A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction

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Abstract

Multidrug resistance is a major problem in successful cancer chemotherapy. Various mechanisms of resistance, such as ABC transporter-mediated drug efflux, have been discovered using established model cancer cell lines. While characterizing a drug-resistant breast cancer cell line, MCF7/AdVp3000, we found that fatty acid synthase (FASN) is overexpressed. In this study, we showed that ectopic overexpression of FASN indeed causes drug resistance and that reducing the FASN expression increased the drug sensitivity in breast cancer cell lines MCF7 and MDA-MB-468 but not in the normal mammary epithelial cell line MCF10A1. Use of FASN inhibitor, Orlistat, at low concentrations also sensitized cells with FASN overexpression to anticancer drugs. The FASN-mediated drug resistance appears to be due to a decrease in drug-induced apoptosis from an overproduction of palmitic acid by FASN. Together with previous findings of FASN as a poor prognosis marker for breast cancer patients, our results suggest that FASN overexpression is a new mechanism of drug resistance and may be an ideal target for chemosensitization in breast cancer chemotherapy. [Mol Cancer Ther 2008;7(2):263–70]

Introduction

Breast cancer is the second leading cause of cancer death for women worldwide. One of the major challenges for a better prognosis of breast cancer patients is the ineffectiveness of chemotherapy or the problem of drug resistance. To investigate the mechanisms of drug resistance, many drug-resistant cancer cell lines have been established by selecting cancer cells with various anticancer agents. One series of such drug-resistant breast cancer cell lines was derived from MCF7 by stepwise selections using increasing concentrations of Adriamycin in the presence of verapamil (1, 2). The final product, MCF7/AdVp3000, showed an extremely high level of resistance, and the overexpression of ABCG2, a member of the ABC transporter family, was thought to be the mechanism of drug resistance in this cell line (3, 4). However, a recent genomic profiling of ABC transporters (5) and a proteomic analysis (6) showed that the drug-selected MCF7/AdVp3000 cells overexpress other ABC transporters, such as ABCC3, as well as numerous other proteins, including 14-3-3σ, which also contribute to the drug resistance of MCF7/AdVp3000 cells.

During our search for potential candidates that are in part responsible for the drug resistance phenotype of MCF7/AdVp3000 cells, we accidentally found that fatty acid synthase (FASN) was overexpressed in MCF7/AdVp3000 cells. In this study, we showed that the elevated FASN expression functionally contributes to increased drug resistance levels to Adriamycin and mitoxantrone not only in MCF7/AdVp3000 cells but also in the breast cancer cell line MDA-MB-468. These results are consistent with the clinical observation that breast cancer patients with a higher level of FASN had poorer prognosis compared with the ones with lower levels (7–9). We also found that selective inhibition of FASN expression in normal breast epithelial cell line MCF10A1 did not affect the drug response level. These findings led us to conclude that FASN overexpression likely causes drug resistance and may be an ideal chemosensitization target for combinational therapy in the treatment of drug-resistant breast cancers.

Materials and Methods

Materials

All electrophoresis reagents, precast slab gels, and polyvinylidene fluoride membranes were from Bio-Rad. Adriamycin, mitoxantrone, verapamil, sulforhodamine B (SRB), and DTT were from Sigma. FuGene 6 and Orlistat were from Roche Applied Sciences. Cell culture media IMEM, Opti-MEM, DMEM, DMEM/F-12 (50:50), and typsin-versene mixture were from BioSources International, Media Tech, or Cambrex. Monoclonal antibody against FASN and anti–poly(ADP-ribose) polymerase (PARP) antibody were from BD Biosciences. LipofectAMINE 2000 reagents and G418 were purchased from Invitrogen. All other chemicals of molecular biology grade were from Sigma or Fisher Scientific.

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Cell Cultures and Transfection

Human breast cancer cell line MCF7 and its drug-resistant derivative cell lines MCF7/AdVp10, MCF7/AdVp100, and MCF7/AdVp3000 were cultured as described previously (1, 2, 6, 10). To maintain the drug resistance phenotype of MCF7/AdVp10, MCF7/AdVp100, and MCF7/AdVp3000 cells, 10, 100, and 3,000 ng/mL Adriamycin were included, respectively, together with 5 μg/mL verapamil. The MDA-MB-468 cell line was cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL strepto-mycin. MCF10A1 cells were cultured in DMEM/F-12 (50:50) with 10% equine serum, 10 μg/mL insulin, 25 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, and 100 ng/mL cholera toxin.

To establish MCF7/AdVp3000 stable cell clones with reduced FASN expression, a plasmid that expresses a short hairpin RNA (shRNA) targeting FASN with a sequence of AACCCTGAGATCCCAGCGCTG or a scrambled control sequence was transfected into MCF7/AdVp3000 cells using FuGene 6 transfection reagents. Twenty-four hours following transfection, the cells were subjected to G418 selection at 800 μg/mL for 2 weeks. Individual stable clones selected were expanded for further analyses.

