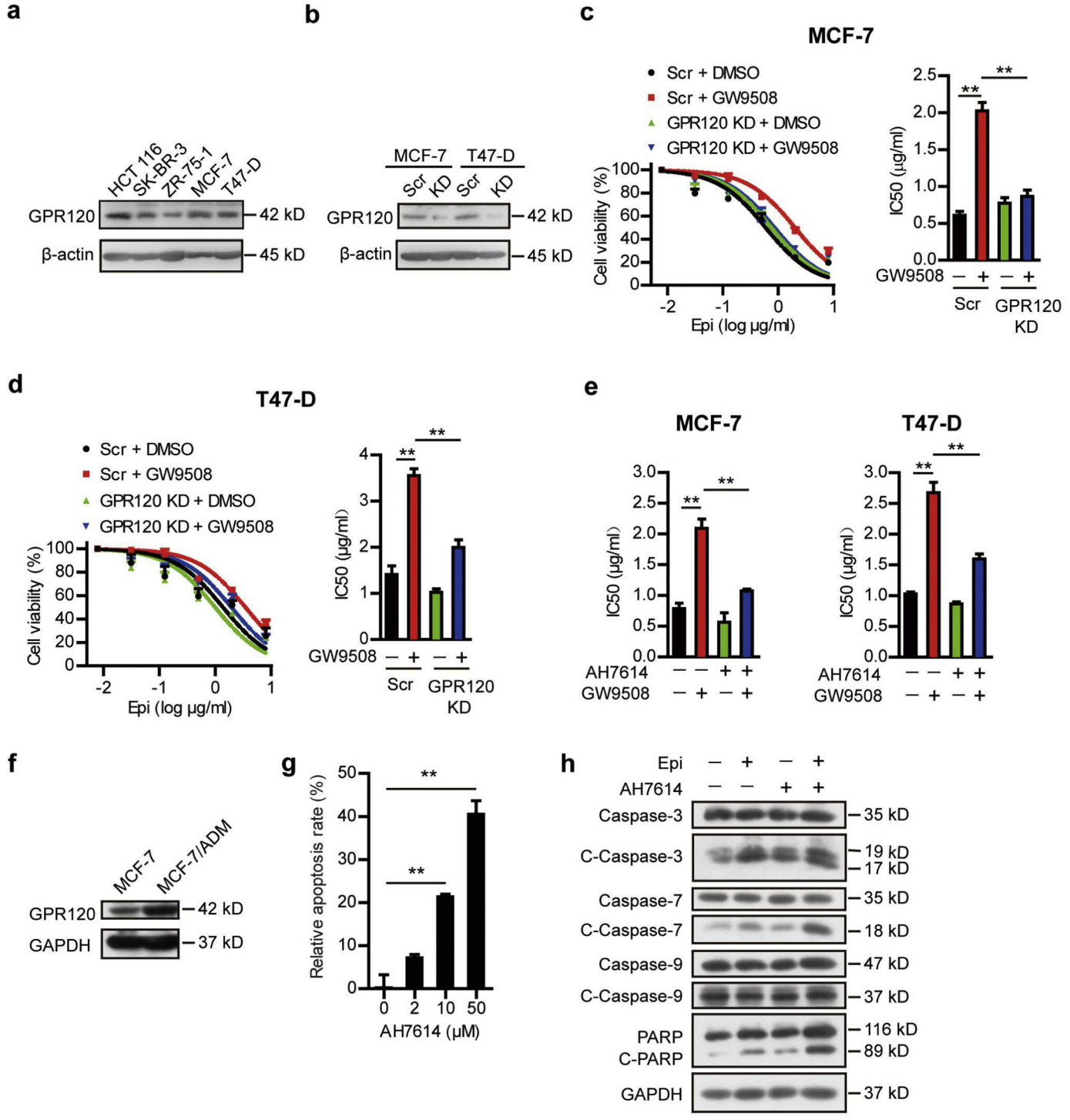

Fig. 2. GPR120 activation reduces the sensitivity of breast cancer cells to epirubicin. a, GPR120 expression in a panel of human breast cancer cell lines measured by western blotting and HCT116 cells as control. b, GPR120 expression in MCF-7 and T47-D cells transfected with shRNA targeting GPR120 or with negative control vector was evaluated by western blotting. c and d, MCF-7 and T47-D cells transfected with shRNA targeting GPR120 or with negative control vector were treated with GW9508 and different concentrations of epirubicin. Cell viability was evaluated by the WST-1 assay. Cell viability curves and IC50 values were presented. e, MCF-7 and T47-D cells were pretreated with the selective GPR120 antagonist AH7614 for 30 min and then with GW9508 and different concentrations of epirubicin. Cell viability was evaluated by the WST-1 assay, and IC50 values were presented. f, GPR120 expression in sensitive (MCF-7) and resistant (MCF-7/ADM) cells was evaluated by western blotting. g, Serum-starved MCF-7/ADM cells were treated with different concentrations of AH7614 for 48 h. Cell viability was evaluated by the WST-1 assay. h, MCF-7/ADM cells were treated with 20 μg/ml epirubicin or 50 μM AH7614 or a combination of both, and apoptosis-associated molecules were evaluated by western blotting. Values were displayed with mean ± SEM. Statistical analysis was carried out by one-way ANOVA. $^{**}P<.01$.

(Supplementary Fig. 2c). Conversely, we employed an adriamycin-resistant subline of MCF-7 (MCF-7/ADM), which was also resistant to many other anticancer drugs, including paclitaxel, epirubicin, vincristine, and mitoxantrone [20]. As determined by the values of IC50, MCF-7/ADM cells were more resistant to epirubicin than were MCF-7 cells (Supplementary Fig. 2d).

