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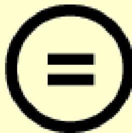

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Anticancer Effect of Chloroform Fraction from Broccoli (*Brassica oleracea* L.) Sprout on Breast Cancer Stem Cells

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Advanced Convergence Technology and Science

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브로콜리(**Brassica oleracea L.**) 새싹 클로로포름 분획물의 유방암 줄기세포에 대한 항암 효과

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이 논문을 이학 석사학위 논문으로 제출함

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Anticancer Effect of Broccoli (*Brassica oleracea* L.) Sprout Chloroform Fraction on Breast Cancer Stem Cells

Ji-Soo Kim

(Supervised by Professor Somi Kim Cho)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Interdisciplinary Graduate Program in Advanced Convergence Technology and Science

2022. 08.

This thesis has been examined and approved.

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Advanced Convergence Technology and Science

Graduate School
Jeju National University

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1. ABSTRCT

Broccoli (*Brassica oleracea L.*) sprout is a cruciferous vegetable that contain a variety of bioactive components. In this study, crude methanol extract (ME) of broccoli sprout was fractioned by hexane, chloroform, ethyl acetate, butanol, and water to compare the anti-proliferative effect on breast cancer cells. Among the fractions, hexane fraction (HF) and chloroform fraction (CF) decreased the viability of MCF-7/SCs like breast cancer stem cells (BCSCs), and the CF had the highest antiproliferative activity (IC_{50} = 69.47 mg/mL). The CF also suppressed the stemness characteristics of MCF-7/SCs and induced apoptotic cell death. The most abundant characteristic peak in CF was identified as oleic acid (area = 35.05%) by gas chromatography-mass spectrometry (GC-MS). We found that oleic acid, a main component in the CF, exhibited antiproliferative activity in MCF-7/SCs and MDA-MB-231/IR cells compared to the parental MCF-7 and MDA-MB-231 cells. Oleic acid induced the apoptosis and impaired characteristics of BCSCs. Furthermore, oleic acid inhibited migration and invasion ability of MCF-7/SCs and MDA-MB-231/IR cells in a dose- and time- dependent manner and suppressed the expression of EMT-related markers including Slug, Snail, ZEB1 and Vimentin. This oleic acid increased the low-level reactive oxygen species (ROS) in BCSCs while decreasing expression of glutathione peroxidase 1 (GPx1). Mechanistically, reduction of GPx1 expression by oleic acid inhibited FAK/NF-κB activation. This study indicates that the CF and oleic acid, a major component of broccoli sprouts, contribute to its potent anticancer activity against BCSCs.

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2. INTRODUCTION

Among the various vegetables, cruciferous vegetables are rich in beneficial bioactive compounds such as glucosinolates, phenolic acids, fatty acids, coumarins, carotenoids, and kaempferol, as well as other minor compounds (Manchali S et al., 2012). Along with this, as interest in sprouted vegetables increases, the possibility of extensive research on broccoli sprout belonging to the cruciferous family is also being shown (KS Lee et al., 2014). Broccoli (*Brassica oleracea L.*) sprout is rich in antioxidant vitamins and phenolic compounds, and contains significantly more glucoraphanin and indolic glucosinolates than broccoli florets (Gao J et al., 2014). Actually, broccoli sprout extract with high antioxidant activity contains a large amount of isothiocyanate, a metabolite of glucoraphanin, and exhibits excellent antioxidant activity along with various phenolic compounds (Jang HW et al., 2015). In addition, broccoli sprout is well known for their anticancer properties (M Nestle, 1998). According to a previous study, broccoli sprout extract caused cell death through cell cycle arrest and apoptosis in lung cancer cells, hepatocellular carcinoma cancer cells and colorectal cancer (TN Le et al., 2019), and also showed antiproliferative activities in prostate cancer cells (L Tang et al., 2006). Broccoli sprouts extract, which have anticancer effects on several cancers, had a high compositional ratio of oleic acid and linoleic acid such as unsaturated fatty acids, which potentially contribute to their anticancer activities (JJ Lee et al., 2009; P Pasko et al., 2018).

Oleic acid is a monounsaturated fatty acid found in the fat component of various plants, as well as broccoli sprouts (AM Tindall et al., 2020). Previously reported studies showed that oleic acid was effective in inducing cell proliferation inhibition in hepatocellular carcinoma cells and colorectal cancer cells (C Carrillo et al., 2012; Giulitti F et al., 2021), and affected several signaling pathways regulating cancer cell activation (C Carrillo et al., 2012).

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However, in breast cancer cells, oleic acid has shown opposing roles by promoting metastasis or cancer growth (S Li et al., 2014). The conflicting role of oleic acid is still a problem to be solved, and research on the mechanisms involved in breast cancer cells is still insignificant, so research needs to be continued. Therefore, elucidating the effect of oleic acid, a substance derived from broccoli sprout extract, on breast cancer stem cells along with broccoli sprout extract, can be an effective suggestion for breast cancer stem cells (BCSCs) treatment.

Breast cancer is the most common cancer among women worldwide, and cancer treatment for poor prognosis is an important issue (Heer E et al., 2020). Breast cancer can be initiated and maintained by a cellular subcomponent that displays stem cell properties (Charafe-Jauffret E et al., 2009). Cancer stem cells have superior ability to self-renew, invade and migrate compared to normal cancer cells through active metabolism by specific protein expression patterns (Prieto-Vila M et al., 2017). For example, $CD44^{+}/CD24^{-}$, aldehyde dehydrogenase (ALDH)-positive are used as phenotypes in BCSCs (C-J O. Conor et al., 2018), and multidrug resistance-associated protein 1 (ABCC1/MRP1), and P-glycoprotein/multidrug resistance protein 1 (ABCB1/MDR1) are overexpressed in cancer stem cells (Ji XW et al., 2019). By elevated expression levels of these markers, breast cancer stem cells acquired resistance to chemotherapy and radiation therapy and contributed to tumorigenesis and metastasis (S Palomeras et al., 2018; J He et al., 2018). Therefore, eliminating the BCSC population by targeting these markers could improve the effectiveness of current treatment strategies.

The contents and activities of biological substances vary depending on the extraction conditions, including the solvent used (Chavan U et al., 2001). Extraction using an appropriate solvent can provide a basic basis for various evaluations of plants. In general, through methanol extraction, which can bring both hydrophilic and hydrophobic components, it is

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possible to identify components by fraction by proceeding stepwise extraction from a solvent with a low polarity to a solvent with a high polarity (AR Abubaker et al., 2020).

In this study, we evaluated the antiproliferative activities of solvent fractions prepared in a stepwise manner from the crude methanol extract (ME) of broccoli sprout, and for the first time, we compared the antiproliferative and stemness-inhibitory effects of solvent fraction of broccoli sprout on BCSCs. Furthermore, we investigated a mechanistically pathway by which oleic acid, which accounts for the large content of the solvent fraction, affects BCSCs.

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3. MATERIALS AND METHODS

3-1. Samples and chemicals

Hot air-dried broccoli sprout powder was purchased from Damaonherb Co. (Yeongcheon, South Korea). Methyl alcohol, Hexane, Chloroform, Ethyl acetate and n-butanol were purchased from DaeJung Chemicals & Metals Co. (Siheung, South Korea). Oleic acid was purchase from Sigma Aldrich Co. (St. Louis, MO, USA). PARP, c-PARP, Caspase-3 and -9, MRP1, CD44, Snail, Slug, MMP9, ZEB1, Vimentin, FAK, pFAK, NF-κB, pNF-κB antibodies were purchased from Cell Signaling Technology Co. (Danvers, MA, USA). GPx1 antibody were purchased from Gene Tex Co. (Irvine, CA, USA).

3-2. Solvent fractionation

Broccoli sprout powder (100 g) was extracted with 2 L of 80% methanol, sonicated three times, and filtered using Whatman No. 2 filter paper. The extract was evaporated at 40°C in a vacuum rotary evaporator and lyophilized to generate crude ME. Dried ME powder (25 g) was suspended in 500 mL of distilled water and fractionated by n-hexane, chloroform, ethyl acetate, n-butanol, and water (1:1) in a stepwise manner. Each fraction was evaporated at 40°C and lyophilized to generate the hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), butanol fraction (BF), and residual water fraction (WF). Each extract was DMSO to 200 mg/mL for use in subsequent experiments.

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3-3. Cell culture

Human breast cancer MCF-7 cells and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The BCSC line MCF-7/SCs were sorted from MCF-7 cells based on $CD44^+$ and $CD24^-$, and characterized as described previously (NB To et al., 2020). The MDA-MB-231/IR cells were established from MDA-MB-231 cells after 25 cycles of 2 Gray irradiation for 5 weeks and characterized as previously reported (SY Koh et al., 2019). MDA-MB-231 cells, MDA-MB-231/IR cells, MCF-7 cells and MCF-7/SCs were cultured in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 (Thermo Fisher Scientific Co.,Waltham, MA, USA) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained at 37°C in an atmosphere containing 5% CO_2.

