

Exogenous lipids promote the growth of breast cancer cells via CD36

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Abstract. Cancer cells present sustained *de novo* fatty acid (FA) synthesis with increased production of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). This change in FA metabolism is associated with overexpression of stearoyl-CoA desaturase 1 (SCD1), which catalyses the transformation of SFAs into MUFAs (e.g., oleic acid). In this study, we provide new evidence that SCD1 inhibition leads to the anti-proliferation effect of breast cancer cells through induction of apoptosis, cell cycle arrest and migration prevention. However, the antitumor effect of the SCD1 inhibitor can be reversed by exogenous oleic acid. We hypothesize that, in addition to *de novo* synthesis, cancer cells may uptake exogenous FAs actively. CD36, also known as FA translocase (FAT), that functions as a transmembrane protein and mediates the uptake of FAs, is observed to be highly expressed in breast cancer tissues. Furthermore, the anti-proliferation effect caused by the SCD1 inhibitor can not be reversed by exogenous oleic acid supplementation in CD36 knockdown breast cancer cells. Our study revealed that the lipid metabolism of breast cancer is regulated not only by *de novo* lipogenesis but also by the availability of lipids outside cancer cells. Consistent with FA synthesis, FA uptake and transport will be another important target pathway for anticancer therapy, and the FA channel protein CD36 may provide a promising therapeutic target. Lipogenesis combined with FA transport will be a new orientation for antitumor therapy.

Introduction

Breast cancer is one of the most common neoplasms and the leading cause of cancer-related deaths in women worldwide (1). Unfortunately, almost all factors known with certainty to cause this disease cannot be easily prevented, such as genetics and the age at which a woman has a child. Therefore novel preventive and therapeutic approaches based on new molecular targets are warranted. Epidemiological studies indicate that obesity and high-fat diets are considered to be risk factors and associated with poor prognosis in breast cancer patients, independently of menopausal status, tumor stage, and hormonal status (2,3).

Moreover, as is known, cancer cells exhibit metabolic alterations characterized by increased glycolysis and lipogenesis, which meet the need of macromolecules and energy to support rapid growth and proliferation of cancer cells. The most important and fundamental role of FAs is as building blocks for newly-synthesized membrane phospholipids. In addition, FAs can also be used to produce protumorigenic signaling lipids and be used for mitochondrial β -oxidation to produce ATP (4). Thus, we assumed that blocking the sources of FA acquisition has the potential to starve cancer cells and induce apoptosis.

Cancer cells present not only increased *de novo* lipid biosynthesis but also modified membrane lipid composition. Monounsaturated fatty acids (MUFAs) represent important precursors that form complex lipids including phospholipids, cholesterol esters, and glycerides, which are the main component of membranes. Thus, a suitable balance of saturated fatty acids (SFAs), the end-product of *de novo* FA synthesis (5) and MUFAs is critical for membrane composition affecting membrane fluidity, signal transduction and gene expression (6). Stearoyl-CoA desaturase 1 (SCD1) is a critical enzyme which catalyzes the conversion of SFAs into MUFAs. Recent evidence suggests that the expression of SCD1 is aberrantly increased in many types of cancer including lung, colon and renal carcinoma relative to the corresponding normal tissues (6,7), and SCD1 inhibition has been shown to attenuate cancer cell

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growth (8). However, recent studies revealed that the cytotoxic effects caused by FA synthesis inhibition can be reversed by exogenous FA supplementation. This indicates that aside from *de novo* FA synthesis, FA transport and uptake are indeed an important and underappreciated aspect of lipid metabolism in cancer. Furthermore, in the anatomy of the mammary gland, adipocytes represent one of the most prominent cell types, thus, cancerous breast glands are embedded in the mammary fat pad (9). Mammary adipocytes store and secrete FAs, adipokines, and have the potential to influence neighboring cells by paracrine and endocrine mechanisms. Mammary adipocytes appear capable of translocating stored lipids to breast cancer cells as another key source of FAs (9,10). Well then, how are FAs transferred from adipocytes to cancer cells? Evidence shows that FAs especially long-chain fatty acids (LCFAs) are actively transported across the cell membrane by specialized proteins instead of passive diffusion (11). The protein-mediated import of LCFAs is of greatest significance when the metabolic requirements for LCFAs are high or when the level of FFAs is low (12). Although, several proteins have been implicated in facilitating FA uptake, CD36 is the best characterized as an FA translocase (FAT) which enhances LCFA uptake by overexpression or translocation from intracellular stores to the plasma membrane (13). Accordingly, we hypothesized that besides *de novo* lipogenesis, breast cancer cells can also uptake exogenous FAs via the transmembrane channel FAT/CD36, which was found to be overexpressed in the majority of breast cancer tissues in our study. The therapeutic efforts aimed to starve cancer cells to death thus suppressing both FA synthesis and uptake pathways.

In this study, we investigated the role of SCD1 and CD36 in tumor viability by pharmacologic inhibition or genetic expression silencing. Our results revealed that breast cancer cells are highly dependent on the activity of SCD1 in the absence of exogenous MUFA. Moreover, the data demonstrated that breast cancer cells can also uptake exogenous MUFA via CD36. Inhibition of both SCD1 and CD36 resulted in significant antitumor synergy in breast cancer. Collectively, these results strongly suggest that SCD1 and CD36 represent viable targets for the development of novel anticancer agents.

Materials and methods

Materials. MCF-7 human breast cancer cell line was acquired from the American Type Culture Collection (ATCC). Normal human skin fibroblasts were obtained from the Laboratory of Clinical Research Center in Hebei General Hospital. Small molecule SCD1 inhibitor MF-438 was purchased from Merck Millipore (catalog #569406, Darmstadt, Germany). Oleic acid and palmitate acid were obtained from Sigma-Aldrich (catalog #O1383, St. Louis, MO, USA). FA-free bovine serum albumin (BSA) was from Equitech-Bio (catalog #BAH66, Kerrville, TX, USA). CellTiter 96 Aqueous One Solution cell proliferation assay was purchased from Promega (MTS; catalog #G3580, Madison, WI, USA). Hoechst 33342 staining kit was obtained from Coolaber (catalog #SL7130, Beijing, China).

Cell culture. MCF-7 cells and normal human skin fibroblasts were cultured in RPMI-1640 medium supplemented with 10%

fetal bovine serum (FBS) (both from Hyclone, Logan, Utah, USA), 100 U/ml streptomycin, 100 U/ml penicillin at 37°C, 5% CO₂, and 100% humidity. For assays, MCF-7 cells were incubated in RPMI-1640 medium with 2% FBS for compound treatment and siRNA treatment.

Small molecule inhibitor and fatty acid treatment. MF-438 was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Oleic acid and palmitate acid were dissolved in 75% ethanol to a concentration of 100 mM, then diluted in phosphate-buffered saline (PBS) containing 10% FA-free BSA (Equitech-Bio). Both FA-BSA (or BSA) and MF-438 (or DMSO) were pre-diluted in assay culture medium. For experiments of FA supplementation, FA bound to BSA (ratio 2:1) was added to the media at a final concentration of 100 μM.

RNA interference. siRNA duplexes targeting human CD36 (5'-GGAAAGUCACUGCGACAUG-3') (14) were synthesized, as well as the negative control (scr) (5'-UUCUCCGAACGUGUCACGUTT-3') and were both obtained both from Sigma-Aldrich. MCF-7 cells were transfected with siRNA at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The transfection efficiency was confirmed by real-time PCR and western blot analysis.