To establish MCF7 stable clones with FASN overexpression, the cDNA encoding human FASN was engineered into pcDNA3 and transfected into MCF7 cells using FuGene 6. Two days following transfection, 10% of the transfected cells were replated and selected with 800 μg/mL G418 for 2 weeks, and stable clones were propagated for further analysis.

Small Interfering RNA Preparation and Transfection

Small interfering RNA (siRNA) targeting FASN (AACCCTGAGATCCCAGCGCTG) and the negative control siRNA with scrambled sequence (scrambled siRNA) were synthesized by Ambion as described previously (11). For siRNA transfection, 5×10^5 MDA-MB-468 cells or MCF10A1 cells were plated in six-well plate for 24 h followed by transfection with siRNAs using Lipofect-AMINE 2000 reagent as described previously (5, 6, 12). Briefly, 5 μL LipofectAMINE 2000 was diluted with 100 μL Opti-MEM and incubated at room temperature for 5 min. siRNA (200 pmol) was added to 100 μL Opti-MEM and then mixed with the diluted LipofectAMINE 2000 reagent followed by incubation at room temperature for 25 min. The siRNA-LipofectAMINE 2000 reagent complex was added drop-wise into the culture containing 0.8 mL fresh medium. At different days following transfection, cell lysates were prepared for FASN detection.

Cell Lysate Preparation and Western Blot Analysis

Cells were washed with PBS and then lysed in a buffer [1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.1% SDS] for 30 min at 4°C with occasional agitation. The cell lysates were sonicated briefly followed by centrifugation (16,000 × g, 4°C) for 15 min to remove insoluble materials. The protein concentration of cell lysates was determined using a Bio-Rad protein assay kit.

Western blot analysis was done as described previously (13–15). Briefly, cell lysates were separated by SDS-PAGE followed by transfer to a polyvinylidene fluoride membrane. The blot was then probed with an antibody to FASN or PARP followed by reaction with horseradish peroxidase–conjugated secondary antibodies. The signal was captured by X-ray films using enhanced chemiluminescence.

FASN Activity Assay

FASN activity was determined using a protocol as described previously (16). Briefly, 96 μg particle-free supernatant of cell lysate was mixed with a buffer containing 200 mmol/L potassium phosphate (pH 6.6), 1 mmol/L DTT, 1 mmol/L EDTA, 0.24 mmol/L NADPH, and 30 μmol/L acetyl-CoA in a final volume of 0.2 mL and the reaction was monitored at 340 nm for 3 min to measure background NADPH oxidation. After the addition of 50 μmol/L malonyl-CoA, the reaction was assayed for an additional 15 min to determine the FASN-dependent oxidation of NADPH. The rates of $A_{340\ nm}$ change were corrected for the background rate of NADPH oxidation. FASN activity was expressed as nmol NADPH oxidized/min/mg protein.

Cytotoxicity Assay

The cytotoxicity of anticancer drugs was determined using the SRB colorimetric assay as described previously (6). Briefly, cells were seeded in 96-well plates and cultured for 24 h before drugs were added and cultured continuously until the control cells become confluent before the SRB assay. For the study of palmitic acid effect, anticancer drugs were added to the cells after incubation with 100 μmol/L palmitic acid for 24 h as described previously (17). For the study of Orlistat effect, Adriamycin was added to the cell in the absence or presence of 60 μmol/L Orlistat and cells were then incubated at 37°C for 3 days before the SRB assay (18). For the SRB assay, the culture medium was removed and the cells were fixed and stained by addition of 0.4% (w/v) SRB in 1% acetic acid solution and incubated at room temperature for 20 min. The plates were then washed three to five times with 1% acetic acid and then air-dried at room temperature. The bound SRB was then solubilized with 10 mmol/L Tris, and the $A_{570\ nm}$ was determined using a 96-well plate reader (MRX; Dynex Technologies). IC$_{50}$ was defined as the concentration of drugs required to kill 50% of the cells relative to the control condition without drugs.

Cell Growth Analysis

Cell growth rate was determined as described previously (19). Briefly, MCF7 and its derivative stable clones over-expressing FASN were plated at 150 cells per well, whereas MCF7/AdVp3000 and its derivative stable clones with reduced FASN expression were plated at 250 cells per well in triplicate in 96 wells. Plates were collected every other day followed by the removal of the medium and the fixation of cells with 100 μL/well of 1% glutaraldehyde for 30 min at room temperature. The plates were then washed thrice with 200 μL PBS and air-dried followed by the addition of 100 μL/well of crystal violet solution (0.2%

crystal violet in 20% methanol), incubated for 30 min at room temperature, washed extensively with water, and air-dried again. To each well of the air-dried plates, 10% acetic acid (200 μL) was added and incubated for 1 h with shaking. Finally, $A_{570\ nm}$ was determined.