3-4. MTT assay

MDA-MB-231 cells, MDA-MB-231/IR cells, MCF-7 cells and MCF-7/SCs were seeded (5,000/well) in 96-well plates and incubated for 24 h. Cells were treated with the fractions or oleic acid for 24 h and exposed to 100 μL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 2 h. Next, 150 μL of DMSO was added to each well to solubilize formazan, and the plates were shaken for 30 min in the dark. Absorbance at 570 nm was evaluated using a microplate reader. IC_{50} values were calculated using GraphPad Prism 7.0 software.

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3-5. Hoechst 33342 staining

MCF-7/SCs and MDA-MB-231/IR cells were seeded into wells (1×10^4/well) and incubated for 24 h. Next, cells were treated with the CF or oleic acid for 24 h, stained with Hoechst 33342 solution (Invitrogen Inc., Waltham, MA, USA) (10 μg/mL) for 10 min in the dark, and visualized using a fluorescence microscope (×100) (IX73; Olympus Corporation, Tokyo, Japan).

3-6. Colony formation assay

MCF-7/SCs and MDA-MB-231/IR cells (400/mL) were seeded for 24 h and treated with CF or oleic acid for 10 days. Colonies were washed twice with PBS, fixed with 4% paraformaldehyde, and stained with 2% crystal violet for 30 min.

3-7. Mammosphere formation assay

For the mammosphere formation assay, we used the MammoCult Human Medium (StemCell Technologies Inc., Vancouver, Canada). and followed company's guidelines described. Simply, 2×10^4 cells were seeded in a non-coated 6mm dish with oleic acid. Cells were incubated for 10 days to form mammospheres, and observed under a phase-contrast microscope.

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3-8. Flow cytometry

Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin, NJ), as described previously (SY Koh et al., 2019). Identical numbers of cells (1 × 10^5/dish) were seeded for 24 h and treated with the CF or oleic acid of oleic acid for 24 h. To assay ALDH activity, the ALDEFLUOR Assay Kit (StemCell Technologies Inc., Vancouver, Canada) was used according to the manufacturer's instructions. Diethylaminobenzaldehyde (DEAB), an inhibitor of ALDH, was used as the negative control. PE-conjugated anti-human CD24 and FITC-conjugated anti-human CD44 antibodies (BD Pharmingen, San Diego, CA) were added to 100 μL of immunofluorescence staining buffer and incubated for 10 min at 4°C. The cells were washed with PBS and the $CD44^+/CD24^-$ cell population was analyzed. To identify apoptotic cells, the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA) was used following the supplier's instructions. Briefly, cells were suspended in annexin V-FITC (1:20 dilution in 1× binding buffer) and propidium (PI; 1:50) and analyzed within 30 min.

3-9. Wound Healing assay

MCF-7/SCs and MDA-MB-231/IR cells (2 × 10^5/mL) were seeded in six-well plates until 95% confluence, and a scratch wound was made in each well using a sterile pipette tip. Next, the cells were treated with the CF or oleic acid at non-lethal concentrations and incubated. Wounds were visualized using an inverted phase-contrast microscope at 4× magnification.

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3-10. Invasion assay

The upper chamber of 24-well Transwell plates (Corning, Corning, NY) was filled with Matrigel and normal culture medium. After the gel had solidified, each well was loaded with MCF-7/SCs and MDA-MB-231/IR cells (2.5×10^5) in serum-free medium, with or without CF of oleic acid, and the lower chamber was loaded with culture medium. After 24 h, the cells were fixed with 4% paraformaldehyde and stained with 2% crystal violet. The cells were observed under a phase-contrast microscope.

3-11. GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) of CF was carried out using the Shimadzu GCMS-QP-2010 Plus instrument in the Bio-Health Materials Core-Facility of Jeju National University, with a DB-5MS GC column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). The injection volume of 1 μL (100 μg/mL dissolved in methanol) was delivered in splitless mode. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The temperature ranged from 80 to 300°C (80°C hold for 5 min; 80–280°C at 5°C/min for 10 min; and 280–300°C at 10°C/min for 10 min). The total run time was 67 min, and mass spectra were detected using W9N08 Wiley library ver. 9.0 at a similarity cut-off of 90%.

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3-12. Quantitative Real Time PCR

Total RNA was extracted from cancer cells using TRIzol (Invitrogen Inc., Waltham, MA, USA) reagent and reversed-transcribed into cDNA using ImProm-II^{TM} Reverse Transcriptase (Promega, Madison, WI, USA) following the manufacturer's instructions. Then, two-step quantitative real-time PCR was performed (Thermal Cycler Dice Real-Time System; Takara, Shiga, Japan) using a TOPreal™ qPCR 2× PreMIX kit (Enzynomics, Daejeon, Korea). The cycling conditions were as follows: initial hold at 95 °C for 15 min, and 40 cycles at 95 °C for 10 s and 60 °C for 30 s. A dissociation curve was generated (95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s). GAPDH was used as an endogenous control. Expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method.

3-13. Western blot assay

MCF-7/SCs and MDA-MB-231/IR cells were prepared at 4×10^5 in 100 mm dishes. After incubation for 24 h and treatment with the CF or oleic acid, cells were lysed with RIPA lysis buffer and protein concentrations in the lysates were quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific Co., Waltham, MA, USA). The lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Except for β-actin (1:10,000), the primary antibodies were used at 1:1,000 dilutions, and secondary antibodies (anti-rabbit and -mouse IgG) were used at 1:5,000 dilutions. Protein bands were developed using the ECL Plus Kit (Biosesang Co., Seongnam, South Korea).

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3-14. Immunofluorescence

The cells were inoculated in a 6-well plate with cover glass slide in advance. After treatment with oleic acid for 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. The cells were washed with PBS twice, permeabilized with 0.1% Triton X-100 (Thermo Fisher Co., Waltham, MA, USA) and BSA at room temperature for 15 min, and finally incubated with the primary antibody (diluted in the above 0.1% Triton X-100 and BSA) overnight. The next day, the cells were washed with PBS. Subsequently, the cells were incubated with fluorescent secondary antibody in PBS for 1 h in dark condition, washed with PBS, and dyed with Hoechst 33342 for 10 min. The cover glass slide was sealed with glycerol, observed, and photographed under the laser confocal microscope (Leica Co., Wetzlar, Germany).

3-15. Statistical analysis

Groups were compared using GraphPad Prism 7.0 software, Student's *t*-test, and one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Data are expressed as means ± standard deviation (SD) of three replicates and statistical significance was set at $p < 0.05$. Pearson's rank correlation was performed using the 'correlation' tool in Prism 7.0 software.

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4. RESULTS

4-1. Antiproliferative activities of chloroform fraction on breast cancer stem cells

To evaluate the antiproliferative effect of the five fractions of broccoli sprout against breast cancer cells, MTT assay was performed. The MCF-7/SCs used are breast cancer stem cells (BCSCs) with stem cell characteristics constructed from MCF-7 (NB To et al., 2020). As shown in Figure 1, cell viability was reduced significantly in MCF-7/SCs by the HF and CF, but no cytotoxicity was indicated in MCF-7, a parental cell, and Fibroblast of the normal cell, at the same concentration. The CF had the lowest IC_{50} value (69.47 μg/mL) among the fractions (Table 1). These data suggest that among the fractions of broccoli sprout extract, the CF is a potential fraction capable of inhibiting BCSCs. Therefore, we decided to determine whether CF impairs stem cell characteristics of MCF-7/SCs, stem cell-like.