Cell growth assays. For MF-438 IC₅₀ determinations, MCF-7 cells were plated in 96-well plates at a density of 8,000 or 10,000 cells/well in 10 or 2% FBS, respectively for 24 h. Then cells were treated with MF-438 or DMSO control only. After 48-h treatment with the inhibitor, the cell viability was assessed using an MTS assay according to the manufacturer's protocol. The percentage of inhibition for wells treated with MF-438 was determined relative to DMSO alone. FA-BSA was added to the media at 100, 200 or 300 μM, and the percentage of inhibition with MF-438 was evaluated again. Then, MCF-7 cells were transfected with CD36-siRNA and the aforementioned steps were repeated.

Cell apoptosis and death analysis. Apoptosis morphology was observed by fluorescence microscope (DMI3000B; Leica, Germany) after Hoechst 33342 staining. Briefly, exponentially growing MCF-7 cells or cells transfected with CD36-siRNA were seeded into a 24-well plate (1×10⁵ cells/well) for 24 h. Then cells were treated with MF-438 at approximately the IC₅₀ concentration or DMSO in the absence or presence of 100 μM of oleic acid for 48 h. The supernatant was discarded and adhered cells were exposed to Hoechst 33342 at 37°C in dark for 20 min. Finally all specimens were observed under fluorescence microscope. Apoptosis cells were identified as cells with condensed and fragmented nuclei. Cell apoptosis and the death rate were determined by propidium iodide staining (PI; Sigma-Aldrich) using flow cytometry (FACSCalibur; Becton Dickinson; BD Biosciences, Franklin Lakes, NJ, USA). MCF-7 cells transfected with CD36-siRNA or not were seeded in a 25-cm culture flask for 24 h. Then adhered and floating cells were collected 48 h after MF-438 treatment or DMSO control with or without 100 μM of oleic acid. PI (1 mg/ml) was added to the cells for 10 min. Positive PI-stained cells were considered as late apoptosis and dead cells.

Cell cycle analysis. MCF-7 cells or cells transfected with CD36-siRNA were treated with MF-438 or DMSO for 48 h in the absence or presence of 100 μ M of oleic acid. At the end of the incubation period, the cells were trypsinized and fixed with 70% ethanol, then resuspended in PI solution (50 μ g/ml of PI, 100 μ g/ml of RNase A and 0.2% Triton X-100) for 30 min at room temperature. The cell cycle distribution was determined using a flow cytometer.

Cell scratch test. MCF-7 cells or cells transfected with CD36-siRNA were plated uniformly in 6-well plates with 80-90% confluence in culture medium containing 2% FBS. Scratches were made at the bottom of the plates using the tip of a 200- μ l pipette. The following day then washed with PBS in order to remove the sloughing cells. Then, the cells were treated with MF-438 or DMSO control in the absence or presence of 100 μ M of oleic acid. Images of the scratches were acquired using a microscope after 24 and 48 h. The scratch closure rate was assessed using image processing software (Image J; NIH, USA). The area between the cells was assessed from 6-8 different regions on a single scratch.

Oil red O staining. In order to stain intracellular lipid deposits, MCF-7 cells or cells transfected with CD36-siRNA were cultured on coverslips in 6-well plates with 100 μ M of oleic acid for 48 h. Then, the cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Sigma-Aldrich). Images were acquired using a microscope.

RNA isolation and quantitative real-time RT-PCR. Total RNA was extracted from cells using standard TRIzol (Invitrogen) for RNA isolation. The RNA concentration was determined by assessing the absorbance of a diluted sample at 260 nm using a UV spectrometer (ND 2000; Thermo Fisher Scientific, Waltham, MA, USA) method. Reverse transcription of RNA was carried out according to the instructions of the EasyScript First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). Quantitative PCR reactions were performed on an ABI PRISM 7300 instrument (Applied Biosystems Life Technologies, Foster City, CA, USA) using SYBR-Green I GoTaq[®] qPCR Master Mix (Promega). PCRs were carried out in a total of 20 μ l as follows: one cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. Then the PCR products were analyzed by melting curve to confirm the specificity of amplification. The gene expression from each sample was analyzed in triplicate and GAPDH was used as an internal control. The results are expressed as the relative gene expression using the $\Delta\Delta$ Ct method. Specific primers designed for amplification of CD36 (forward, 5'-CGGAAGTGTGGCT CAT-3' and reverse, 5'-GGTCTCCAAGTGGCATTAGAA-3') and GAPDH (forward, 5'-GGATGATGTTCTGGAGAGCC-3' and reverse, 5'-CATCACCATTCCAGGAGC-3') were verified by NCBI BLAST database.

Western blot analysis. Cells were washed twice with ice-cold PBS and scraped into 1 ml of RIPA lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China) with protease inhibitors of PMSF. Lysates were clarified by centrifugation (12,000 rpm for 10 min) and the supernatants were collected. Equal amounts of

protein (50 μ g) underwent SDS-PAGE and then were electro-transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA), which were then sealed at room temperature for 2 h. The membranes were then incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: SCD1 (catalog sc-14715), and CD36 (catalog sc-7309) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the internal control β -actin (Cell Signaling Technology, Inc. Danvers, MA, USA). Then, the membranes were washed and incubated with the appropriate HRP-conjugated secondary antibody (Beijing CoWin Bioscience Co., Ltd., Beijing, China) in PBST for 2 h at room temperature. The membranes were then washed three times and reacted with chemiluminescent agent for 5 min. Then they were ECL labeled, exposed, and displayed. Quantification of the resulting images was performed by densitometry with Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) and the final readings were normalized against β -actin.

Construction of tissue microarray and immunohistochemistry. The clinical study was approved by the Ethics Committee of Hebei General Hospital. Sixty-five breast cancer specimens from resected breast tissue were obtained at the Hebei General Hospital between 2010-2013 following patient informed consent. A tissue microarray (TMA) of breast cancer (65 cases) and adjacent normal breast tissues (37 cases) was prepared by our team manually. A representative area was selected from an H&E section and the corresponding area was marked on the surface of the paraffin-embedded breast cancer tissue block. Then, a paraffin tissue punch was used to extract a 1.8-mm core sample from the selected area, which was placed into a recipient block and linked to a database that contained clinicopathological data. TMA sections (4 μ m) were stained using standard immunohistochemistry (IHC) techniques for expression and localization of SCD1 and CD36 according to the supplier's protocol. ImmPRESS anti-goat or anti-mouse peroxidase polymer detection systems along with a NovaRED kit as a substrate were used for the peroxidase-mediated reaction.