Drug Accumulation Assay

The drug accumulation assay was done as described previously with modifications (20). Briefly, 5×10^5 cells were resuspended in 0.5 mL PBS with 20 μmol/L mitoxantrone and incubated at 37°C for 30 min. Cells were then washed twice with PBS followed by analysis using flow cytometry on a Becton-Dickinson FACSCalibur. The data were then analyzed using Cell Quest Pro (BD Biosciences).

Results

Identification of FASN as an Overexpressed Protein Correlative to Drug Resistance in Breast Cancer Cells

While performing SDS-PAGE analyses comparing protein profiles between the parental MCF7 and its drug-resistant derivative MCF7/AdVp3000 cells, we found an overexpressed protein of ~260 kDa in the drug-resistant MCF7/AdVp3000 cells (Fig. 1A). This protein band was exercised from the gel and subjected to analysis using matrix-assisted laser desorption/ionization-time-of-flight

Figure 1. Overexpression of FASN in drug-resistant MCF7 cells. **A,** SDS-PAGE profile of cell lysates of MCF7 and its drug-resistant derivative MCF7/AdVp3000 cells. Proteins (20 μg), each from MCF7 and MCF7/AdVp3000 cells, were separated by a 4% to 15% gradient precast SDS-PAGE and stained with Coomassie blue. *Arrowhead,* a high molecular weight protein overexpressed in MCF7/AdrVp3000 cells, which was identified as FASN by mass spectrometry (access no. G01880). **B,** FASN activity in MCF7 and MCF7/AdVp3000 cells. FASN activity was determined as described in Materials and Methods ($n = 5$; $P = 0.0001$). **C,** FASN expression in MCF7 and the stepwise-selected drug-resistant derivative cell lines. Proteins (20 μg), each from MCF7, MCF7/AdVp10, MCF7/AdVp100, and MCF7/AdVp3000 cells, were separated by SDS-PAGE followed by Western blot analyses using FASN antibody. GAPDH was used as a loading control. The relative level of FASN was determined by measuring the intensity of the FASN band using an online software Scion image.

mass spectrometry and peptide matching/protein searching of the National Center for Biotechnology Information database using the ProFound search engine. The only likely matching candidate was found to be human FASN with a similar molecular weight (275 kDa), a satisfying Z score (1.69), and 5% peptide coverage. The next candidate, ELKS protein, in the database was too small in size (109 kDa) with too low a Z score (1.06) for positive identification.

To verify that functional FASN is overexpressed in the drug-resistant MCF7/AdVp3000 cells, we determined FASN activity in MCF7 and MCF7/AdVp3000 cells. As shown in Fig. 1B, the FASN activity in MCF7/AdVp3000 cells was increased ~5-fold compared with that in MCF7 cells. Next, we determined the FASN expression level using a Western blot. As shown in Fig. 1C, the FASN level in MCF7/AdVp3000 cells is ~2-fold higher than that in the MCF7 cells.

To determine if the elevated FASN expression potentially contributes to the drug resistance of MCF7/AdVp3000 cells, we did a correlative analysis of FASN expression with drug resistance. During the stepwise selection used to generate MCF7/AdVp3000 cells, other MCF7 derivatives (MCF7/AdVp10 and MCF7/AdVp100) with low and intermediate drug resistance levels, respectively, were also generated (1, 2, 10). As shown in Fig. 1C, the FASN protein level increased as the drug resistance level of the MCF7 derivative cells increased. These results suggest that the elevated FASN expression may contribute to the increased drug resistance in the drug-selected MCF7 cell lines.

FASN Overexpression Causes Drug Resistance in Breast Cancer Cells

To determine if FASN overexpression causes drug resistance, we first determined if the elevated FASN expression contributes to the drug resistance phenotype of MCF7/AdVp3000 cells. For this purpose, FASN expression in MCF7/AdVp3000 cells was knocked down using shRNA followed by analysis of changes in drug resistance using a SRB assay. A hairpin DNA segment targeting FASN was cloned into pRNA-U6.1/Neo vector (GeneScript), which is expected to constitutively express FASN shRNA. The FASN shRNA construct was then transfected into MCF7/AdVp3000 cells followed by selection of stable clones and analysis of FASN expression using Western blot. As shown in Fig. 2A, the FASN expression was decreased in two independent stable clones (Si18 and Si19) compared with the control clone (Scr) transfected with scrambled shRNA. Analysis of these clones for their responses to anticancer drug treatment showed that knocking down FASN expression significantly decreased the resistance level of MCF7/AdVp3000 cells to both Adriamycin and mitoxantrone (Fig. 2B).