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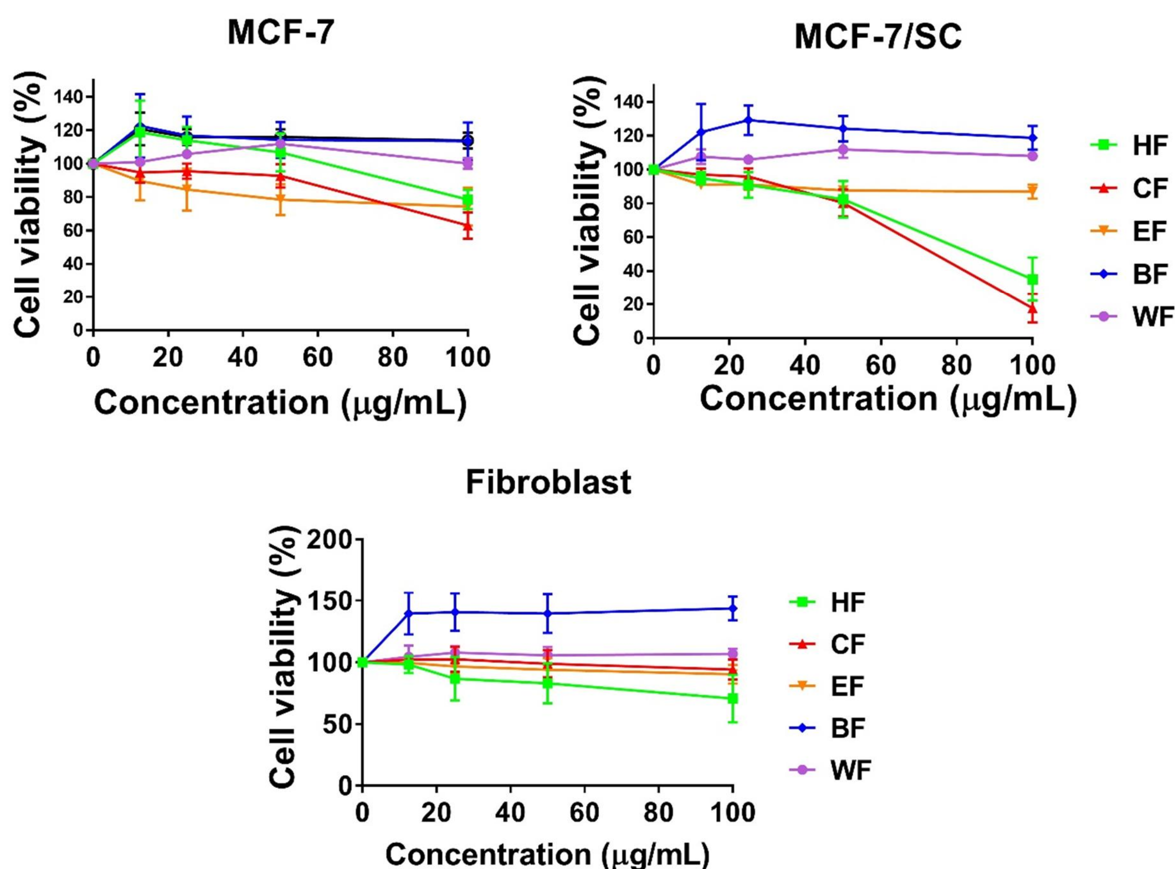

Figure 1. The antiproliferative potential of broccoli-sprout fractions. Cell viability of MCF-7 cells, MCF-7/SCs and Fibroblast cells was measured by MTT assay after treatment with the indicated solvent fractions for 24 h. Data are from three independent experiments (N = 3).

Table 1. IC_{50} (μg/mL) values of solvent fractions of broccoli sprout.

IC_{50} (μg/mL)	MCF7	MCF7-SC	Fibroblast
Hexane	>100	81.53	>100
Chloroform	>100	69.47	>100
Ethyl acetate	>100	>100	>100
Butanol	>100	>100	>100
Water	>100	>100	>100

IC_{50} values are half-maximal inhibitory concentrations from three independent experiments (N = 3)

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4-2. Effect of chloroform fraction on the stemness characteristics

The growth inhibitory effect of the CF on MCF-7/SCs was confirmed by colony formation assay (Figure 2A). FACS analysis showed a marked decrease in the $CD44^{+}/CD24^{-}$ population, the phenotype of BCSCs, on MCF-7/SCs treated with CF at 100 μg/mL, at both 24 and 48 h (Figure 2B). In addition, ALDH activity, a metabolic cancer stem cell marker (Fillmore C et al., 2007), was markedly reduced by non-lethal concentrations of CF (Figure 2C). In addition, the migration and invasion abilities of MCF-7/SCs were suppressed by CF (Figure 2D and 2E). Western blotting showed that the levels of β-catenin, CD44, and MRP1 were significantly decreased in MCF-7/SCs treated with CF in a dose-dependent manner (Figure 2F). These results suggest that CF impairs stemness characteristics of MCF-7/SCs.

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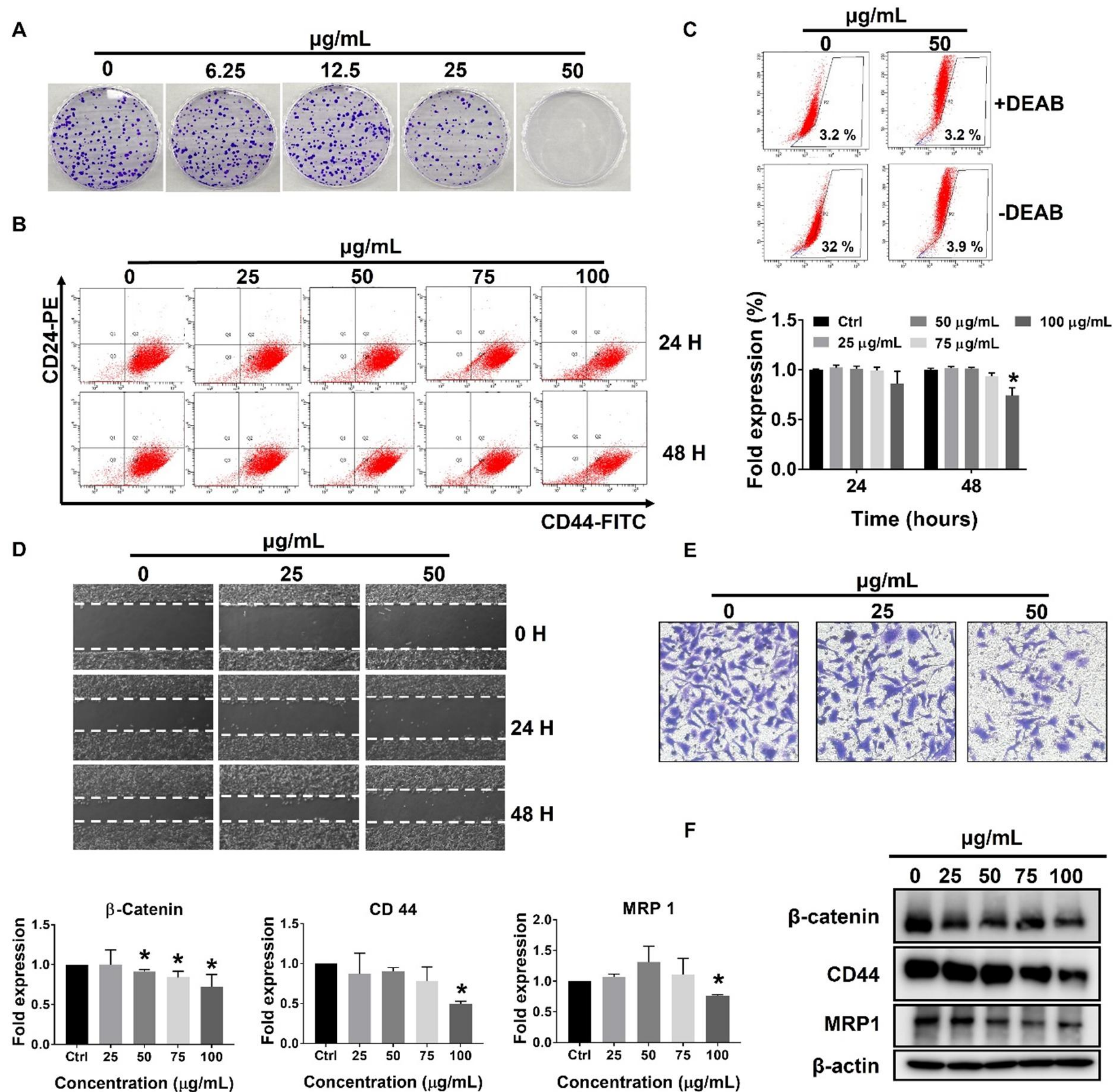

Figure 2. CF attenuates stem like-cell characteristics in MCF-7/SCs as a BCSCs. (A) Colonies were formed via CF treatment for 10 days (B) $CD44^{+}/CD24^{-/low}$ population measured by FACS after 24 or 48 h of treatment with CF. (C) $ALDH^{+}$ population after CF treatment for 24 h determined using an Aldefluor assay kit; negative control, diethylaminobenzaldehyde (DEAB). (D) Migrated cells after 24 or 48 h of treatment with CF by wound healing assay. Phase-contrast micrographs (100× magnification). (E) Cell invasion determined using Transwells after 24 h of treatment with CF (0–50 μg/mL). Phase-contrast micrographs (100× magnification). (F) Stemness markers after 24 h of treatment with CF detected by Western blotting. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). *$P < 0.05$ for each group compared with the control.

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4-3. Induction of apoptosis by the chloroform fraction

Apoptosis is characterized by morphological changes such as condensed and fragmented chromatin with apoptotic bodies (Vassalli G, 2019; Saraste A et al., 2000). Hoechst 33342 staining revealed condensed nuclei in CF-treated MCF-7/SCs, but not in control cells (Figure 3A). The Annexin V/PI staining showed the CF induced early and late apoptotic cell death in a time- and dose-dependent manner (Figure 3B). Furthermore, the levels of apoptosis-related proteins detected by western blotting showed that the Bax to Bcl-2 ratio decreased in a dose-dependent manner. Furthermore, the CF reduced the levels of pre-caspases-9, -3, and -7, whereas the proteolytically cleaved PARP level increased significantly in a concentration-dependent manner (Figure 3C). These data suggest that CF induces cell death of MCF-7/SCs via apoptosis.