Statistical analysis. All experiments were repeated at least three times. Values are represented as the means \pm SD. Statistical analyses were performed with the SPSS statistical package (SPSS 16.0 software). Statistical significance was assessed by ANOVA (post-hoc used the Student-Newman-Keuls method) and unpaired Student's t-tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SCD1 inhibition results in a serum dependent decrease of cell proliferation in MCF-7 cells. MF-438 is a small molecule that specifically inhibits SCD1 enzymatic activity. To determine the effects of MF-438 on proliferation, MCF-7 cells were treated with MF-438 from 100 nmol/l to 100 μ mol/l and the cell viability was assessed after 48 h. MF-438 revealed a significant dose-dependent proliferation inhibition in MCF-7 cells. Fig. 1A shows concentration response curves of MCF-7 cells treated in 10 or 2% FBS conditions. Under the 10% FBS condition, the IC₅₀ value was determined to be 16.7 μ mol/l, whereas the IC₅₀ in 2% FBS was 3.7 μ mol/l. The growth viability of MCF-7 cells

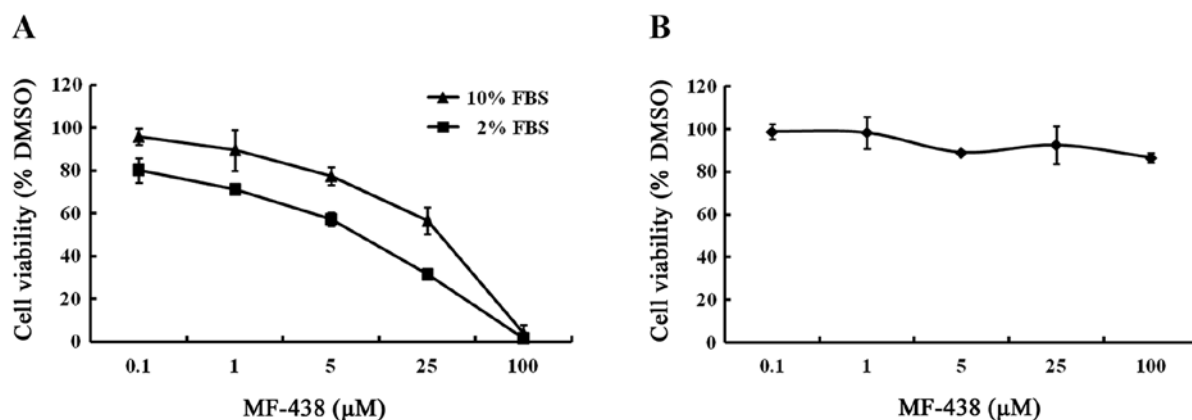


Figure 1. Anti-proliferation of SCD1 inhibitor in breast cancer cells and normal human fibroblasts. Percentage of inhibition of the cell viability at different concentrations of MF-438 treatment for 48 h relative to the DMSO control. The cell viability was determined using the MTS assay in 3 independent replicates at each dose level. Error bars represent the SD from the mean. (A) Pharmacological inhibition of SCD1 potentially inhibits MCF-7 cell proliferation in decreased serum conditions. (B) Pharmacological inhibition of SCD1 has no effect on normal human fibroblasts. SCD1, stearoyl-CoA desaturase 1; DMSO, dimethyl sulfoxide.

Table I. IC₅₀ values for the inhibition of the cell viability by MF-438 in MCF-7 cells cultured in media with 10 or 2% FBS, and various concentrations of oleic acid or palmitic acid added to 2% FBS.

Culture media	IC ₅₀ (μM) of MF-438
2% FBS	3.7
10% FBS	16.7
100 μM oleic acid	20.0
200 μM oleic acid	32.2
300 μM oleic acid	38.5
100 μM palmitate acid	0.3

FBS, fetal bovine serum.

was only slightly slowed when cultured in 2% FBS without compound treatment relative to the 10% serum concentration. These results revealed that cells grown in decreased FBS were more sensitive to growth inhibition of the SCD1 inhibitor.

SCD1 inhibition does not impair the proliferation of normal human fibroblasts. To investigate the influence of the SCD1 inhibitor on normal cells, human fibroblasts were incubated for 48 h, enough time to ensure at least one population doubling, with MF-438 at concentrations ranging from 100 nmol/l to 100 μmol/l in medium containing 10% FBS. As displayed in Fig. 1B, the SCD inhibitor did not impair the growth of normal human fibroblasts. This demonstrates that normal cells have much less requirement for endogenously produced MUFA than cancer cells.

MUFA rescue cell proliferation impaired by SCD1 inhibition in MCF-7 cells. To confirm that MCF-7 cells sensitive to SCD1 inhibition in decreased serum conditions are related to limiting availability of MUFAs, we investigated whether the anti-growth effects of the SCD1 inhibitor could be rescued by the addition of exogenous FA. MCF-7 cells were incubated

with 5 μM of MF-438 for 48 h in the presence of either MUFA or SFA. Addition of BSA alone did not alter the dose response of cells to MF-438. However, supplementation with oleic acid-BSA revealed a dose-dependent rescue of cell viability to MF-438 treatment. To extend this result, we also assessed the effects of supplementing media with SFA palmitic acid, the substrate of SCD1. The results revealed that far from protecting cells from growth inhibition by the SCD1 inhibitor, addition of palmitic acid produced a modest viability decrease at the concentrations used (Fig. 2A). The cellular IC₅₀ values for growth inhibition by MF-438 were determined in different conditions and are shown in Table I. These results indicate that the major viability impact observed in SCD1 inhibition is due to depletion of MUFAs, the downstream products of SCD1, while on the other hand, part of this effect may be induced by the resulting accumulation of SFAs, the upstream substrates of SCD1. SCD1 activity or MUFAs protect cells against cytotoxicity of SFAs.

CD36 depletion results in a non-MUFA-dependent decrease in breast cancer cell proliferation. Both the expression of CD36 mRNA (24 h after transfection) and protein (48 h after transfection) were markedly suppressed in CD36-depleted MCF-7 cells (Fig. 3). Then, these cells were incubated with 5 μM of MF-438 or DMSO vehicle for 48 h in the absence or presence of 100 μM of oleic acid. As shown in Fig. 2B, MF-438 treatment also exhibited a dose-dependent inhibition of cell proliferation. CD36-depletion treatment slightly decreased cell proliferation compared with normal MCF-7 cells, in other words, CD36 depletion increased the cell sensitivity to MF-438. However, supplementation with oleic acid did not exhibit an obvious rescue effect of cell viability to MF-438 in CD36-depleted MCF-7 cells. This revealed that the anti-proliferative effects of MF-438 could not be reversed by exogenous oleic acid when the CD36 gene was depleted.

Oleic acid rescues the cell apoptosis induced by SCD1 inhibition. To further examine the anti-proliferation effects of MF-438 on apoptosis, MCF-7 cells were treated with 5 μM of MF-438 for 48 h. Hoechst staining revealed that the apoptotic