Next, we did a reverse experiment to further investigate the role of FASN in drug resistance. The parental MCF7 cells were transfected with FASN cDNA for ectopic overexpression. Stable clones were established for analyses of FASN expression and drug resistance. As shown in Fig. 2C, two table clones (F3 and F20) clearly have a higher level of FASN compared with the control vector-transfected
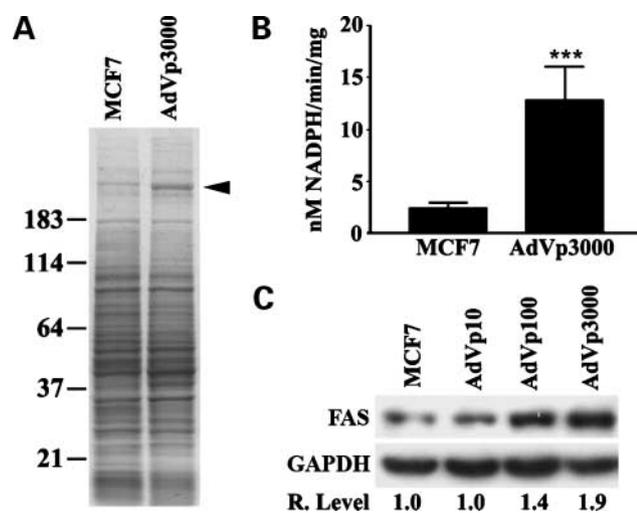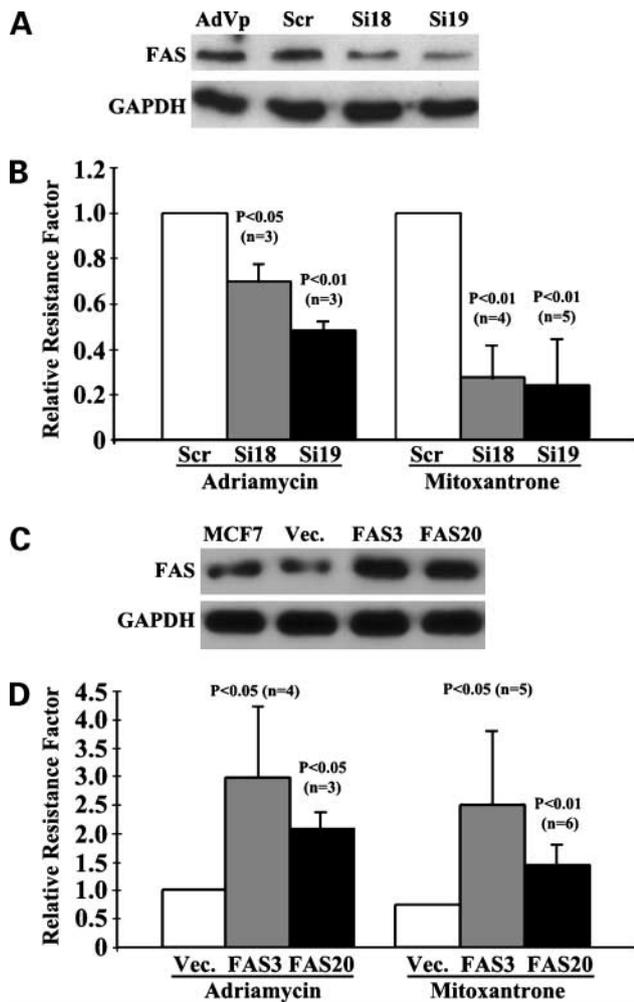
Figure 2. Effect of altering FASN expression on cellular responses to anticancer drugs. **A** and **C**, Western blot analyses of FASN expression. Drug-resistant MCF7/AdVp3000 cells were stably transfected with shRNA targeting FASN for silencing with scrambled siRNA-transfected cells as a control (**A**) and the parental sensitive MCF cells were stably transfected with FASN cDNA for overexpression with vector-transfected cells as a control (**C**). Cell lysates from two stable clones harboring FASN shRNA (*Si18* and *Si19*), two stable clones for FASN overexpression (*F3* and *F20*), a control stable clone harboring a shRNA with scrambled sequence (*Scr*), and a control stable clone harboring vector (*Vec.*) were analyzed for expression of FASN using Western blot. GAPDH was used as a loading control. **B** and **D**, effect on drug resistance. The stable clones with reduced or enforced FASN expression were tested for their response to Adriamycin or mitoxantrone using SRB assay.

MCF7 cells. As shown in Fig. 2D, the stable clones with FASN overexpression are also significantly more resistant to Adriamycin and mitoxantrone than the vector-transfected MCF7 control cells. Taken together, we conclude that the increased FASN expression in MCF7/AdVp3000 cells likely contributes to the drug resistance phenotype of this cell line.

To rule out the possibility that the FASN-mediated drug resistance is specific to MCF7 cells, we did a similar FASN knockdown experiment using MDA-MB-468 breast cancer cells, which express a high level of endogenous FASN. As shown in Fig. 3A, the transient FASN knockdown in MDA-MB-468 cells using siRNA persisted for 8 days. The effect of FASN knockdown on drug response of MDA-MB-468 cells was determined next using SRB assay. As shown in Fig. 3B, MDA-MB-468 cells transfected with FASN siRNA are much more sensitive to Adriamycin and mitoxantrone than the cells transfected with scrambled siRNA controls. Thus, FASN overexpression-mediated drug resistance is not restricted to MCF7 cells.