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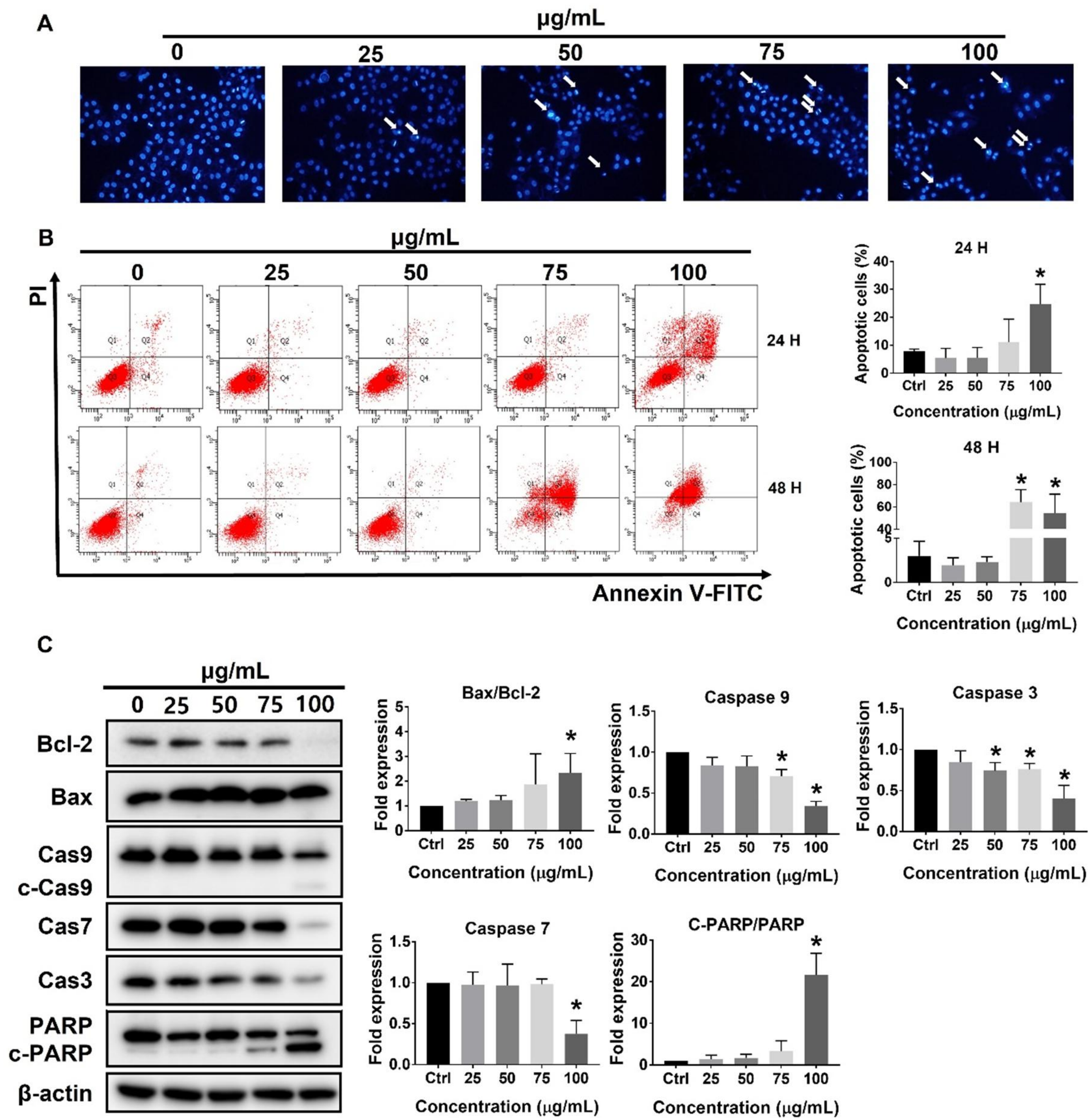

Figure 3. CF induces apoptosis of MCF-7/SCs. (A) Apoptotic nuclei observed by Hoechst 33342 staining after 24 h of treatment with CF. (B) Cells were treated with CF for 24 h and stained with annexin V-FITC/PI. (C) Apoptosis markers after 24 h of treatment with CF by Western blotting. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). *$P < 0.05$ for each group compared with the control.

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4-4. GC-MS analysis of the chloroform fraction

Gas chromatography-mass spectrometry (GC-MS) is a mass spectrometry method that matches and screens an unknown compound with a library through a volatile gas obtained by oven at high temperature (David J Beale et al., 2018). So, the CF was analyzed by GC-MS to identify its major constituents. The dominant compound in CF was oleic acid (35.05 ± 3.65 %), an unsaturated fatty acid (Table 2). Oleic acid has been shown to be involved in cancer growth inhibition in various cancer cell lines (C Carrillo et al., 2012). The results of this analysis suggest that we speculate that oleic acid contained in CF may contribute to suppression of BCSCs, MCF-7/SCs.

Table 2. Major compounds in CF detected by GC-MS.

No.	Compound	Area % [a]	Similarity	RT
1	2-Decenal, (E)-	0.08 ± 0.01	93	13.625
2	*Hexadecamethylcyclooctasiloxane*	0.64 ± 0.40	94	23.41
3	Myristic acid	0.08 ± 0.00	95	26.172
4	Methyl palmitate	3.26 ± 3.06	96	26.69
5	Cyclopentadecanone, 2-hydroxy-	0.91 ±0.06	92	29.945
6	Pentadecanoic acid	5.85 ±0.24	92	30.425
7	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	1.15 ±0.08	96	32.899
8	Methyl oleate	3.15 ± 0.25	94	33.037
9	Methyl elaidate	1.70 ± 0.00	95	33.144
10	Oleic acid	35.05 ± 3.65	92	34.029
11	Oleamide	1.90 ± 0.00	92	37.508
12	Cyclopropaneoctanoic acid, 2-octyl-, methyl ester	1.52 ±0.05	91	39.913
13	2-Palmitoylglycerol	0.98 ± 0.00	90	39.976
14	9-Octadecenylamide	0.20 ±0.06	92	43.95

RT, retention time

[a] Means and standard deviations are from three independent experiments (N = 3).

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4-5. Oleic acid, a major component in chloroform fraction inhibits proliferation of MCF-7/SCs and MDA-MB-231/IR cells

To determine whether oleic acid, a major component of CF revealed through GC-analysis, contributes to cytotoxicity against 4 types of breast cancer, MTT assay was performed. MCF-7 cells and MCF-7/SCs, MDA-MB-231 cells and MDA-MB-231/IR cells were used, and MDA-MB-231/IR cells, a resistant breast cancer cell constructed from MDA-MB-231 cells, were have shown differentiation from parental cells in a previous study (Yen T-K Nguyen et al., 2020). As illustrated in Figure 4A, among the breast cancer cell lines treated with oleic acid, only MCF-7/SC and MDA-MB-231/IR cells remarkably decreased cell viability. It was non-toxic to normal Fibroblast cells together with MCF-7 and MDA-MB-231 cells. Furthermore, oleic acid significantly reduced colony formation in MCF-7/SCs and MDA-MB-231/IR cells in dose-dependent manner (50-200 μM), but not in parental cells, MCF-7 and MDA-MB-231 (Figure 4B). The treatment of oleic acid indicated more sensitive cell growth inhibitory effect on MCF-7/SCs and MDA-MB-231/IR than MCF-7 and MDA-MB-231. Therefore, the following experiment was conducted to determine the effect of oleic acid on cell death.