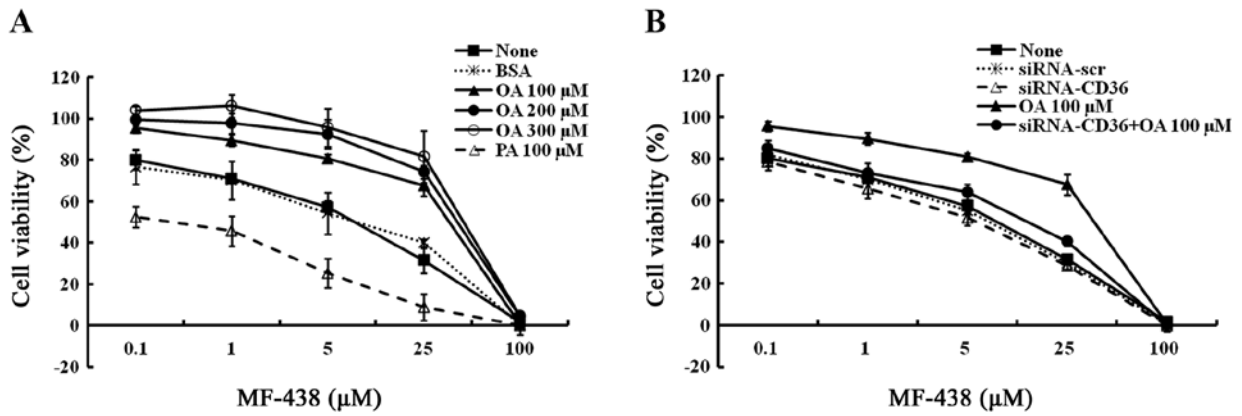


Figure 2. Rescue effect of exogenous oleic acid in MCF-7 cells with or without CD36 expression. (A) Anti-proliferation of the SCD1 inhibitor in MCF-7 cells can be rescued by oleic acid-BSA supplementation but not by BSA alone or palmitic acid-BSA. (B) Anti-proliferation of the SCD1 inhibitor in CD36-depleted MCF-7 cells cannot be rescued by oleic acid supplementation. The cell viability was determined using the MTS assay in 3 independent replicates at each dose level. Data represent the mean ± SD of three independent experiments. Error bars represent the SD from the mean. SCD1, stearyl-CoA desaturase 1; BSA, bovine serum albumin.

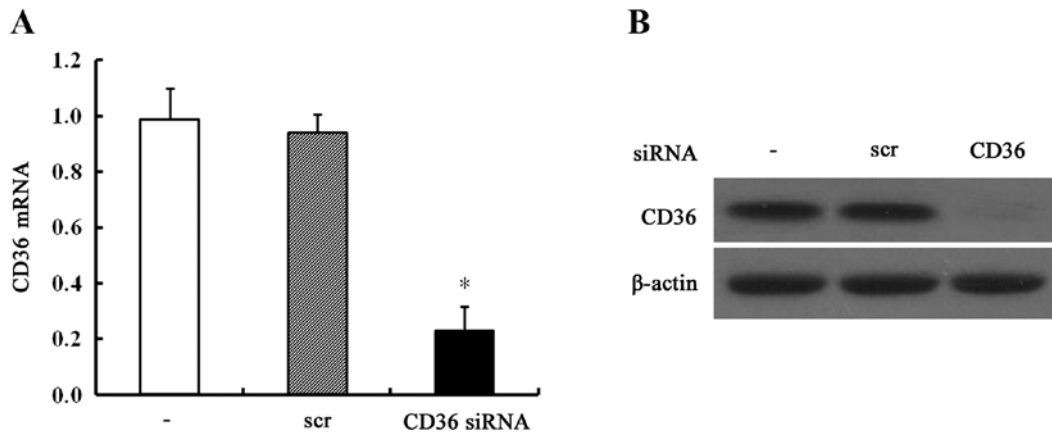


Figure 3. Efficient suppression of CD36 expression. (A) MCF-7 cells were transfected with siRNA control (scr) or with CD36-siRNA at 24 h for CD36 mRNA expression by RT-PCR. Values represent the mean ± SD. *p<0.05 vs. the siRNA scr-treated cells. (B) Efficient suppression of CD36 expression by western blotting at 48 h after transfection with CD36-siRNA.

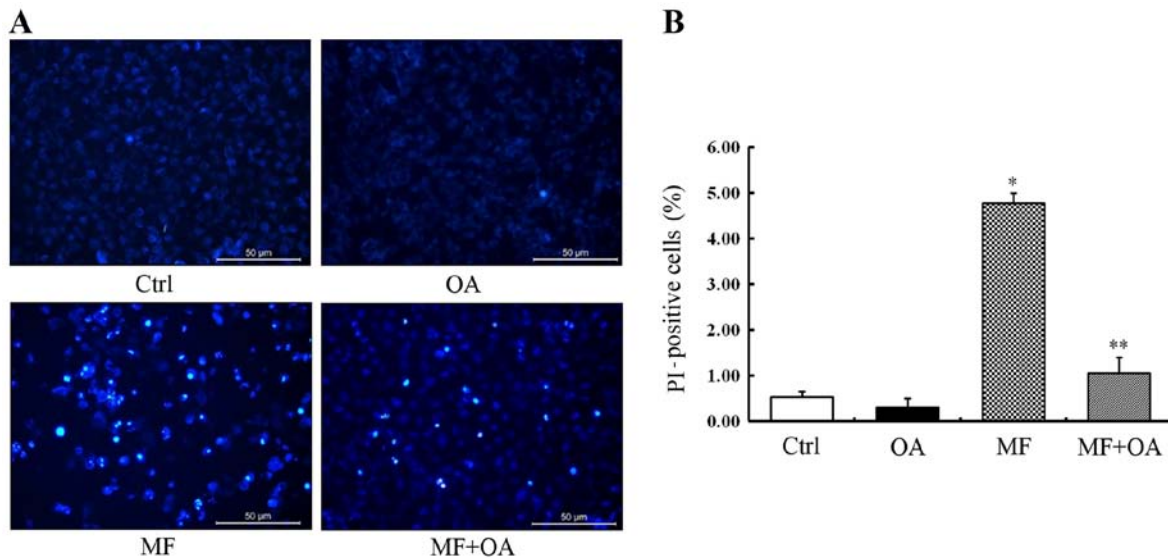


Figure 4. SCD1 inhibition and the rescue effect of oleic acid on apoptosis in MCF-7 cells. (A) Apoptosis morphology was observed by Hoechst 33342 in MCF-7 cells treated with 5 μM of MF-438 for 48 h in the absence (BSA) or presence of 100 μM of oleic acid-BSA. Microscopy images were recorded at an x200 magnification (scale bar, 50 μm). (B) Total cell death was analysed by flow cytometry after PI staining. *p<0.05 vs. the DMSO control, **p<0.05 vs. the MF-treated cells. Data represent the mean ± SD from three independent experiments. SCD1, stearyl-CoA desaturase 1; BSA, bovine serum albumin.