We measured the expression of GPR120 in MCF-7 and MCF-7/ADM cells at protein levels, and the results showed that MCF-7/ADM cells expressed higher levels of GPR120 (Fig. 2f). Importantly, blockade of GPR120 with AH7614 sensitized the cells to drug-induced apoptosis in MCF-7/ADM cells as evidenced by the results of the WST-1 assay (Fig. 2g) and caspase pathway analysis (Fig. 2h). These results collectively indicated that GPR120 promoted the development of chemoresistance in breast cancer cells.

3.3. Intracellular accumulation of epirubicin is reduced by GPR120 through the upregulation of the expression of ABC transporters

The effectiveness of chemotherapy treatment relies largely on intracellular drug concentrations. The chemotherapeutic drug epirubicin is autofluorescent, and its distribution and intensity in cells can therefore be determined through flow cytometry [21]. MCF-7 and MCF-7/ADM cells were treated with 5 μg/ml epirubicin for 24 h. Consistent with the results reported by others [20,22], MCF-7/ADM cells displayed lower mean fluorescence intensity than did MCF-7 cells (Supplementary Fig. 3a). As determined by the values of relative epirubicin fluorescence intensity, we found that GW9508 treatment profoundly reduced fluorescence intensity of intracellular epirubicin (Fig. 3a). In contrast, GPR120 knockdown or pretreament with its antagonist AH7614 in cells inhibited the reduction in epirubicin accumulation caused by GW9508 treatment, further validating that the role of GW9508 was dependent on GPR120 (Fig. 3a-b). Likewise, we showed that AH7614 treatment could also markedly enhance epirubicin accumulation in MCF-7/ADM cells (Supplementary Fig. 3b). Our data indicated that GPR120 promoted cell survival through the reduction of epirubicin accumulation in cells.

The overexpression of ABC transporters that pump out several chemotherapeutic drugs from cells has been shown to cause chemoresistance in many cancers [4]. MCF-7/ADM cells expressed a higher level of ABCB1, ABCC1, and ABCG2 than did MCF-7 cells (Supplementary Fig. 3c). Thus, we hypothesized that activating GPR120 signaling would increase the expression of ABC transporters and thus reduced the accumulation of cytotoxic drugs and protected cells from drug-induced death. As expected, activating GPR120 signaling with GW9508 dramatically induced the expression of ABC transporters, including ABCC1 and ABCG2 (Fig. 3c-d). To determine the functional dependence between GPR120 and ABC transporters, we treated MCF-7 cells simultaneously with PGP-4008, MK-571 or fumitremorgin C, the inhibitors of ABCB1, ABCC1, or ABCG2, respectively, either alone or in combination. As expected, we found that the effect of GW9508 on reducing the fluorescence intensity of intracellular epirubicin was significantly abrogated after treatment with the inhibitors (Fig. 3e-f). Collectively, our results demonstrated that activating GPR120 signaling reduced the intracellular accumulation of epirubicin mainly through the induction of expression of ABC transporters.

3.4. Akt/NF-κB pathway is involved in the regulation of ABC transporters expression by GPR120

We next explored the molecular mechanism by which GPR120 activation promoted the expression of ABC transporters. We examined the effects of GPR120 activation on candidate signaling pathways, including the Akt, NF-κB, ERK, JNK and p38 pathways [9,12]. Among the pathways examined, the Akt and NF-κB pathways were the most regulated in response to GPR120 activation. As shown by western blot analysis, phosphorylation of p65 at Ser536, IκBα and Akt at Thr308 and Ser473 was significantly increased in MCF-7 cells after GW9508 stimulation. This stimulatory effect of GW9508 was suppressed when MCF-7 cells were pretreated with GPR120-siRNA or the selective GPR120 antagonist AH7614 (Fig. 4a). Previous studies have suggested that ABCC1 and ABCG2 can be upregulated by NF-κB p65 [23,24]. Therefore, we measured whether GPR120 activation modulated the targeting or binding of p65 to the promoter regions of ABCC1 and ABCG2. Thus, luciferase reporter constructs with regions encompassing the ABCC1 promoter (−660 to +103) or ABCG2 promoter (−243 to +362) were transiently transfected into MCF-7 cells, and we showed that GW9508 stimulated the transcriptional activity of these promoters (Fig. 4b). Furthermore, ChIP-qPCR assays using anti-p65 antibody revealed that GW9508 stimulation promoted the recruitment of p65 to the gene locus with the ABCC1 or ABCG2 promoter region (Fig. 4c). To further confirm that GPR120 activation increased the expression of ABC transporters through the activation of the Akt/NF-κB pathway, we used the NF-κB inhibitor BAY11–7082 (10 μM) and the Akt inhibitor MK2206 (50 μM) to pretreat MCF-7 cells before GW9508 stimulation. As shown in Fig. 4d, pretreatment with the NF-κB inhibitor and Akt inhibitor significantly reduced the GW9508-induced increase in ABCC1 and ABCG2 expression. Consistently, the intracellular accumulation of epirubicin was increased when NF-κB and Akt were inhibited (Fig. 4e-f). Taken together, these results demonstrated that the Akt/NF-κB pathway played an important role in GPR120- mediated chemoresistance.