To determine if similar effects of FASN expression on drug responses would be observed with noncancerous mammary epithelial cells, we did a similar knockdown experiment using human normal breast epithelial cell line MCF10A1. As shown in Fig. 3C, FASN siRNA was able to knockdown FASN expression in MCF10A1 cells, which persisted for at least 6 days after transfection. However, the drug responses of siRNA-transfected MCF10A1 cells had no significant difference compared with the control cells transfected with scrambled siRNAs (Fig. 3D). This observation is interesting and indicates that FASN may be used as a target for chemosensitization of breast cancer cells without any effect on normal mammary epithelial cells.

To corroborate the above claims, we tested if a FASN inhibitor, Orlistat (18), can be used to reverse FASN-mediated drug resistance. For this purpose, MCF7 stable clones with FASN overexpression were tested for their response to Adriamycin and mitoxantrone in the presence of 60 μmol/L Orlistat, at which concentration no Orlistat toxicity was observed. As shown in Fig. 4, Orlistat was able to reverse FASN-mediated drug resistance in both MCF7 stable clones with FASN overexpression. Thus, FASN inhibitors may be developed as sensitizers in combinational chemotherapy of drug-resistant human cancers.

FASN Expression and Intracellular Drug Accumulation

To understand the mechanism of FASN-mediated drug resistance, we first tested if FASN overexpression affects drug permeability and if it affects ABC transporter-mediated decreases in drug accumulation due to its likely effect on lipid composition of cell membranes. To determine if FASN expression level affects membrane permeability and thus drug accumulation, we compared mitoxantrone accumulation between MCF7 cells with stable overexpression of ectopic FASN and control cells transfected with vector alone. As shown in Fig. 5A, there is no difference in mitoxantrone accumulation between these cells. Thus, FASN overexpression does not affect membrane permeability to mitoxantrone. To determine if FASN expression level affects ABC transporter-mediated decreases in drug accumulation, we took advantage of MCF7/AdVp3000 cells, which were known to overexpress several ABC transporters, including ABCG2 (5), and its derivative stable clones with reduced FASN expression. As shown in Fig. 5B, knocking down FASN expression did not affect mitoxantrone accumulation in MCF7/AdVp3000 cells that overexpress ABCG2 and other ABC transporters, indicating that FASN did not affect the ABC transporter

activity in these cells. We also found that FASN over-expression did not affect cellular responses to anticancer drugs paclitaxel and vinblastine, substrates of multidrug-resistant ABC transporters (data not shown). This finding further corroborates with the conclusion that FASN expression likely does not affect ABC transporter activity.

FASN Expression and Cell Proliferation

Another possible mechanism of FASN-mediated drug resistance is that FASN expression may affect cell growth rate. To test this possibility, we determined the cell growth rate among MCF7-derived stable cell clones compared with their respective controls. As shown in Fig. 5C and D, ectopic overexpression or knocking down FASN expression
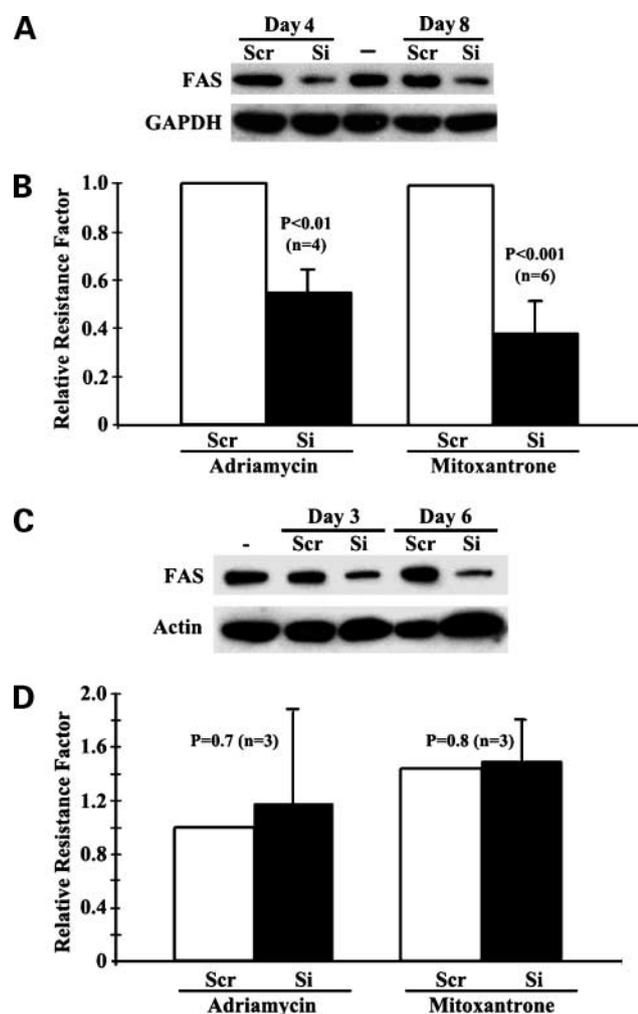