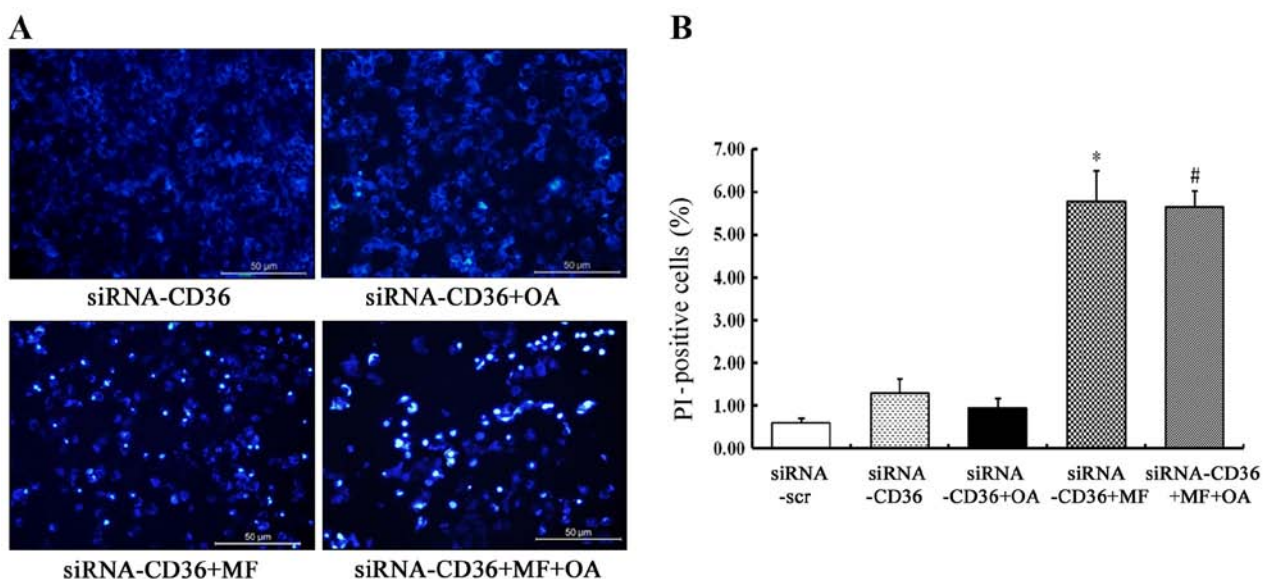


Figure 5. SCD1 inhibition and the rescue effect of oleic acid on apoptosis in CD36-depleted MCF-7 cells. (A) Apoptosis morphology was observed by Hoechst 33342 in CD36 depleted MCF-7 cells treated with 5 μ M of MF-438 for 48 h in the absence (BSA) or presence of 100 μ M of oleic acid-BSA. e, DMSO control; f, oleic acid-BSA; g, MF-438; h, MF-438 with oleic acid-BSA. Microscopy images were recorded at an x200 magnification (scale bar, 50 μ m). (B) Total cell death was analysed by flow cytometry after PI staining. * p <0.05 vs. siRNA-CD36 control cells, # p >0.05 vs. MF-treated cells. Data represent the mean \pm SD from three independent experiments. SCD1, stearoyl-CoA desaturase 1; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide.

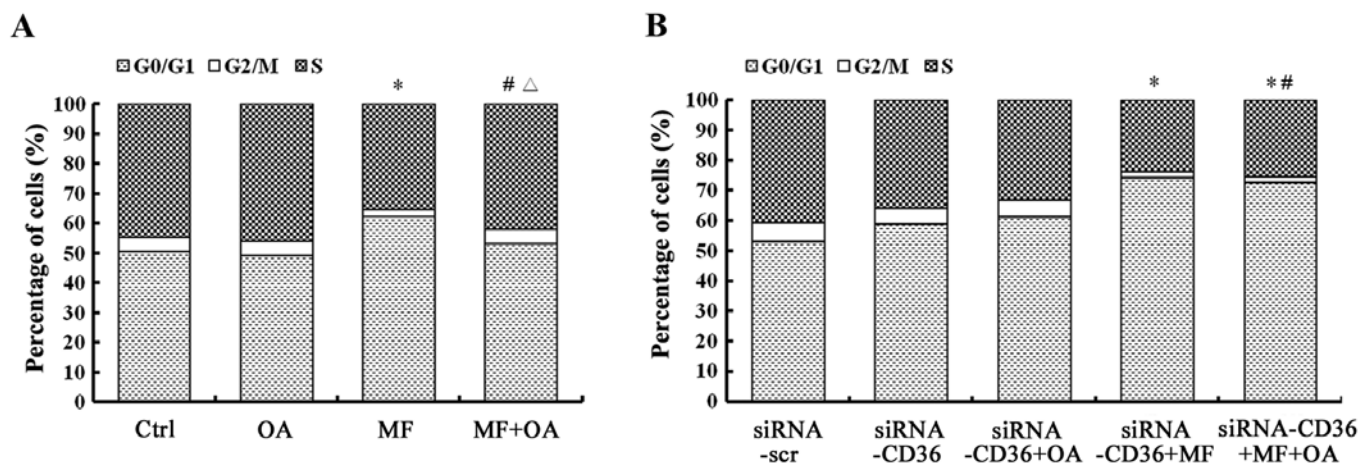


Figure 6. SCD1 inhibition and the rescue effect of oleic acid on the cell cycle in MCF-7 cells with or without CD36 expression. (A) SCD1 inhibitor MF-438 blocked the cell cycle and this was reversed by oleic acid supplementation in MCF-7 cells. The distribution of cells in the cell cycle phases was determined by fluorocytometry. The graph represents the percentage of cells in each phase of the cell cycle. (n=3). * p <0.05 vs. the DMSO control in all the cell cycle phases, # p <0.01 vs. the MF-treated cells in all the phases, p >0.05 vs. the DMSO control. (B) Cell cycle arrest induced by the SCD1 inhibitor cannot be reversed by oleic acid in CD36-depleted MCF-7 cells. * p <0.01 vs the siRNA-CD36 control cells in all the phases, # p >0.05 vs. the MF-treated cells. SCD1, stearoyl-CoA desaturase 1; DMSO, dimethyl sulfoxide.

MCF-7 cells were stained bright blue fluorescence while the normal cells were stained slightly blue (Fig. 4A). The typical apoptotic morphology was observed in most MCF-7 cells treated with MF-438 including cell shrinkage, chromatin condensation, marginalization and fragmentation as well as the apoptotic bodies. Furthermore, the result of PI staining indicated that the rate of late apoptotic and dead cells was significantly higher in the MF-438 treated group ($4.78 \pm 0.22\%$) than the DMSO control ($0.54 \pm 0.12\%$, $p=0.000$) (Fig. 4B).

We then supplied SCD1-inhibited MCF-7 cells with 100 μ M of oleic acid. It was observed that the apoptotic cells were significantly decreased after oleic acid addition. The result of PI staining revealed that exogenous oleic acid alleviated

cytotoxicity and decreased the rate of cell death caused by SCD1 inhibition ($1.06 \pm 0.35\%$ for oleic acid combined with MF-438 vs. $4.78 \pm 0.22\%$ for MF-438 alone, $p=0.000$) (Fig. 4B). Thus, we demonstrated that the pharmacological SCD1 inhibitor-induced apoptotic cell death can be rescued by exogenous oleic acid.

CD36 depletion prevents the rescue effect of oleic acid on SCD1 inhibition-induced cell apoptosis. CD36-silenced MCF-7 cells were incubated with 5 μ M of MF-438 or DMSO for 48 h in the absence or presence of 100 μ M of oleic acid. The typical apoptotic morphology was found in most CD36-depleted MCF-7 cells treated with MF-438 (Fig. 5A). PI staining