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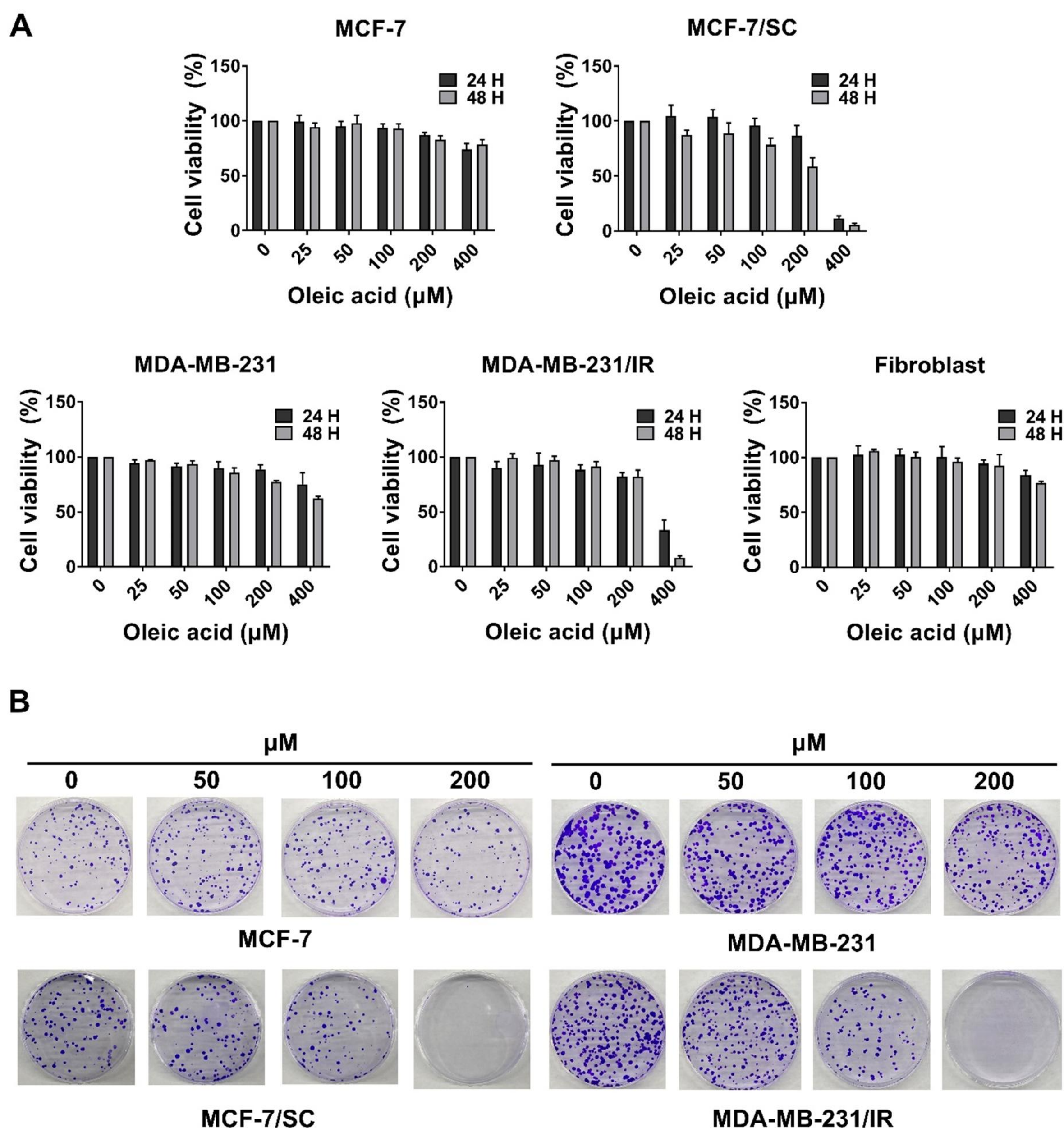

Figure 4. Cytotoxicity to breast cancer stem cells of oleic acid. Cell viability of MCF-7 cells, MCF-7/SCs, MDA-MB-231 cells, MDA-MB-231/IR cells and normal Fibroblast cells was measured by MTT assay after treatment with the indicated concentration for 24 h. (B) Colonies were formed via oleic acid treatment for 10 days. Data are from three independent experiments (N = 3).

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4-6. Oleic acid induces cell death of MCF-7/SCs and MDA-MB-231/IR cells via apoptosis

Apoptosis is a programmed cell death accompanied by morphological features which chromatin is condensed and biochemical mechanisms (S Elmore 2007). Hoechst 33342 staining was performed to determine whether cell death through induction of apoptosis in oleic acid-treated MCF-7/SCs and MDA-MB-231/IR cells. Condensed apoptotic bodies were increased by oleic acid in a dose-dependent manner (Figure 5A). In addition, the Annexin V/PI staining demonstrated that oleic acid led to an increase early and late apoptotic cell proportion of MCF-7/SCs and MDA-MB-231/IR cells (Figure 5B). The expression of apoptosis-related proteins in MCF-7/SCs and MDA-MB-231/IR cells was detected using western blotting (Figure 5C). The pre-caspases-9, -3 ratio decreased, and the levels of cleaved PARP increased significantly in a dose-dependent manner by oleic acid. These data show that oleic acid induces apoptosis in MCF-7/SCs and MDA-MB-231/IR cells.

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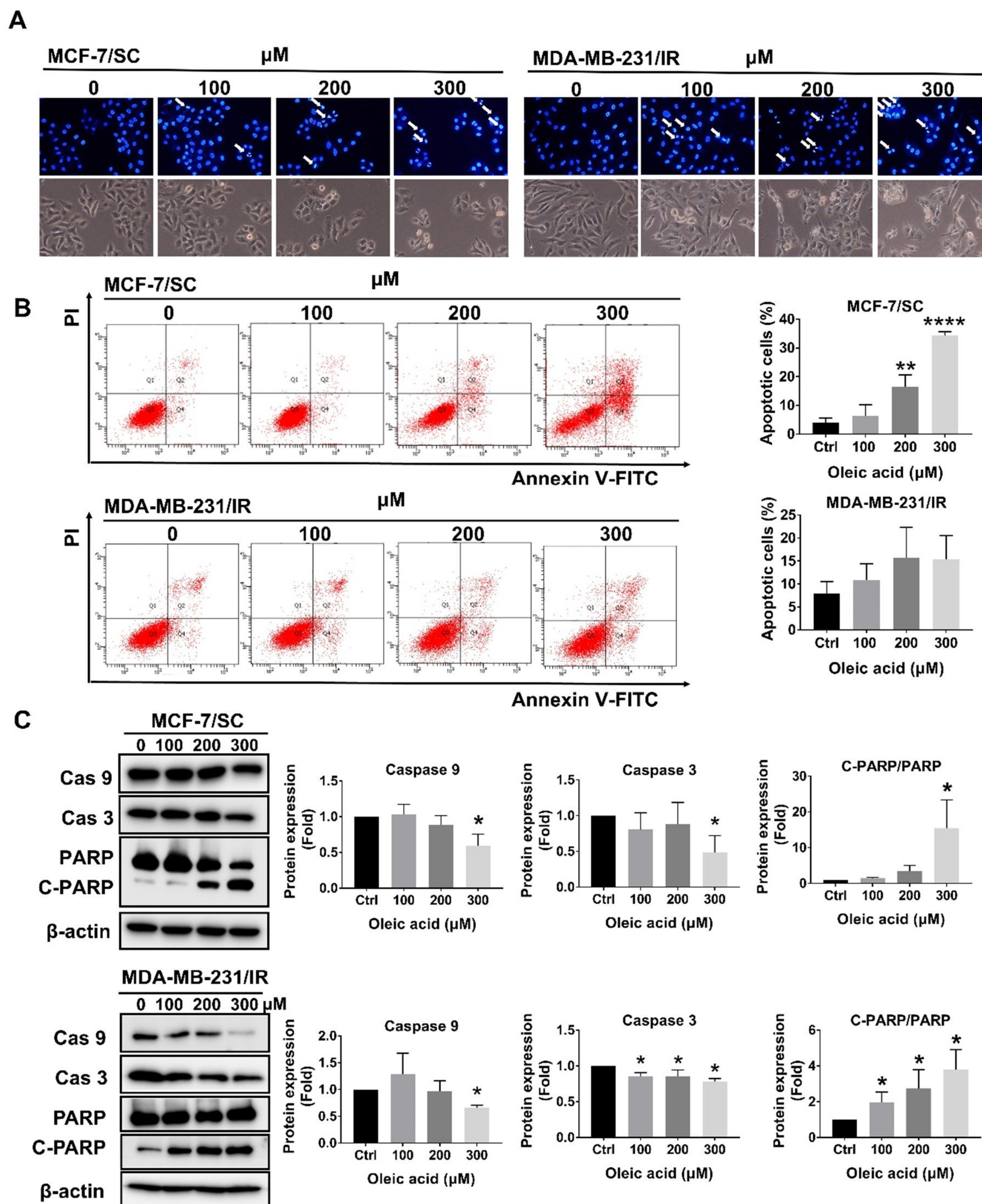

Figure 5. Oleic acid induces apoptosis of MCF-7/SCs and MDA-MB-231/IR cells. (A) Apoptotic nuclei observed by Hoechst 33342 staining after 24 h of treatment with oleic acid. (B) Cells were treated with oleic acid for 24 h and stained with annexin V-FITC/PI. (C) Apoptosis-associated markers after 24 h of treatment with oleic acid by Western blotting. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). *$P < 0.05$ for each group compared with the control.

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4-7. Oleic acid impairs stemness of MCF-7/SCs and MDA-MB-231/IR cells

Mammospheres are biomarkers used to identify signaling pathways involved in maintaining the cancer stem cell phenotype (Saddin K et al., 2013). In MCF-7/SCs and MDA-MB-231/IR cells mammosphere formation and size was markedly reduced by oleic acid (Figure 6A). Also, oleic acid-treated in MCF-7/SCs and MDA-MB-231/IR cells resulted in reductions of the population of $CD44^{+}/CD24^{-}$, an important phenotype of breast cancer cells, and ALDH activity, a metabolic cancer stem cell marker (Figure 6B and 6C). As shown in Figure 6D, oleic acid dose-dependently suppressed the expression of β-catenin, CD44 and MRP1 detected by western blotting. These results suggest that oleic acid is a substance that can target BCSCs by significantly reducing the properties of BCSCs.