3.5. GPR120 promotes the de novo synthesis of fatty acids that serve as GPR120 ligands

FFAs are known to provide an important energy source as nutrients and act as messenger molecules in various cellular processes [7]. During this study, we serendipitously found that activating GPR120 signaling increased the level of fatty acids in MCF-7 cells both inside the cells (Fig. 5a-b) and in the culture supernatant (Fig. 5c-d). Further, GPR120-mediated fatty acid synthesis was mainly attributed to the GPR120-mediated induction of the expression of lipogenesis-associated genes, such as ACACA, FASN, SREBF1 and SREBF2 (Fig. 5e). We also detected the level of fatty acids in the same cell number of MCF-7 and MCF-7/ADM cells. The levels were significantly increased (Supplementary Fig. 4a), and FASN expression was elevated in MCF-7/ADM cells (Supplementary Fig. 4b).

Based on fatty acid profile, several fatty acids such as C14:0, C16:1 (ω7), C16:0, C18:1 (ω9), and C18:0, have been demonstrated to be GPR120 ligands [11]. It has been previously reported that GPR120 activation can increase intracellular Ca^{2+} levels [11]. To determine whether FFAs can activate GPR120, we examined the effects of C18:0 and C18:1 (ω9) on the Ca^{2+} response. C18:0 and C18:1 (ω9) induced an increase in Ca^{2+} concentration which was inhibited by GPR120-siRNA or the selective GPR120 antagonist AH7614 (Supplementary Fig. 4c). We treated MCF-7 cells with C18:0 or C18:1 (ω9) before the addition of epirubicin and found that C18:1 (ω9) treatment significantly reduced the fluorescence intensity of intracellular epirubicin (Fig. 5f). C18:1 (ω9) stimulation also activated the Akt/NF-κB pathway in MCF-7 cells, in a GPR120-dependent manner (Fig. 5g). Pretreatment with the Akt inhibitor significantly reduced the GW9508-induced increase in FASN expression (Fig. 5h). Collectively, these results suggested that GPR120 activation promoted fatty acid synthesis, which acted in an autocrine manner to enhance chemoresistance in breast cancer cells.

3.6. Targeted therapy against chemoresistance via GPR120 is feasible in breast cancer xenografts

To determine the role of GPR120 in chemoresistance, mice bearing MCF-7/ADM xenografts were treated with AH7614 or GPR120-siRNA. Injection of AH7614 or GPR120-siRNA at tumor sites reduced the