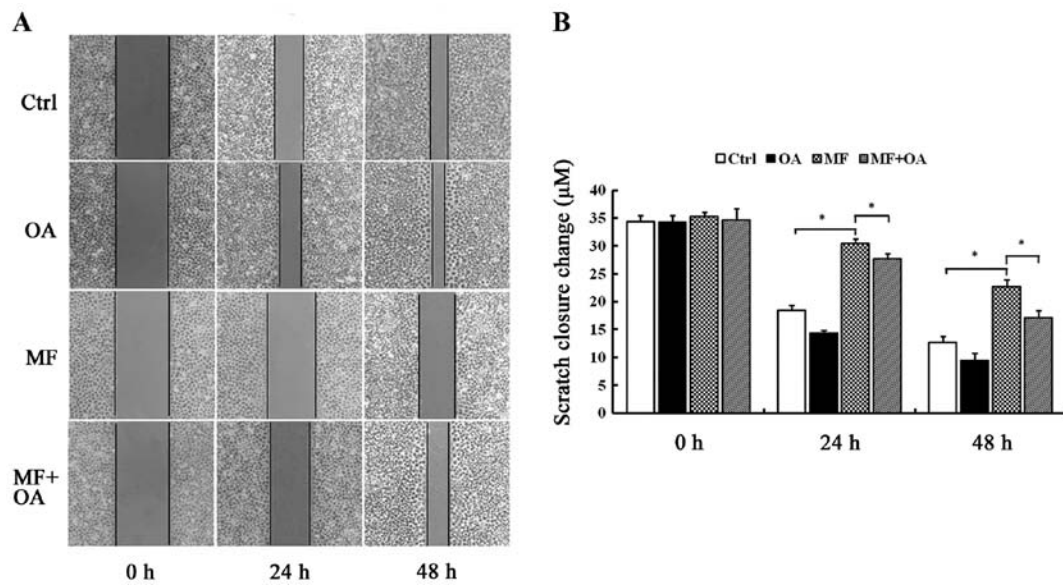


Figure 7. SCD1 inhibition and the rescue effect of oleic acid on cell migration in MCF-7 cells. MF-438 suppresses cell migration and is restored by oleic acid addition. Scratches of the same width were made for each group at 0 h, and then the width of the scratches was measured at 24 and 48 h by Image J software. Data represent the mean \pm SD from three independent experiments. (A) Cell migration ability (scale, 50 μ m; \times 10 magnification). (B) Quantification of wound area changes. * $p < 0.01$. SCD1, stearoyl-CoA desaturase 1.

revealed that the rate of late apoptotic and dead cells was significantly higher when CD36-silenced MCF-7 cells were treated with MF-438 ($5.77 \pm 0.72\%$ for MF-438 vs. $1.30 \pm 0.33\%$ for siRNA-CD36 control, $p = 0.000$) whether oleic acid was added or not ($5.65 \pm 0.38\%$ for oleic acid combined with MF-438 vs. $5.77 \pm 0.72\%$ for MF-438 alone, $p = 0.998$) (Fig. 5B). The result demonstrated that the rescue effect of oleic acid on the SCD1 inhibitor-induced cell apoptosis was exerted only if CD36 was present.

Oleic acid reverses the cell cycle blocked by SCD1 inhibition. To better understand the mechanism involved in growth inhibition by the SCD1 inhibitor, MCF-7 cells were incubated with 5 μ M of MF-438 in serum-decreased media for 48 h and the cell cycle distribution was analyzed by flow cytometry. The results revealed a significant decrease in the population of cells in the S phase (35.3 vs. 44.8%, respectively, $p = 0.001$) and the G2/M phase (2.6 vs. 4.9%, respectively, $p = 0.003$) with MF-438 treatment compared to the DMSO control (Fig. 6A). A concomitant increase in the percentage of cells in the G0/G1 phase was also observed (62.1 vs. 50.4%, respectively, $p = 0.001$). This indicated that SCD1 inhibition specifically targeted the progression of the cell cycle at the level of the synthetic phase.

However, when 100 μ M of oleic acid-BSA was supplemented in the aforementioned cells, the population of cells in the S phase (42.1 vs. 44.8%, respectively, $p = 0.315$), G2/M phase (4.8 vs. 4.9%, $p = 1.0$) and the G0/G1 phase (53.1 vs. 50.4%, $p = 0.466$) were all reversed in the MF-438-treated group compared to the DMSO control (Fig. 6A). Oleic acid reversed the changes of the cell cycle caused by the SCD1 inhibitor, suggesting that the lipid components of serum, possibly MUFA, were able to ensure the SCD1 inhibition of cells through mitosis.

CD36 depletion prevents the rescue effect of oleic acid on the restoration of the cell cycle arrest of SCD1 inhibited

cells. CD36-depleted MCF-7 cells were also incubated with MF-438 for 48 h in the absence or presence of oleic acid. Fig. 6B also revealed a significant decrease in the population of CD36-depleted cells in the S phase (24.0 vs. 40.5%, respectively, $p = 0.000$) and the G2/M phase (1.7 vs. 6.4%, respectively, $p = 0.001$) with MF-438 treatment compared to the siRNA-CD36 control, and a concomitant increase in the percentage of cells in the G0/G1 phase (74.4 vs. 53.2%, respectively, $p = 0.000$). Oleic acid supplementation did not alter the cell cycle profile, with the percentage of S phase 25.3 vs. 24.0% ($p = 0.370$), G2/M phase 2.0 vs. 1.7% ($p = 0.986$), and G0/G1 phase 72.7 vs. 74.4% ($p = 0.357$), respectively in oleic acid combined with MF-438 treatment compared to MF-438 treatment alone (Fig. 6B). The results revealed that the cell cycle arrest caused by the SCD1 inhibitor cannot be reversed by exogenous oleic acid when the CD36 gene was silenced.

Oleic acid restores the migration ability decreased by SCD1 inhibition. The results (Fig. 7A) revealed that the scratches of the cells treated with MF-438 were wider than the DMSO control at the same time-point (30.52 ± 0.73 vs. 18.47 ± 0.90 μ m at 24 h $P = 0.000$; 22.80 ± 1.10 vs. 12.70 ± 1.04 μ m at 48 h $P = 0.000$). However, when oleic acid was added to the cells treated with MF-438, the scratches became significantly narrower compared to MF-438 group (27.81 ± 0.81 vs. 30.52 ± 0.73 μ m at 24 h $P = 0.000$; 17.18 ± 1.25 vs. 22.80 ± 1.10 μ m at 48 h $P = 0.000$) (Fig. 7B). This indicated that the SCD1 inhibitor was able to suppress migration in MCF-7 cell line, however the addition of oleic acid restored the migration ability.

CD36 depletion prevents the rescue effect of oleic acid on the restoration of the migratory ability of SCD1 inhibited cells. The scratches of the CD36-silenced MCF-7 cells treated with MF-438 were wider than the DMSO control (38.30 ± 0.77 vs. 35.20 ± 0.91 μ m at 24 h $P = 0.002$; 38.14 ± 0.66 vs. 25.47 ± 1.27 μ m at 48 h $P = 0.000$). When oleic