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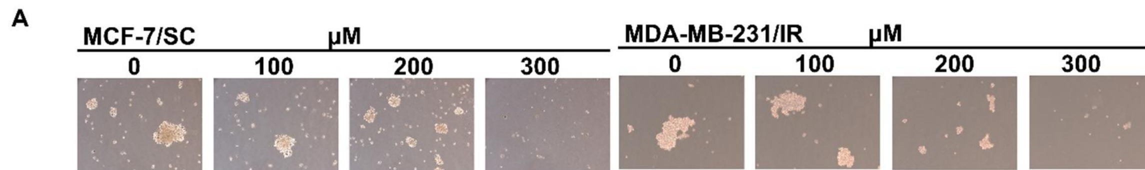

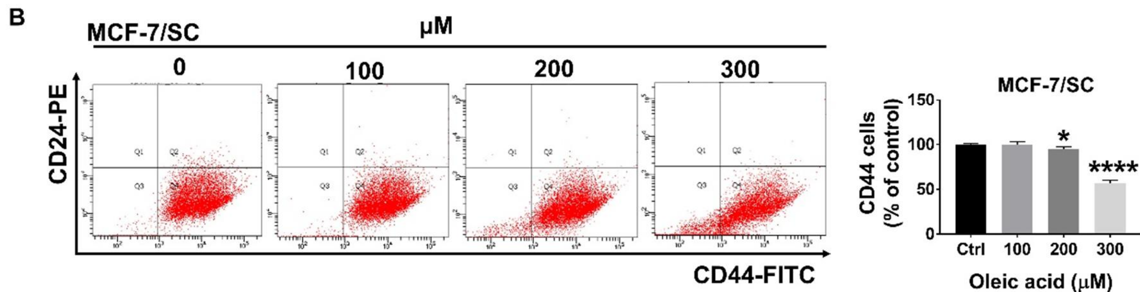

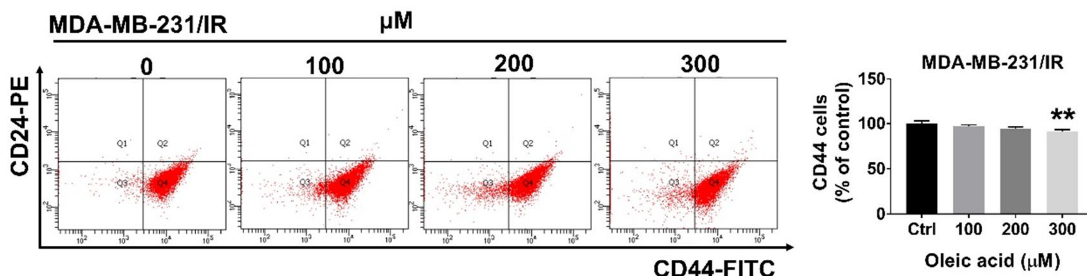

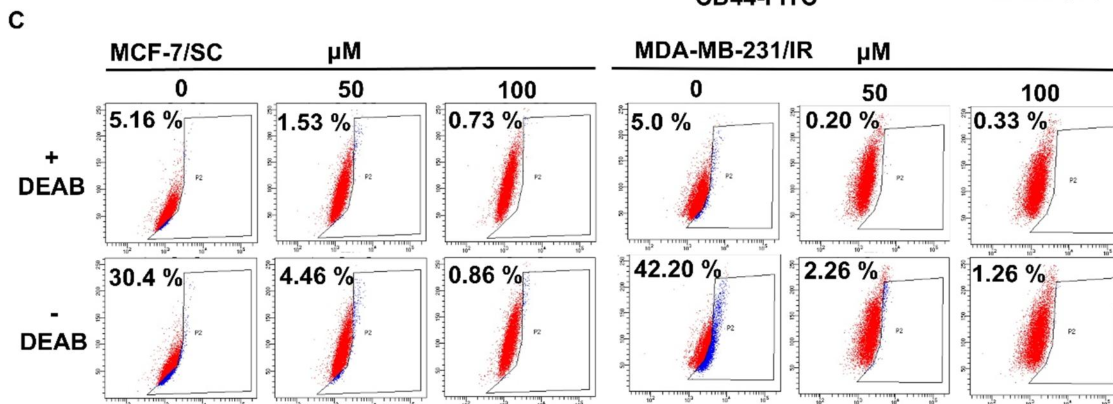

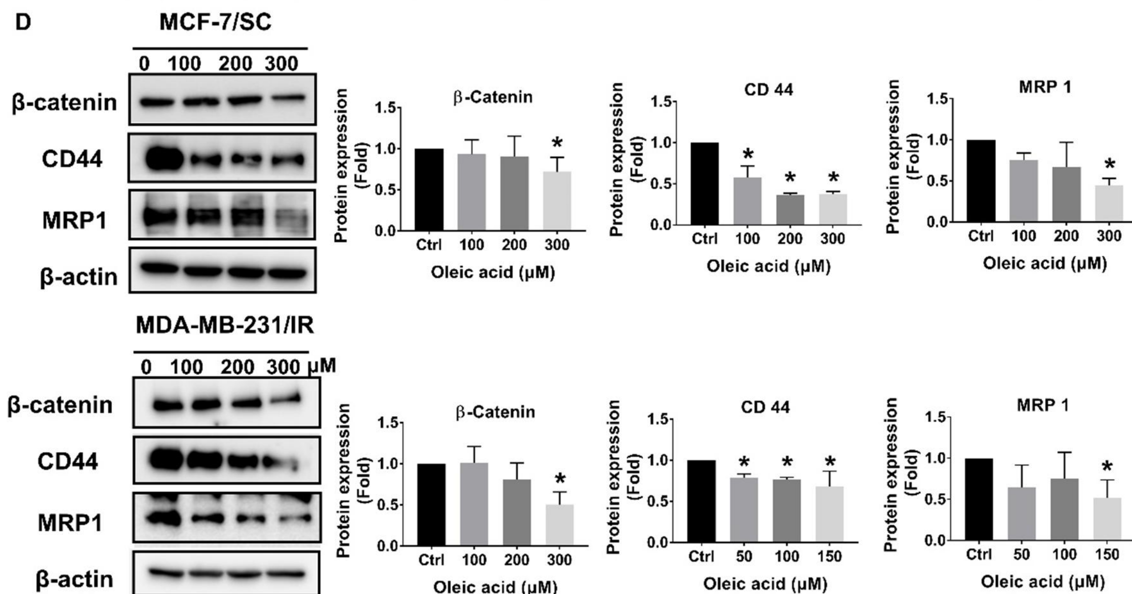

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Figure 6. Oleic acid attenuates stem like-cell characteristics in MCF-7/SCs and MDA-MB-231/IR cells. (A) Mammosphere were formed with oleic acid treatment for 10 days. (B) $CD44^{+}/CD24^{-/low}$ population measured by FACS after 24 h of treatment with oleic acid. (C) $ALDH^{+}$ population after oleic acid treatment for 24 h determined using an Aldefluor assay kit; negative control, diethylaminobenzaldehyde (DEAB). (D) Stemness-markers were detected by Western blotting after 24 h of treatment with oleic acid. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). *$P < 0.05$ for each group compared with the control.

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4-8. Oleic acid reduces migration and invasion abilities of MCF-7/SCs and MDA-MB-231/IR cells

Metastatic and invasive properties are acquired by contributing from the epithelial-to mesenchymal transition (EMT) programs (Gray W. Pearson., 2019). The effect of oleic acid on MCF-7/SCs and MDA-MB-231/IR cells attenuated migration and invasion ability at non-lethal concentrations (Figure 7A and 7B). The qRT-PCR results demonstrated that the expression of EMT-associated genes, MMP9, Slug, Snail and Vimentin, was decreased by oleic acid (Figure 7C). Furthermore, the reduction of protein expression of MMP9, Slug, Snail, Vimentin and ZEB1 by western blotting supports the evidence that oleic acid affects migration and invasion contributed by the EMT programs (Figure 7D). These data show that oleic acid reduces metastasis and invasion of BCSCs and retards the growth of BCSCs.