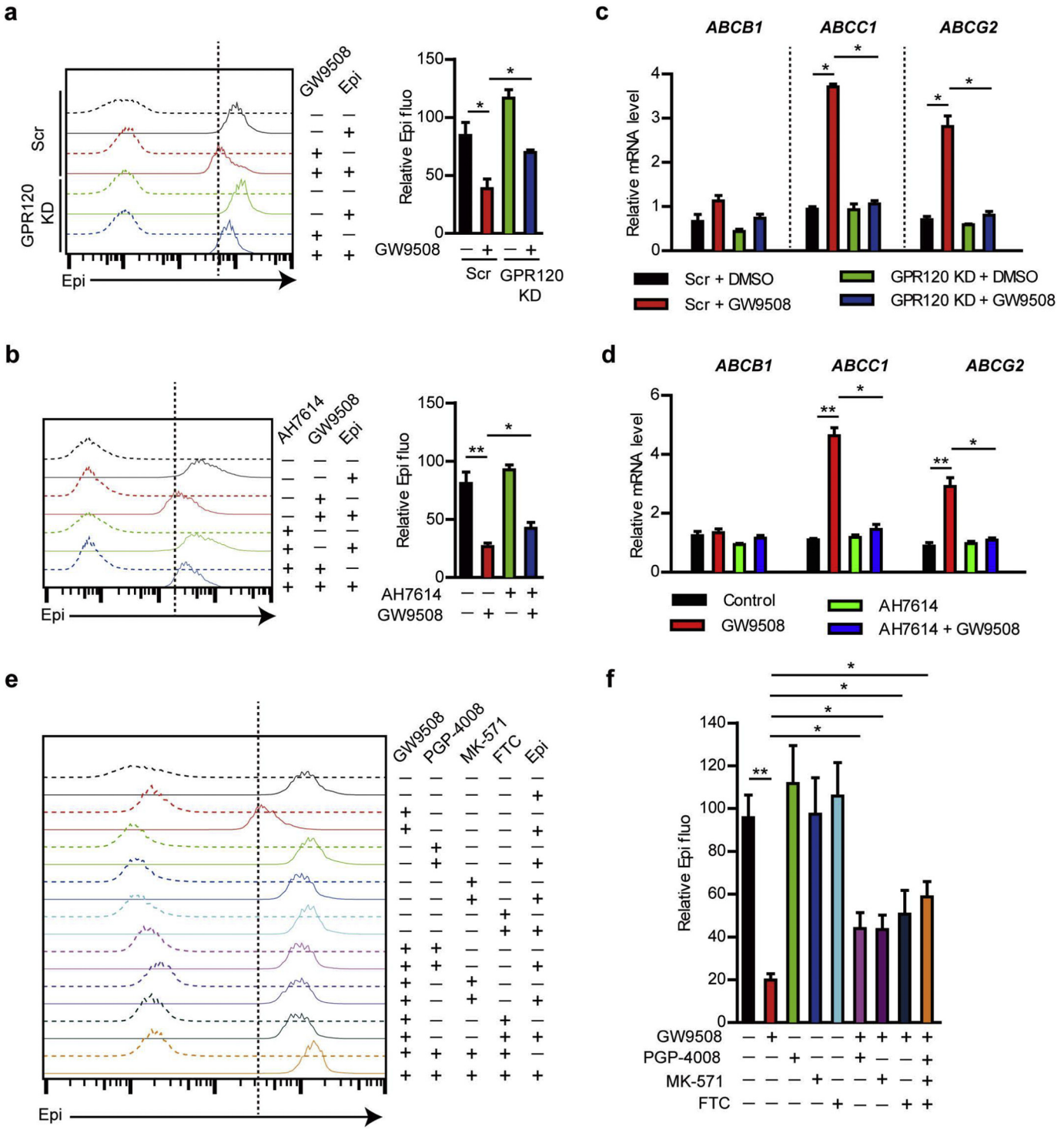

Fig. 3. Activation of GPR120 signaling reduces the intracellular accumulation of epirubicin and upregulates the expression of ABC transporters. a, MCF-7 cells transfected with shRNA targeting GPR120 or with negative control vector were pretreated with GW9508 for 24 h before the addition of 5 μg/ml epirubicin. Two hours after the addition of epirubicin, fluorescence intensity of intracellular epirubicin was evaluated by flow cytometry. Relative epirubicin fluorescence intensity was presented. b, MCF-7 cells were pretreated with 50 μM AH7614 for 30 min before the addition of GW9508. After 24 h, the cells were further treated with 5 μg/ml epirubicin for 2 h. Fluorescence intensity of intracellular epirubicin was evaluated by flow cytometry. Relative epirubicin fluorescence intensity was presented. c, MCF-7 cells transfected with shRNA targeting GPR120 or with negative control vector were treated with GW9508 for 24 h, and the cells were harvested for the analysis of ABC transporters (ABCB1, ABCC1, and ABCG2) mRNA expression. d, MCF-7 cells were pretreated with 50 μM AH7614 for 30 min before the addition of GW9508. After a 24 h stimulation, the cells were harvested for the analysis of ABC transporters (ABCB1, ABCC1, and ABCG2) mRNA expression. e and f, MCF-7 cells were simultaneously treated with GW9508 and 5 μM PGP-4008, an inhibitor of ABCB1, or 25 μM MK-571, an inhibitor of ABCC1, or 5 μM fumitremorgin C, an inhibitor of ABCG2, or the combination of the three inhibitors. After 24 h treatment, the cells were further treated with 5 μg/ml epirubicin for 2 h. Fluorescence intensity of intracellular epirubicin was evaluated by flow cytometry. Relative epirubicin fluorescence intensity was presented. Values were displayed with mean ± SEM. Statistical analysis was carried out by one-way ANOVA. $^{*}P < .05$, $^{**}P < .01$.