Figure 4. Effect of FASN inhibitor, Orlistat, on FASN-mediated drug resistance. Two stable MCF7 clones with overexpression of FASN were treated with Adriamycin in the absence or presence of 60 μmol/L Orlistat for 3 d followed by SRB assay. Orlistat significantly reduced the resistance of these cells to Adriamycin.

does not affect the growth rate of these cells. Thus, the drug responses mediated by FASN overexpression are unlikely to occur by affecting cell growth rate.

FASN Overexpression Causes Resistance to Drug-Induced Apoptosis Possibly by Overproducing Palmitic Acid

To determine if the overexpression of FASN causes cellular resistance to drug-induced apoptosis, we analyzed cleavage of PARP, a 115-kDa protein substrate of caspases during execution of apoptosis. The cleavage of PARP by caspases yields an 85-kDa fragment. As shown in Fig. 6A, the 85-kDa PARP cleavage product was produced in the vector-transfected MCF7 control cells following treatment with 10 μmol/L Adriamycin for 48 h. However, in three stable MCF7 clones that overexpress FASN, the drug-induced PARP cleavage was inhibited, indicating that these cells with FASN overexpression are resistant to drug-induced apoptosis.

Because FASN overexpression likely causes overproduction of palmitate, we wondered if simply increasing the cellular palmitate level would increase the level of drug resistance. To test this hypothesis, we treated MCF7 cells with or without palmitate and tested their responses to Adriamycin and mitoxantrone using SRB assay. As shown in Fig. 6B, the palmitate-treated cells are significantly more resistant to both Adriamycin and mitoxantrone compared with the vehicle-treated control cells. However, the degree of increase in resistance is less than that induced by FASN overexpression (30-40% increase compared with 2- to 3-fold increase). This limited increase in resistance by palmitate may be due to difficulties in delivering palmitate into cells or due to other unknown mechanisms involved.

Figure 3. FASN silencing and its effect on drug response of MDA-MB-468 and MCF10A1 cells. **A** and **C**, Western blot analysis of FASN expression. MDA-MB-468 (**A**) and MCF10A1 (**C**) cells were transiently transfected with FASN siRNA (*Si*) or a negative control scrambled siRNA (*Scr*) followed by preparation of cell lysates at various days after transfection for Western blot analyses. **B** and **D**, effect of FASN silencing on drug resistance. MDA-MB-468 (**B**) and MCF10A1 (**D**) cells transfected with FASN siRNA or control scrambled siRNA were tested for their resistance to Adriamycin or mitoxantrone using SRB assay.
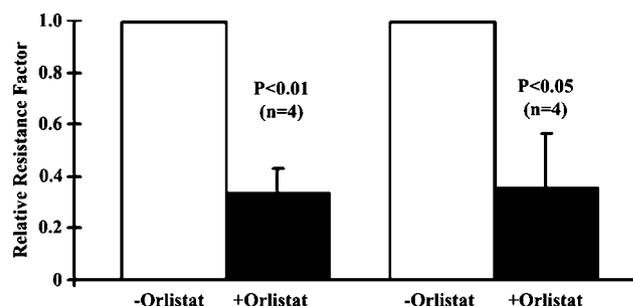
Discussion

In this study, we found a new mechanism of drug resistance due to the overexpression of FASN in a series of Adriamycin-selected breast cancer cell lines. We showed that the FASN overexpression caused drug resistance in these cells possibly by overproducing palmitate to increase resistance to drug-induced apoptosis. We also showed that

FASN-mediated drug resistance is not specific to MCF7 cells and that inhibiting FASN expression in normal breast epithelial cells does not affect their drug responses. The latter finding is interesting and suggests that FASN can be used as a target for developing chemosensitizing agents in combinational therapy without affecting normal cells.

FASN catalyzes the synthesis of palmitate by condensing malonyl-CoA and acetyl-CoA using NADPH. It contains seven catalytic domains and a phosphopantotheine prosthetic group on a single polypeptide (8). FASN expression is minimal in most normal human tissues, except lactating breast and cycling endometrium (8). Previously, FASN was identified as the oncogenic antigen-519 (7). It gradually became clear that FASN expression is high in a biologically aggressive subset of human carcinomas, including breast cancers (8, 21). FASN overexpression and hyperactivity were also found to correlate with poor prognosis (8, 9). Together with our data presented in this study, these observations strongly suggest that overexpression of FASN likely plays an important role in drug resistance and thus poor prognosis of breast cancer patients.