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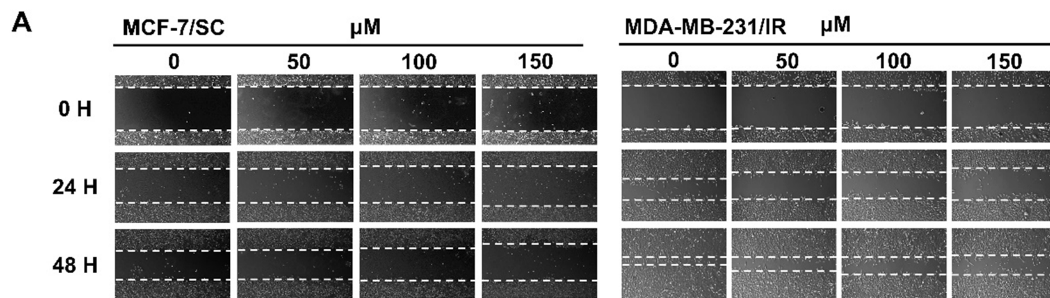

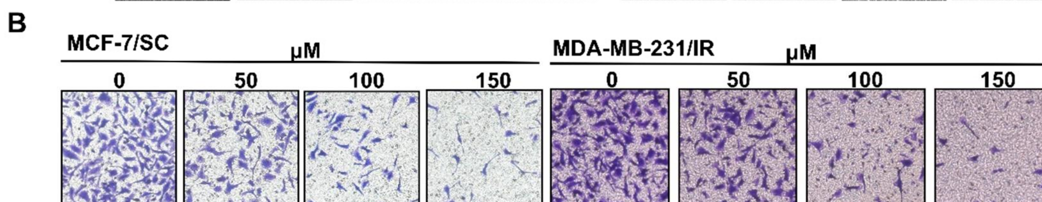

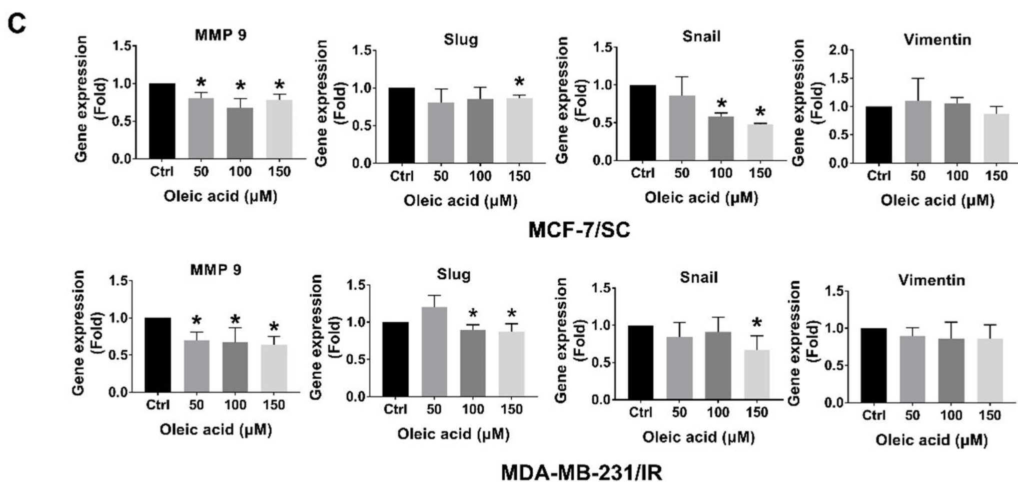

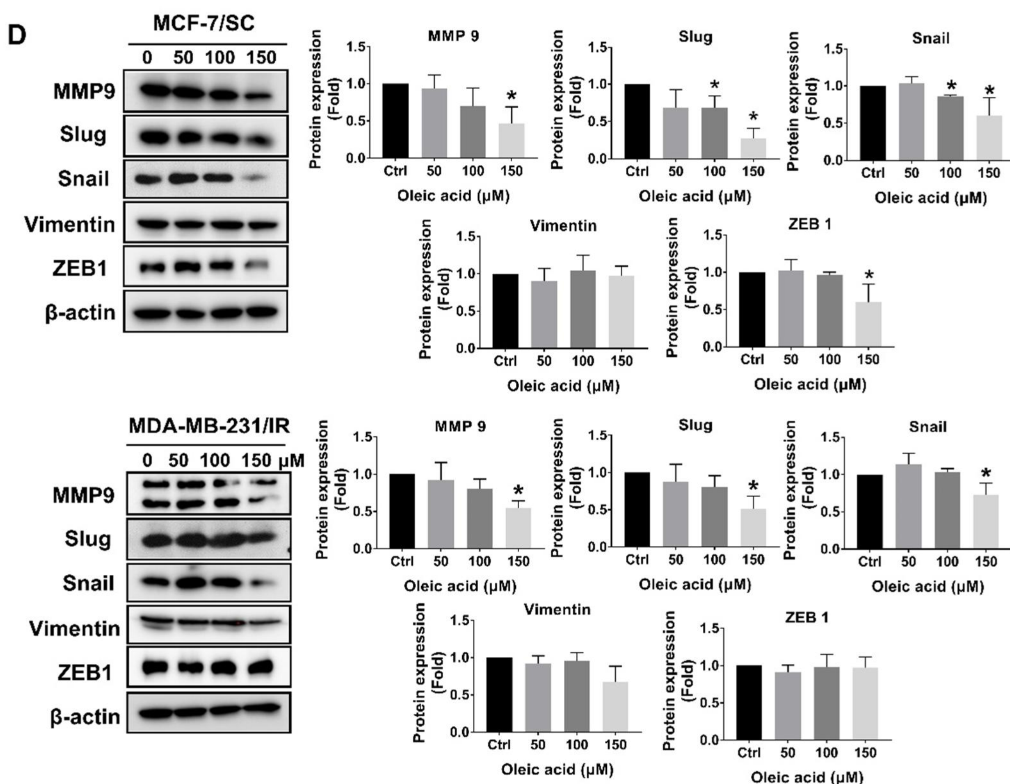

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Figure 7. Oleic acid reduces migration and invasion abilities of MCF-7/SCs and MDA-MB-231/IR cells. (A) Migration observed by Wound healing assay after 24 h or 48 h of treatment at non-lethal concentration. (B) Invasive cell was determined using trans-well after oleic acid treatment for 24 h. (C) The expression of EMT-related gene mRNA was detected by qRT-PCR. (D) EMT-related markers were detected after 48 h of treatment with oleic acid by Western blotting. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). $*P < 0.05$ for each group compared with the control.

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4-9. Oleic acid increases ROS levels of MCF-7/SCs and MDA-MB-231/IR cells while inhibiting GPx1

The stemness of cancer cells is significantly correlated with low levels of reactive oxygen species (ROS) that induce oxidative stress (X Shi et al., 2012). FACS analysis indicated that MCF-7/SCs and MDA-MB-231/IR cells had lower ROS levels compared to the parental cells, MCF-7 and MDA-MB-231 (Figure 8A). Oleic acid promoted ROS accumulation of MCF-7/SCs and MDA-MB-231/IR cells in a dose- and time-dependent manner (Figure 8B). Glutathione peroxidase (GPx) uses reduced glutathione to promote the breakdown of free radicals that cause oxidative stress (R Margis et al., 2008). Moreover, these GPxs regulate the ability to maintain stem cells in cancer stem cells (Y Jiao et al., 2017). Therefore, we confirmed the gene expression for GPx family, an antioxidant enzyme. As seen in Figure 9B, of GPx family 1-4, only GPx1 showed the higher basal gene expression ratio in both MCF-7/SCs and MDA-MB-231/IR cells than parental cells (Figure 8C). Correlating with the ROS results, the gene and protein expression of GPx1 were reduced after oleic acid treatment (Figure 8D and 8E). These results suggest that oleic acid may induce oxidative stress and affect cancer stem cell growth.

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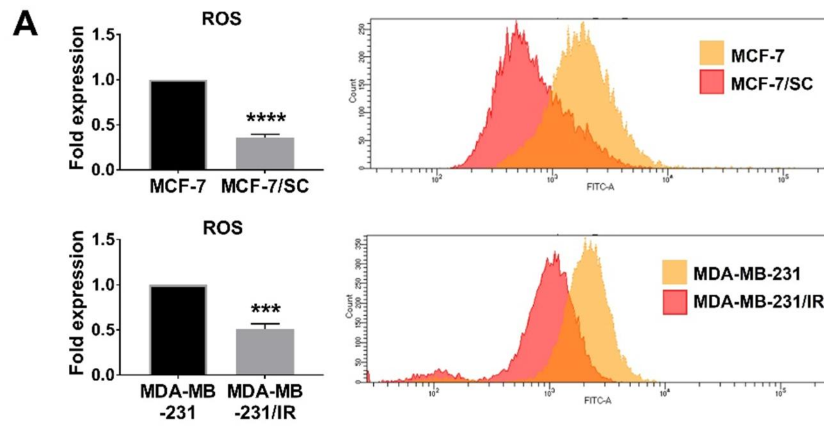