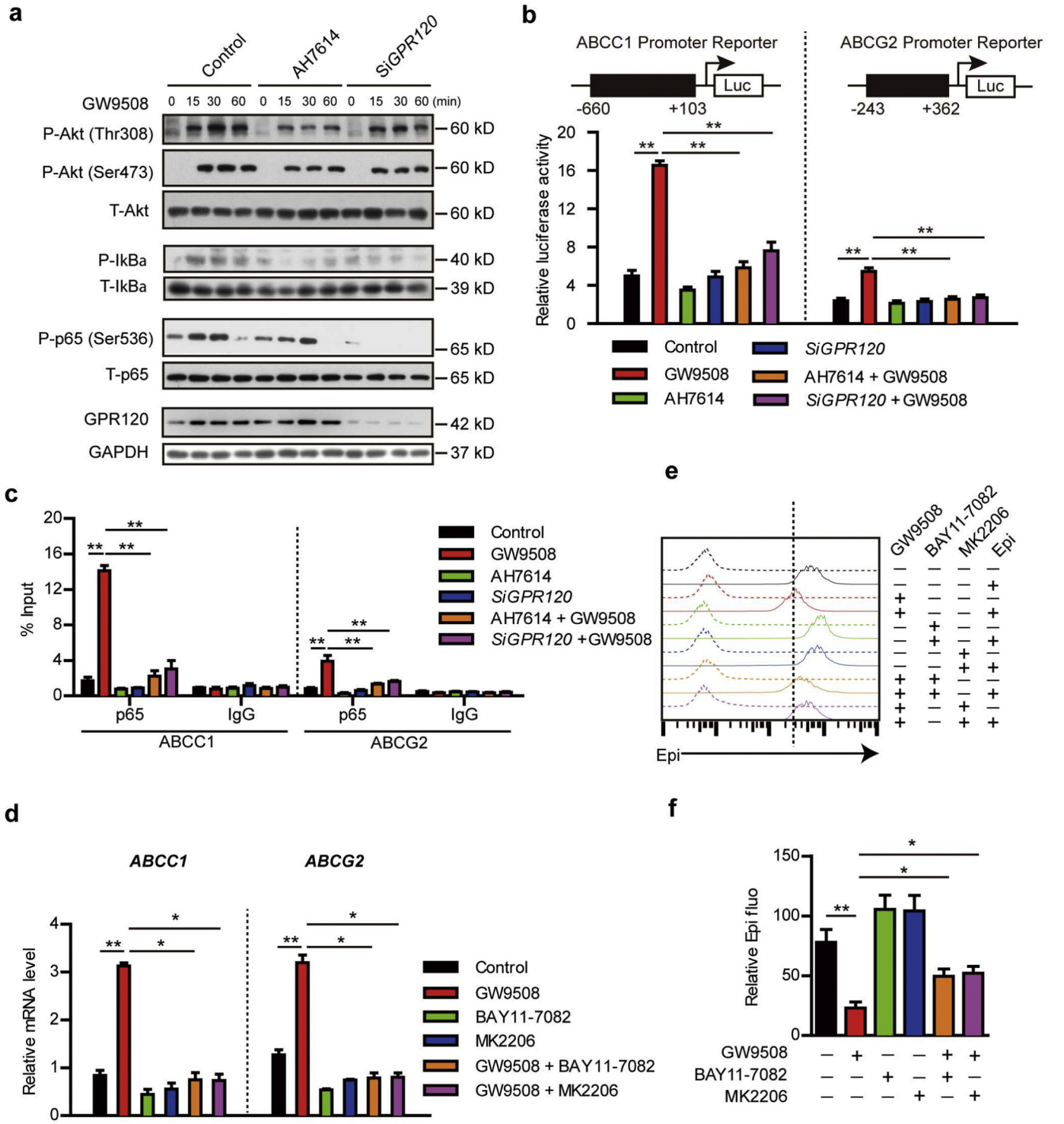

Fig. 4. Akt/NF-κB pathway is involved in the regulation of ABC transporters expression by GPR120. a, MCF-7 cells were pretreated with 50 μM AH7614 for 30 min or transfected with GPR120-siRNA 48 h in advance. Next, the cells were further treated with GW9508 for the indicated time (15, 30, or 60 min). Levels of signaling molecules were evaluated by western blotting. b, MCF-7 cells were transiently co-transfected with 2.5 μg of reporter constructs and 100 ng of the Renilla luciferase vector pRL-TK. Dual-Luciferase reporter assays were carried out following treatment with GW9508 for 1 h. c, MCF-7 cells were treated with GW9508 for 1 h. ChIP assay was performed, and p65 recruitment to the ABCC1 and ABCG2 regulatory regions was examined by real-time PCR. d, MCF-7 cells were simultaneously treated with GW9508 and BAY11–7082 or MK2206. After 24 h, the cells were harvested for the analysis of ABCC1 and ABCG2 mRNA expression. e and f, MCF-7 cells were simultaneously treated with GW9508 and BAY11–7082 or MK2206. After 24 h, the cells were further treated with 5 μg/ml epirubicin for 2 h. Fluorescence intensity of intracellular epirubicin was evaluated by flow cytometry. Relative epirubicin fluorescence intensity is presented. Values were displayed with mean ± SEM. Statistical analysis was carried out by one-way ANOVA. $^{*}P < .05$, $^{**}P < .01$.

tumor growth, and treatment with AH7614 or GPR120-siRNA in combination with epirubicin resulted in a further reduction of tumor growth (Fig. 6a). At the end of 20 days of treatment, the tumors were removed for the comparison of tumor size and tumor weight. Treatment with AH7614 or GPR120-siRNA in combination with epirubicin significantly reduced tumor size (Fig. 6b) and tumor weight (Fig. 6c). Western blot analysis revealed that AH7614 or GPR120-siRNA decreased ABCG2 and FASN expression in tumor tissues (Fig. 6d). Our