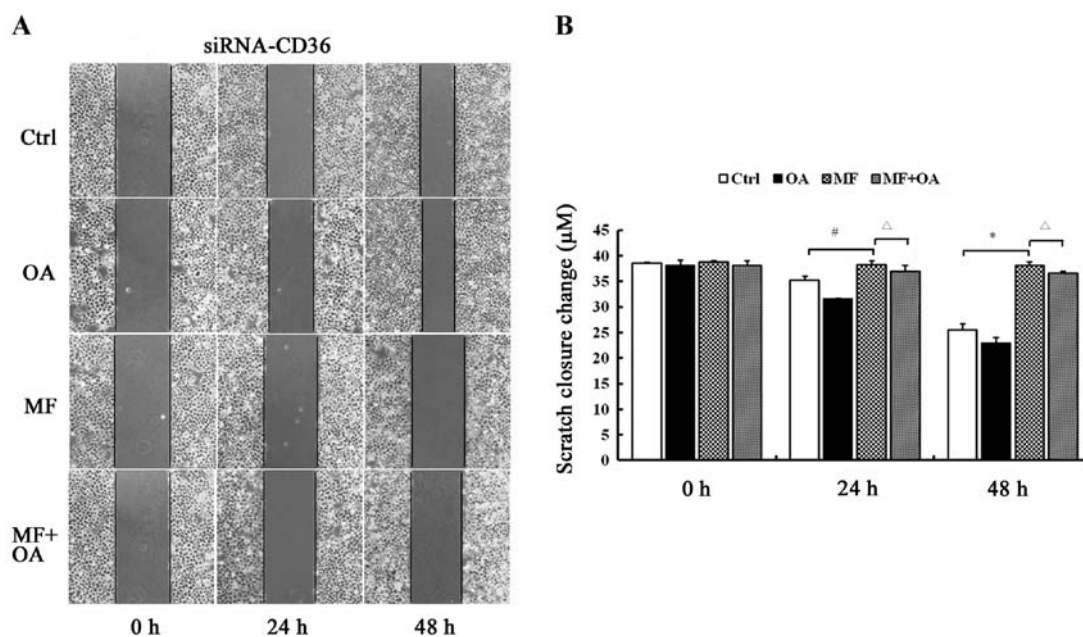


Figure 8. SCD1 inhibition and the rescue effect of oleic acid on the cell migration in CD36-depleted MCF-7 cells. Decreased migration caused by the SCD1 inhibitor cannot be reversed by oleic acid in CD36-depleted MCF-7 cells. Scratches of the same width were made for each group at 0 h, and then the width of the scratches was assessed at 24 and 48 h by Image J software. Data represent the mean \pm SD from three independent experiments. (A) Cell migration ability (scale, 50 μ m; \times 10 magnification). (B) Quantification of the wound area changes. # p <0.05, * p <0.01, Δ p >0.05. SCD1, stearoyl-CoA desaturase 1.

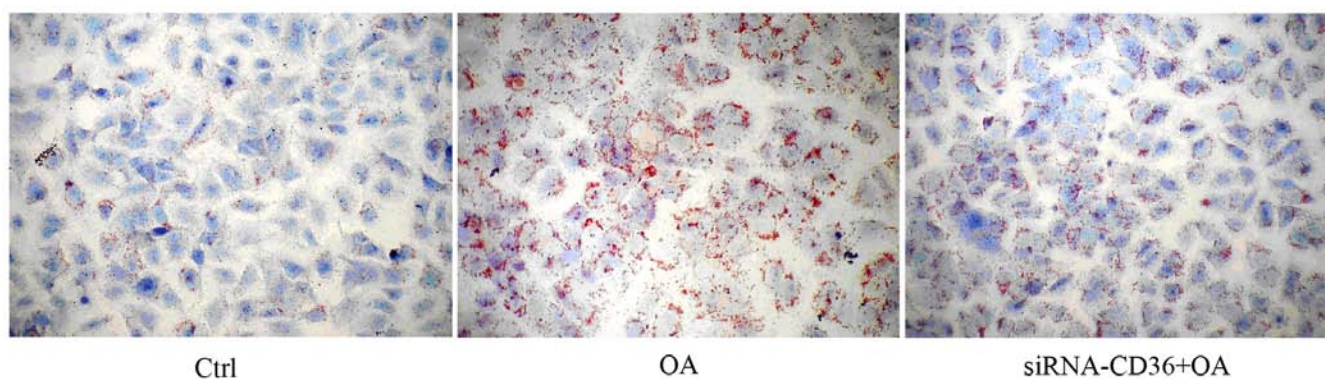


Figure 9. CD36 mediates exogenous oleic acid uptake in MCF-7 cells. Oil red O staining for intracellular lipid deposits in MCF-7 cells and CD36-depleted MCF-7 cells cultured with 100 μ M oleic acid. (\times 200 magnification).

acid was supplemented, there were no obvious changes in the width of the scratches compared to MF-438-treated group (37.02 ± 1.17 vs. 38.30 ± 0.77 μ m at 24 h $P=0.685$; 36.67 ± 0.33 vs. 38.14 ± 0.66 μ m at 48 h $P=0.545$) (Fig. 8). Exogenous oleic acid did not reverse the migration ability in CD36-depleted MCF-7 cells.

Overall, these results indicated that blockade in SCD1 activity could not enable MCF-7 cells to get through the early stages of the cell cycle, leading to cell apoptosis and making cells migrate more slowly. Exogenous oleic acid markedly reversed the effect caused by SCD1 inhibition but only if CD36 was present.

Breast cancer cells can use exogenous FAs for tumor growth.

To further determine whether breast cancer cells also uptake exogenous FAs via CD36 as an alternative pathway to obtain lipids, we added oleic acid in the media of MCF-7 cells and CD36-silenced MCF-7 cells. It was observed that more

cytoplasmic lipid droplets accumulated in MCF-7 cells but not in CD36-silenced MCF-7 cells (Fig. 9) after oleic acid supplementation.

SCD1 and the CD36 protein are highly expressed in human breast cancer. IHC was performed to detect SCD1 and the protein level of CD36 in human breast cancer specimens and adjacent normal breast tissues. SCD1 exhibited diffuse cytoplasmic staining and was highly expressed in nearly all breast cancer samples, whereas it was low or not expressed in adjacent normal breast tissues (Fig. 10). Similarly, the majority of breast cancer samples were also stained for CD36 mainly at the plasma membrane (Fig. 10).

Discussion

Cancer cells are distinct from normal cells based partly on their unique metabolic status, one aspect of which is an

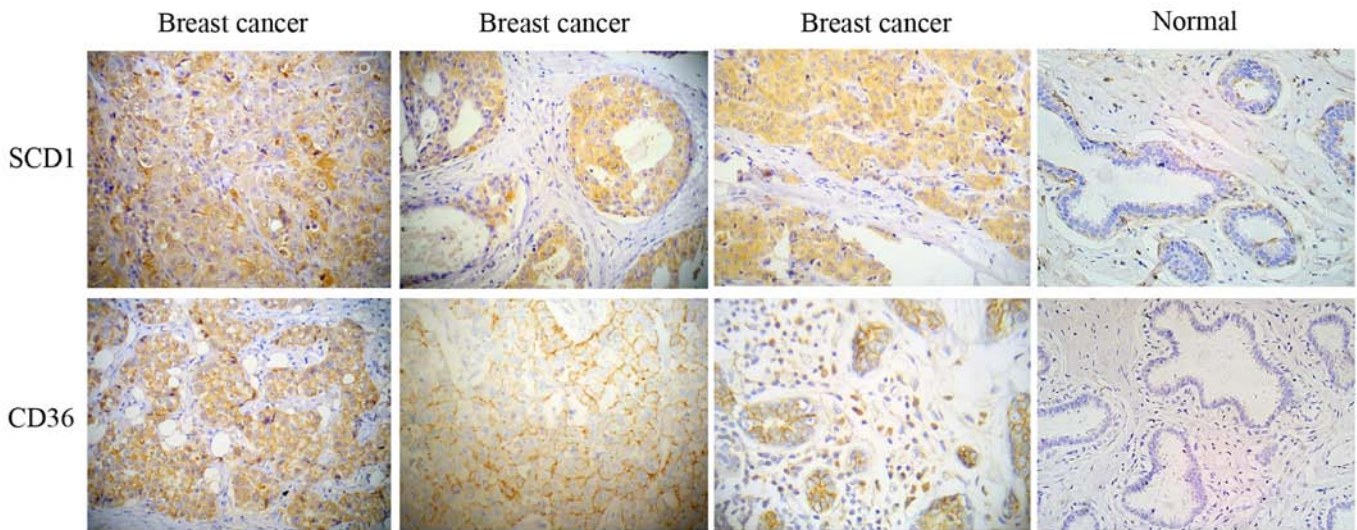


Figure 10. Expression of SCD1 and CD36 in human breast cancer samples. Immunohistochemical evidence that SCD1 and CD36 is highly expressed in a large number of human breast cancer samples. Representative photomicrographs of positively-stained tissue from breast cancer specimens as well as the respective normal breast tissues (x200 magnification). SCD1, stearyl-CoA desaturase 1

unusual requirement for FA metabolism to sustain cell division and proliferation, fulfill energy requirements and provide metabolites for anabolic processes. Numerous studies have documented that LCFAs influence the proliferation of cancer cells both *in vivo* and *in vitro* (8). The critical role of lipids in cancer cell proliferation has led to a number of proposed strategies for treating cancer through inhibition of lipid availability.