Previously, it has been shown that inhibiting FASN expression using siRNA or inhibiting FASN activity using inhibitors C75 or cerulinin causes apoptosis of breast and prostate cancer cell lines (11, 16, 22, 23). Interestingly, we did not observe apoptosis of drug-resistant breast cancer

cells following reduction of FASN expression using shRNA. The reason for the difference between these studies is currently unknown. It is noteworthy, however, that the parental sensitive MCF7 cells express less FASN than the drug-resistant derivative cells, yet they are perfectly alive. It is thus possible that the reduction of FASN expression in the drug-resistant cells by shRNA in the current study is not large enough to induce apoptosis as observed in the previous studies using regular cancer cell lines (11, 16, 22, 23).

The mechanism of FASN-mediated drug resistance is not yet known. There are several possible ways that FASN can affect cancer cell responses to drugs. Firstly, the newly synthesized fatty acids by FASN in cancer cells are saturated and monounsaturated. Therefore, the increased lipid synthesis in cancer cells may change not only the quantity in cell membranes required for cell growth but also the lipid composition of the membrane (24, 25). These changes in membrane composition may decrease the permeability of the membrane to anticancer drugs and thus the accumulation that in turn causes drug resistance. They may also increase the activity of ABC transporters, such as ABCG2, which causes drug resistance by actively effluxing anticancer drugs and hence effectively reduces cellular drug accumulation. However, we have ruled out these possibilities (see Fig. 5). It is also unlikely that ABCG2
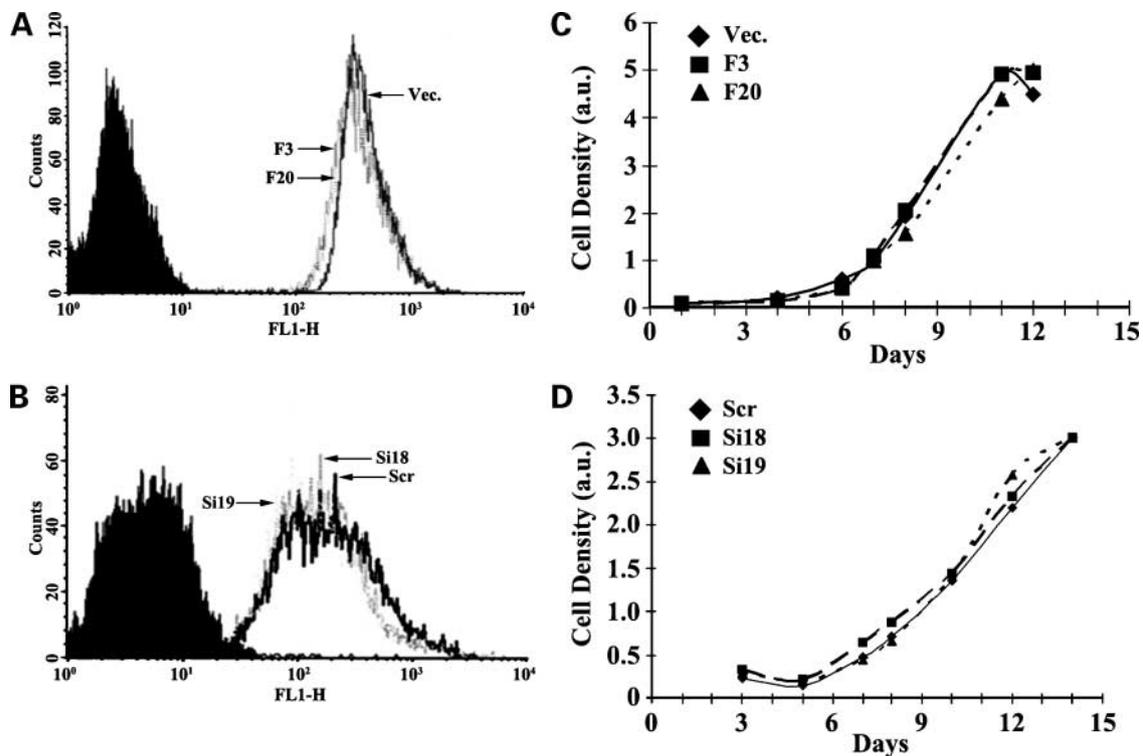