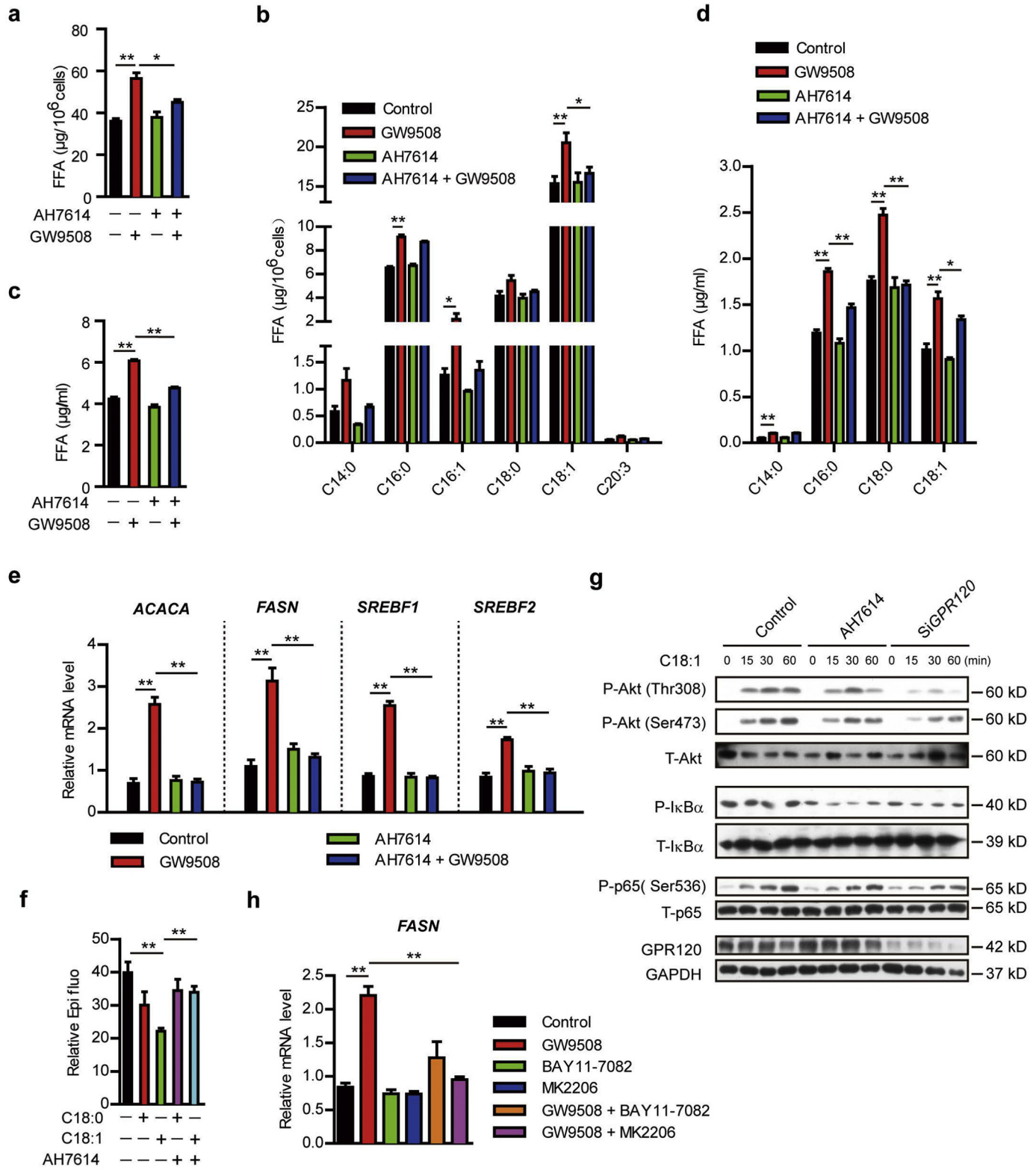

Fig. 5. GPR120 promotes the de novo synthesis of fatty acids that serve as GPR120 ligands. MCF-7 cells were pretreated with AH7614 for 30 min before the addition of GW9508. After 24 h, the cells or culture supernatants were harvested for the analysis of fatty acid components and lipogenesis-associated molecules. a and b, Fatty acid components in cells are presented. c and d, Fatty acid components in culture supernatants are presented. e, Expression of lipogenesis-associated molecules (ACACA, FASN, SREBF1, and SREBF2) was analyzed by quantitative real-time PCR. f, MCF-7 cells were treated with C18:0 or C18:1(ω9) for 24 h before the addition of 5 μg/ml epirubicin. Two hours after the addition of epirubicin, fluorescence intensity of intracellular epirubicin was evaluated by flow cytometry. g, MCF-7 cells were pretreated with AH7614 for 30 min or transfected with GPR120-siRNA 48 h in advance. Next, the cells were further treated with C18:1(ω9) for the indicated time (15, 30, or 60 min). Levels of signaling molecules were evaluated by western blotting. h, MCF-7 cells were simultaneously treated with GW9508 and BAY11–7082 or MK2206. After 24 h, the cells were harvested for the analysis FASN mRNA expression. Relative epirubicin fluorescence intensity is presented. Values were displayed with mean ± SEM. Statistical analysis was carried out by one-way ANOVA. $^{*}P < .05$, $^{**}P < .01$.

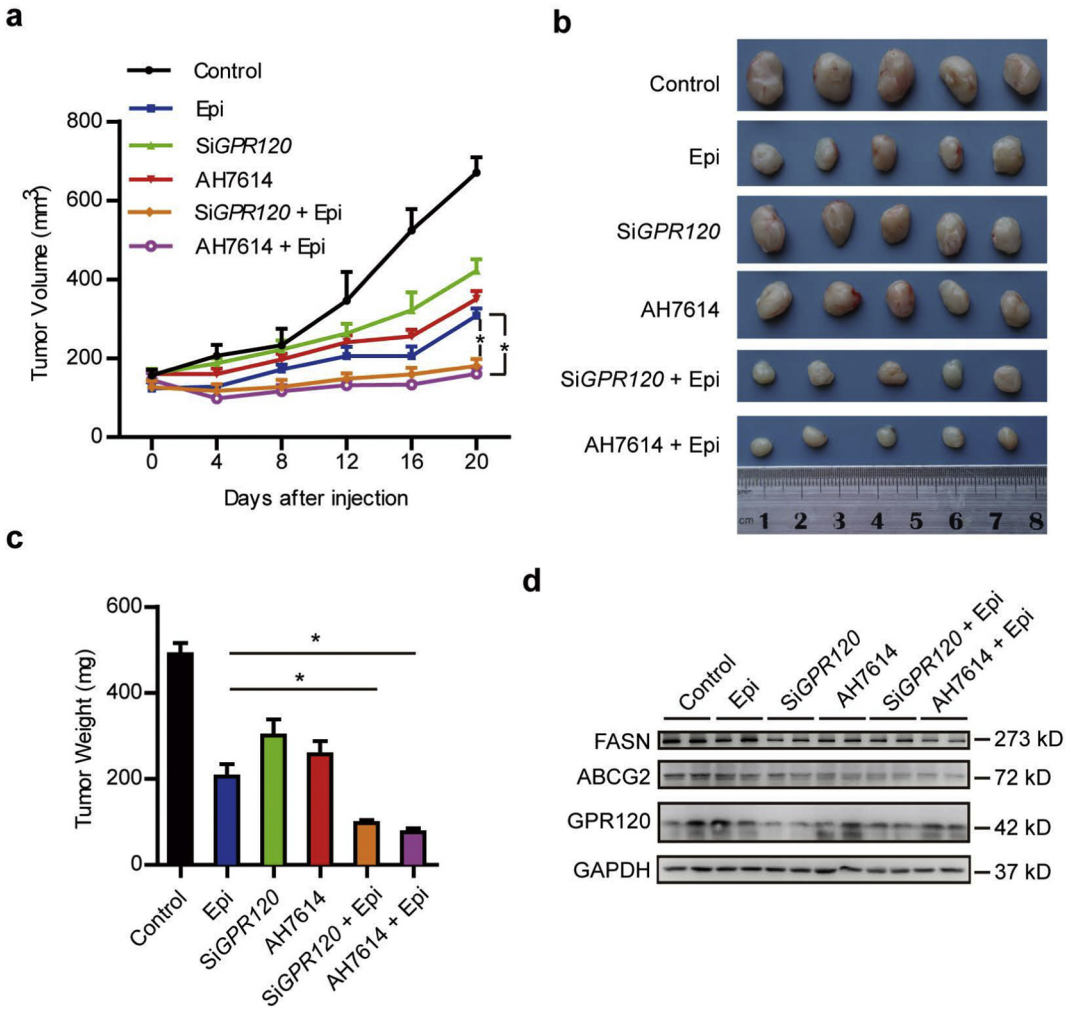

Fig. 6. Antitumor effect of GPR120 antagonists in MCF-7/ADM tumor xenografts. A xenograft model of human breast tumors was established by injecting MCF-7/ADM cells into BALB/c nude mice, and mice carrying xenograft tumors were subjected to different treatments. a, Tumor volumes over the 20 days of treatment are presented. b and c, Representative images and weights of harvested tumors after 20 days of treatment are displayed. d, Expression of GPR120, ABCG2 and FASN in tumor xenografts was evaluated by western blotting. Values were displayed with mean ± SEM. Statistical analysis was carried out by one-way ANOVA. $^{*}P < .05$, $^{**}P < .01$.