Thus the FA synthesis pathway has been an attractive cancer target for quite some time, and attention is primarily focused on FA synthase with the production of long-chain SFAs (15). However, MUFAs play a more important role in dynamic or rapidly dividing cancer cells and are a major constituent of biological structures such as membranes, and can also function as or modify signaling molecules. SCD1 is the critical enzyme in the synthesis of MUFAs, oleic and palmitoleic acid, from SFA, stearic and palmitic acid. SCD1 may represent a final rate-limiting point in the FA synthesis pathway. We revealed in the present study that loss of SCD1 activity yielded pronounced growth inhibition in breast cancer MCF-7 cells while producing no notable effects in normal cells. This was also observed in colon and lung cancer cells *in vitro* (16,17). In the present study, we provided new evidence that SCD1 controls breast cancer cell proliferation through induction of apoptosis, cell cycle arrest and migration prevention.

Moreover, our data revealed that the anti-proliferative effect of the SCD1 inhibitor was more sensitive under low serum conditions. Furthermore, this growth inhibition could be reversed by oleic acid in all aspects aforementioned but not palmitic acid which even produced the opposite effect. These results imply that the major viability impact is attributable to an SCD1-inhibition-mediated oleic acid deficiency, while on the other hand, partly due to the buildup of intracellular palmitic acid. Although it appears that SFA enhanced the anti-proliferative effect of SCD1 inhibition in MCF-7 cells, human normal cells are more sensitive to the toxicity of excess SFA than cancer cells which may cause cardiovascular diseases and fatty liver called lipotoxicity. SCD1, overex-

pressed in cancer cells but not normal cells, appears to be part of a safeguard mechanism for cancer cells to confront the SFA-mediated toxicity.

While cancer cells are characterized by persistent cell division, the number of proliferating cells is also affected by the rate of cell death. The present results indicate that SCD1 is a critical factor for breast cancer cells to promote cell survival and prevent programmed cell death through its production of MUFA. Furthermore, our observation that breast cancer cells treated with the SCD1 inhibitor are arrested in the G1 phase and that this effect is rescued by oleic acid suggests that MUFA synthesis is required in the early phases of the cell cycle. In fact, we have known for years that a high rate of membrane phospholipid synthesis and turnover occurs during the G1 and early S phases for cell division (18). Our results demonstrated that constant activation of SFA synthesis coordinated with subsequent conversion of SFA into MUFA is required to provide the phospholipid biosynthesis with MUFA substrates for new membrane synthesis before or during the synthetic G1/S phase of the cell cycle. Moreover, the presence of abundant unsaturated lipids in cancer cells may have critical implications for their biological phenotype. Highly expressed SCD1 in cancer cells enriches the membrane phospholipids with MUFA thereby producing a more fluid lipid membrane, which is thought to induce growth factor-activated proliferation, migration and invasion (19,20).

Provided that these observations may be extended to the *in vivo* situation, human breast cancer proliferation involves a complex interaction between genes, hormones, calorogenic nutrients and the microenvironment. Obesity which is manifested as increased circulating FFAs (e.g., oleic acid), and a high fat diet especially a diet rich in oleate may decrease the antitumor effect of SCD1 inhibition and favor mammary tumor progression. Conversely, this indicates that the anti-tumor effect of the SCD1 inhibitor is steadily exerted possibly only in low fat diet conditions or non obese patients. Thus, this may require patients to eat a low fat diet and control their body weight or body fat.

However, many tumors associated with obesity reside near anatomic adipose tissue depots, including renal, pancreatic, colon and breast. For instance, adipocytes are a major component of the stromal microenvironment of mammary ducts and glands where breast cancer arises. Emerging data has revealed that adipocytes participate in the crosstalk with neoplastic cells and promote cancer initiation and progression, however the mechanisms involving the paracrine effects of adipokines and adipocyte products transferred to cancer cells have not been fully clarified yet (21,22). Some studies have shown that adipocytes promote tumorigenesis through providing free FAs to ovarian cancer cells and glutamine to leukemia cells (23,24). From our aforementioned observations, we realize that lipid transport and uptake maybe also be an important and underappreciated aspect of lipid metabolism in breast cancer especially within an adipocyte-rich environment (23). Thus, the fact that SCD1 inhibition fails to have constant effects on tumor growth is not surprising given the ability of cancer cells to uptake unsaturated lipid from their microenvironment.

Unlike glucose, which is water soluble, exogenous FAs are either circulating FA-albumin complexes or LPL-mediated hydrolysis of plasma lipoproteins. CD36, as a transmembrane protein, binds and concentrates exogenous LCFAs at the membrane, facilitating their translocation across the plasma membrane. Previous studies revealed that CD36-deficient mice display changes in LCFA utilization and metabolism consistent with an important role for the molecule in LCFA transport (25-27). The channel protein CD36 mediates the uptake of FAs in some normal tissues with FA metabolism, including adipose tissue and muscle, but is expressed at low levels (28). However, our study revealed that CD36 expression is increased in breast cancer samples, and that cytoplasmic lipid droplets accumulate only in cells in which CD36 exists after exogenous oleic acid addition. This indicates that breast cancer cells may obtain exogenous FAs via CD36 from the microenvironment (e.g., adipocytes) or circulating particles (e.g., diet-derived lipoprotein particles). Thus, alterations in CD36 expression could influence the uptake of exogenous FAs. Our results also confirm that the antitumor effect caused by SCD1 inhibitor can not be reversed by exogenous oleic acid when CD36 gene is depleted. This study provides the first evidence of the effect of CD36 in exogenous FA uptake by breast cancer cells, making CD36 an ideal candidate for therapeutic intervention in patients presenting with breast cancer.

In summary, SCD1 may control the overall rate of lipid synthesis and then affect cell proliferation in breast cancer cells. SCD1 may be a promising molecular target for cancer therapy. Consistent with FA synthesis, FA uptake and transport may be another important target pathway for anticancer therapy. Our study suggests that there was a synergistic cytostatic effect when both pathways were blocked simultaneously. Selective inhibition of key enzymes in endogenous lipogenesis, such as SCD1, combined with an exogenous FA uptake channel protein, such as CD36, may be a potential chemotherapeutic strategy for breast cancer. Although, our investigations reveal a synergistic antitumor response in breast cancer cells to SCD1 inhibition paired with CD36 depletion, the exact mechanism of this synergistic effect is not well defined, and further studies must be conducted in some other cell lines.

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