Figure 5. Effects of FASN expression on drug accumulation and cell proliferation. **A** and **B,** drug accumulation. MCF7 and its two FASN overexpressing stable clone derivatives (**A**) and MCF7/AdVp3000 cell line and its two derivative clones with down-regulated FASN expression (**B**) were tested for their ability to accumulate anticancer drug mitoxantrone. **C** and **D,** cell proliferation. MCF7 and its two FASN overexpressing stable clone derivatives (**C**) and MCF7/AdrVp3000 cell line and its two derivative clones with down-regulated FASN expression (**D**) were determined for their proliferation over a period of 2 wk.
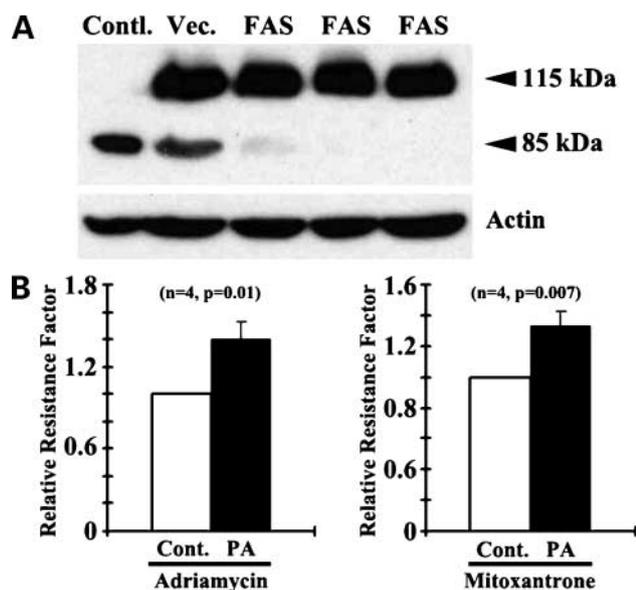
Figure 6. Effect of FASN overexpression on apoptosis and effect of palmitic acid on drug resistance. **A,** PARP cleavage. Different stable MCF7 clones with FASN overexpression and a control clone transfected with vector were treated with Adriamycin followed by analysis of PARP cleavage using a Western blot. *Lane 1,* a control for the cleaved 85-kDa PARP cleavage product. **B,** effect of palmitic acid on drug resistance. MCF7/AdVp3000 cells were first treated with palmitic acid for 24 h followed by treatment with Adriamycin or mitoxantrone and subjected to SRB assay.

plays any role in FASN-mediated drug resistance because knocking down FASN expression in MDA-MB-468 cells or overexpressing FASN in MCF7 cells, both of which do not have detectable ABCG2, changed the drug response level of these cells.

Second, FASN has been suggested to promote cancer cell proliferation (9). It is thus possible that FASN overexpression changes cell growth rate or cell cycle distribution, which in turn causes decreased cellular response to anticancer drugs. However, we showed that changing the FASN expression level did not affect the cell proliferation rate under normal growth conditions. Hence, the change in cell proliferation or cell cycle distribution due to FASN overexpression is not likely to be responsible for FASN-mediated drug resistance.

Third, most anticancer drugs exert their effect by causing cancer cell apoptosis. It has been shown that Adriamycin induces apoptosis in MCF7 cells by generating sphingosine from ceramide (26). In breast cancer cells, ceramide has been shown to be up-regulated when FASN is inhibited (27). It is reasonable to suggest that the altered FASN expression level affects the level or biosynthesis of cellular lipids to protect cancer cells from drug-induced apoptosis. Indeed, supplementation of palmitate to MCF7 cells caused significant increase in drug resistance. Recently, it was also found that knocking down FASN expression in breast cancer cells using siRNA up-regulated the expression of several proapoptotic genes, such as BNIP3, TRAIL,

and DAPK2, in addition to ceramide (27). It is thus tempting to propose that FASN overexpression in drug-resistant cells causes decreased expression of these proapoptotic genes and ceramide levels, which in turn causes drug resistance.

Knocking down FASN expression or inhibiting its activity can sensitize the drug-resistant cancer cells to traditional anticancer drugs. This observation suggests that FASN may be used as a target in combinational therapy. The observation that knocking down FASN expression in normal breast epithelial cells does not affect their drug responses further supports the above idea. However, it is not yet known if inhibiting FASN expression or its function affects bone marrow or other critical normal cells, thus limiting the use of FASN as a target. Recent studies using Orlistat, a FASN inhibitor that sensitized FASN-mediated drug resistance (see Fig. 4), did not cause any adverse effect in nude mice (18), further confirming that FASN may be an ideal target for chemosensitization. Because Orlistat is a Food and Drug Administration–approved drug for treating obesity patients, it may be potentially developed into a sensitizer in combinational therapy for cancers.

In normal cells, FASN expression is tightly regulated by hormonal and nutritional signals, whereas, in cancer cells, FASN overexpression is thought to be the result of inappropriate activation of growth factors such as epidermal growth factor, keratinocyte growth factor, and HER-2 receptor tyrosine kinase (28). These growth factors activate downstream signaling pathways, particularly PI3K/Akt pathway, to transcriptionally activate FASN through activation of SREBP-1a. Also, in prostate cancer cells, FASN protein was shown to be stabilized by USP2a, an ubiquitin-specific protease (29). However, the mechanism for further up-regulation of FASN in drug-resistant cancer cells, compared with their parental sensitive ones, remains to be investigated. Nevertheless, the finding that the FASN expression is increased in the early stepwise-selected breast cancer cell lines suggests that FASN may be an early respondent gene for drug assault.

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