data suggested that GPR120 signaling blockade with AH7614 or GPR120-siRNA in combination with epirubicin could enhance cancer cell sensitivity to the chemotherapy and inhibit tumor progression.

3.7. GPR120 expression is correlated with ABCC1, ABCG2 and FASN expression in human breast cancer tissues

The above results demonstrated a causal link between GPR120 signaling and ABC transporter expression as well as fatty acid synthesis. Thus, we sought to determine whether GPR120 expression is correlated with the expression of ABCC1, ABCG2 and FASN, and the level of FFAs in patients. The data showed that high levels of GPR120 were associated with high levels of ABCC1, ABCG2, FASN and FFAs (Fig. 7a).

As summarized in Fig. 7b, our studies revealed that GPR120 activation induced chemoresistance in breast cancer cells. GPR120 activation could increase ABC transporters expression and fatty acid synthesis. In turn, fatty acids could promote GPR120 activation. Taken together, our data support a positive feedback loop between GPR120 activation and fatty acid synthesis in inducing chemoresistance in breast cancer cells.

4. Discussion

GPR120 functions as a receptor for long-chain fatty acids. Studies on GPR120 mainly focus on its potent anti-inflammatory and insulin-sensitizing effects [25], suggesting that selective GPR120 agonists could become new insulin-sensitizing drugs for the treatment of type 2 diabetes and other human insulin-resistant state-associated conditions in the future. However, our previous studies demonstrated that there is a significant association between GPR120 expression and tumor progression – the expression of GPR120 is increased in tumors with poor pathologic grade and advanced clinical stage [9]. Zhu S et al. reported that GPR120 protein levels were highly expressed in breast cancerous tissues compared to adjacent normal tissues and promoted breast cancer cell growth [26]. In the current study, we aimed to investigate whether GPR120 signaling can promote the development of chemoresistance in breast cancer and to explore the underlying mechanisms. Here, we identified GPR120 as a chemoresistance-promoting receptor in breast cancer cells that increases ABC transporters expression and fatty acid synthesis.

While mechanisms of chemoresistance are multifactorial, the reduction in intracellular drug accumulation is believed to be one of major causes [27]. Several cell membrane transporters have been linked to resistance to commonly used chemotherapeutics via the promotion of drug efflux. ABC transporters are the major transmembrane proteins that mediate active efflux of multiple structurally and mechanistically distinct chemotherapeutic agents [4]. Although there have been ongoing efforts to develop modulators that can either block or inactivate ABC transporters to increase the intracellular concentration of

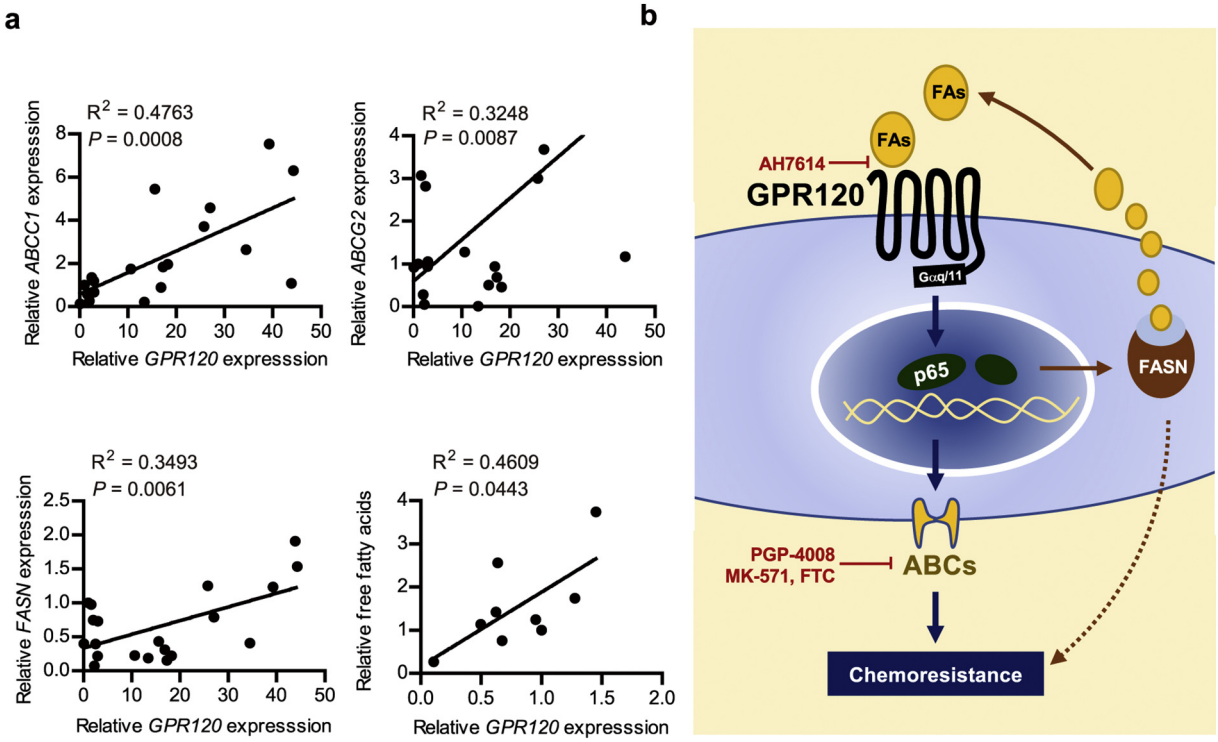

Fig. 7. GPR120 expression is correlated with ABCC1, ABCG2 and FASN expression in breast cancer tissues. a, Correlation analysis between GPR120 expression and ABCC1, ABCG2, and FASN expression at mRNA levels or FFA levels in human breast cancer tissues. b, Proposed mechanism by which GPR120 activation promotes the development of chemoresistance in breast cancer.

anticancer drugs, existing ABC transporters modulators in combination with standard chemotherapeutic drugs have not been approved by the Food and Drug Administration because of toxic effects or low efficacies. Therefore, it would be ideal to explore new compounds that directly block ABC transporters or suppress the expression of these transporters. Here, we found that the blockade of GPR120 signaling with AH7614 or GPR120-siRNA could reduce the expression of ABC transporters, including ABCC1 and ABCG2, and thus increase the concentration of epirubicin in MCF-7 cells.

High levels of fatty acids are common in breast tissues. Recently, a series of orphan GPCRs have been determined to be the receptors of FFAs. GPCRs that are activated by FFAs are categorized according to ligand profiles depending on the length of FFA carbon chains. Medium- and long-chain fatty acids activate GPR40 and GPR120, whereas short-chain fatty acids activate GPR41 and GPR43 [28,29]. It is important to explore the role of fatty acids. In addition to fatty acids of dietary origin, de novo synthesized fatty acids are a key component in the tumor microenvironment. In this study, we found that activating GPR120 signaling could increase the level of fatty acids in MCF-7 cells by inducing the expression of lipogenesis-associated molecules including ACACA, SREBF1, SREBF2, and FASN. Therefore, the increased lipid synthesis in cancer cells may change not only the quantity in cell membranes that is required for cell growth but also the lipid composition of the membrane. These changes in membrane composition may decrease the permeability of the membrane to anticancer drugs and thus the accumulation of these drugs in cells, thereby resulting in drug resistance [30]. These alterations may also increase the activity of ABC transporters [31], which causes drug resistance by actively pumping out anticancer drugs and hence effectively reducing cellular drug accumulation. Based on the fatty acid profile, several fatty acids, such as C14:0, C16:1(ω7), C16:0, C18:1 (ω9), and C18:0, can activate GPR120 signaling [11]. Our data suggest that the autocrine loop mediated by the interactions between fatty acids and GPR120 is involved in the development of chemoresistance.

Although we showed that GPR120 signaling promoted chemoresistance by increasing the expression of ABC transporters and thus decreasing the intracellular accumulation of epirubicin, it is possible that additional mechanisms of chemoresistance also exist. The blockade of GPR120 signaling increased apoptosis in serum-starved MCF-7/ADM cells (Fig. 2g-h) and inhibited tumor growth (Fig. 6c, Control vs. SiGPR120, one-way ANOVA, $^{**}P<0.01$, and Control vs. AH7614, one-way ANOVA, $^{**}P<0.01$). In addition, the increased levels of fatty acids owing to GPR120 activation promoted the development of chemoresistance, which was dependent on GPR120-irrelevant targets, such as GPR40 [32] and peroxisome proliferator-activated receptors (PPARs) [33].

The role of fatty acids, both saturated and unsaturated, in tumors remains controversial [34,35]. Using a GPR120 knockout mouse, H Chung et al. reported that ω-3 fatty acid-mediated antitumor effects were independent of GPR120 model [36]. Jeanine M.L. et al. found that in tumor-bearing mice, endogenous MSCs were activated during treatment with platinum analogs and secreted polyunsaturated fatty acids that protected tumor cells against a range of chemotherapeutic agents [37]. Follow-up studies uncovered that polyunsaturated fatty acids could stimulate a GPR120-induced signaling cascade in splenic macrophages and enhance PLA2-mediated generation and release of a specific isoform of lysophosphatidylcholine to promote chemotherapy resistance [38,39]. Various targets and pathways for fatty acids, including GPR40 [40], PPARs [33], and the Hippo pathway [41], have been investigated and these differences in pathways and targets may account for inconsistencies in the results from different studies.

In conclusion, we established that GPR120 functions as a chemoresistance-promoting receptor in breast cancer. The activation of GPR120 signaling using its ligands in breast cancer cells promoted the resistance against epirubicin-induced cell death by increasing ABC transporter expression and de novo fatty acid synthesis. This suggests that GPR120 might be a promising pharmaceutical target for the treatment of breast cancer chemoresistance, and targeting GPR120 in combination with chemotherapy may overcome breast cancer chemoresistance.

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Declaration of interests

The authors declare that there is no conflict of interest.

Author contributions

Xue Wang, Yanyun Zhang, Hui Wang and Leizhen Zheng designed the research. Xue Wang and Songbing He performed the experiments and wrote the paper. Yuting Gu, Xiao Chu, Min Jin, and Qiwei Wang assisted with experiments and data analysis. Liang Xu, Qiong Wu, Qianjun Zhou, and Bei Wang analyzed clinical samples. Yanyun Zhang, Hui Wang and Leizhen Zheng supervised experiments. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.12.037.

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