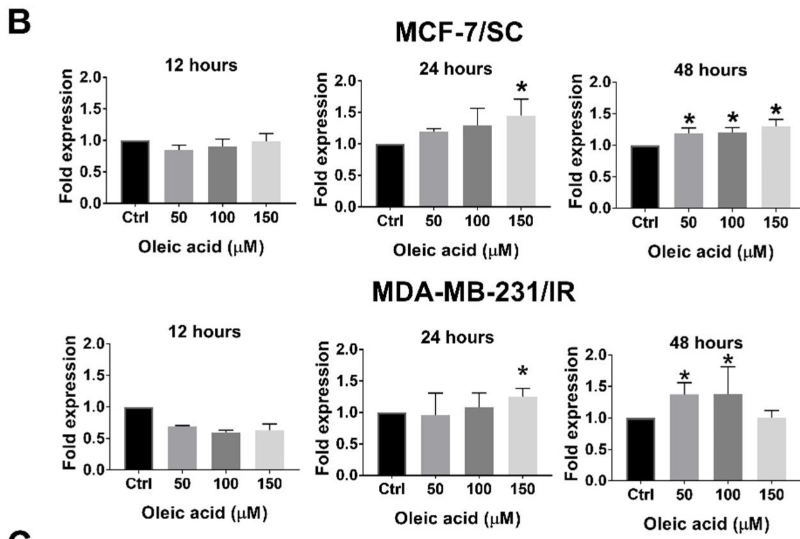

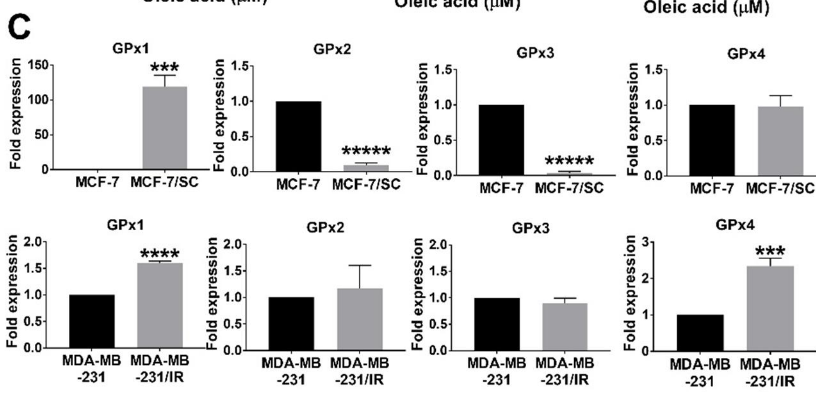

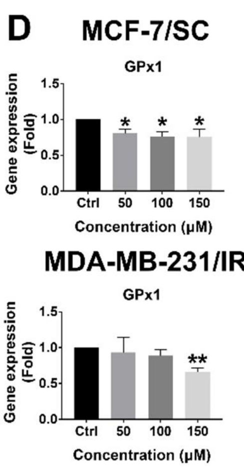

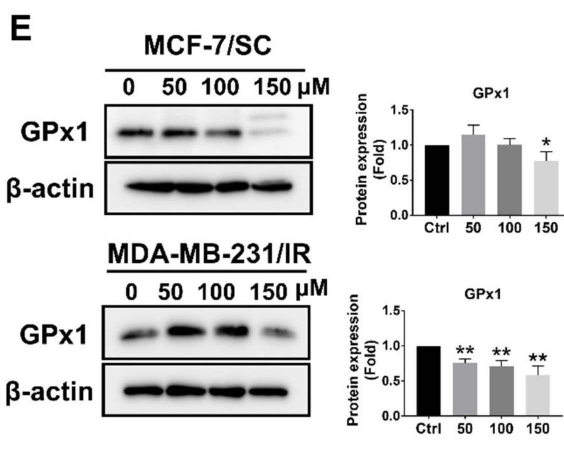

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Figure 8. Oleic acid induces an increase in ROS accumulation in MCF-7/SCs and MDA-MB-231/IR cells. (A) Basal ROS levels of breast cancer cells were determined by FACS. (B) The ROS level of MCF-7/SCs and MDA-MB-231/IR cells was detected after oleic acid treatment for 12, 24 and 48 h by FACS. (C) The expression of GPx family mRNA on breast cancer cells was detected by qRT-PCR. (D) The GPx1 expression of mRNA was analyzed after 48 h of treatment with oleic acid by qRT-PCR. (E) The expression of GPx1 protein was determined by Western blotting after oleic acid treatment. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). $^*P < 0.05$ for each group compared with the control.

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4-10. Oleic acid suppresses FAK/NF-κB signaling in MCF-7/SCs and MDA-MB-231/IR cells

Previous studies demonstrated that GPx1 interacts with focal adhesion kinase (FAK) in breast cancer cells (EK Lee et al., 2020). Moreover, FAK signaling regulates the transcription factor, nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) (T Petzold et al., 2009; X Wang et al., 2012), which enhances cancer stem cell metastatic capacity (VN Kolev et al., 2017; AL.Rinkenbaugh et al., 2016). To explore the molecular mechanisms of oleic acid-induced MCF-7/SCs and MDA-MB-231/IR cells, western blotting was performed. As illustrated in Figure 9A, treatment of oleic acid induced the inhibition of phosphorylated FAK and NF-κB in both cell lines. Additionally, immunofluorescence staining indicated that the nuclear translocation of NF-κB was decreased in MCF-7/SCs and MDA-MB-231/IR cells when treated with oleic acid compared to the control without any treatment (Figure 9B). These data suggest that the activity of NF-κB, a transcriptional activator regulated by FAK, is reduced by oleic acid.

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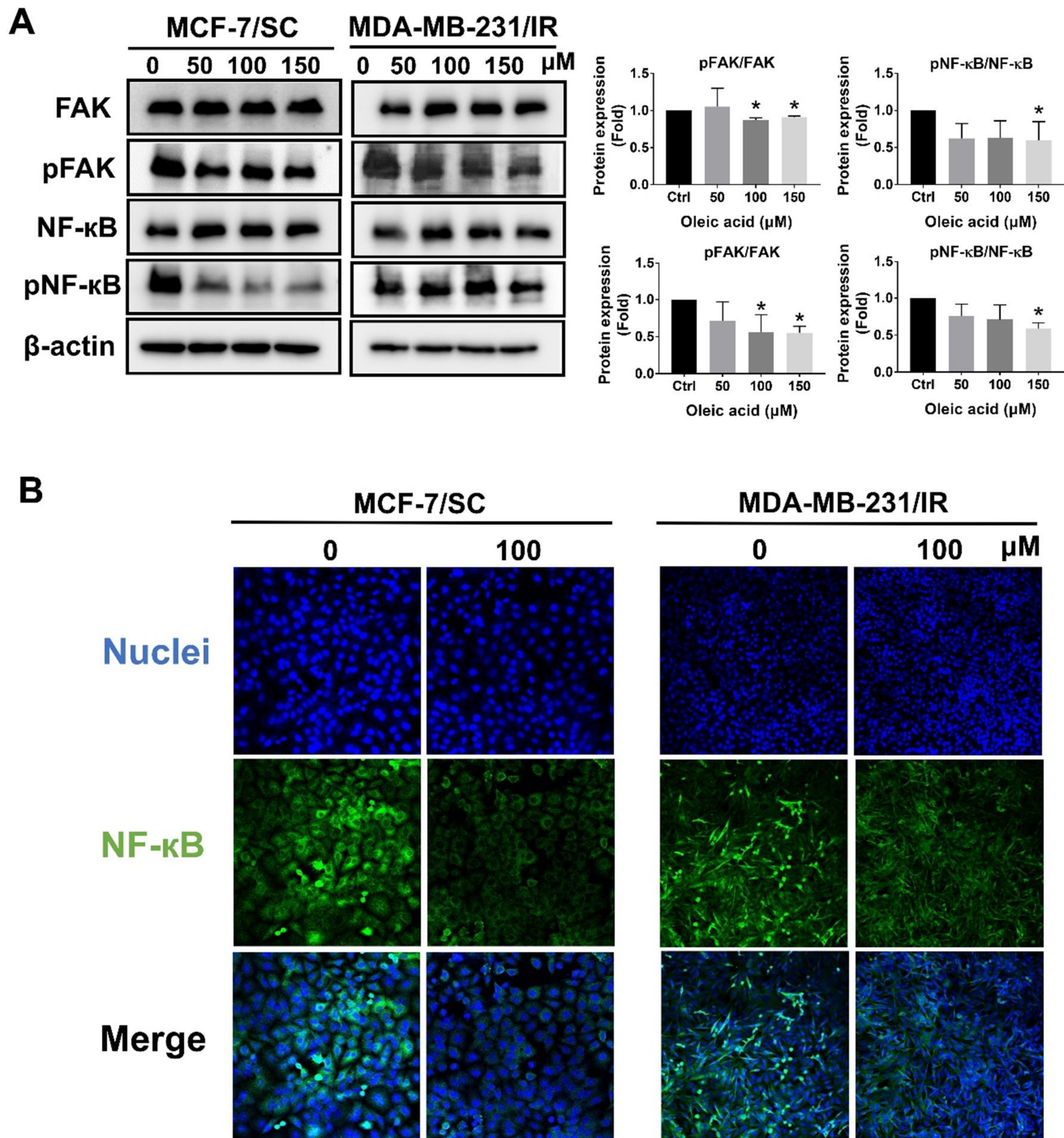

Figure 9. The FAK/NF-κB axis is inactivated by oleic acid in MCF-7/SC and MDA-MB-231/IR cells. (A) The expression of FAK, phosphorylated FAK, NF-κB and phosphorylated NF-κB protein was determined by Western blotting after oleic acid treatment. β-actin was used as the loading control. Data are means ± standard deviation (N = 3). *$P < 0.05$ for each group compared with the control. (B) The intracellular distribution of NF-κB (green) in MCF-7/SCs and MDA-MB-231/IR cells treated with oleic acid 100 μM was determined by Immunofluorescence (IF), and the nucleus was stained with blue. Representative IF images are shown at 200 × magnification.

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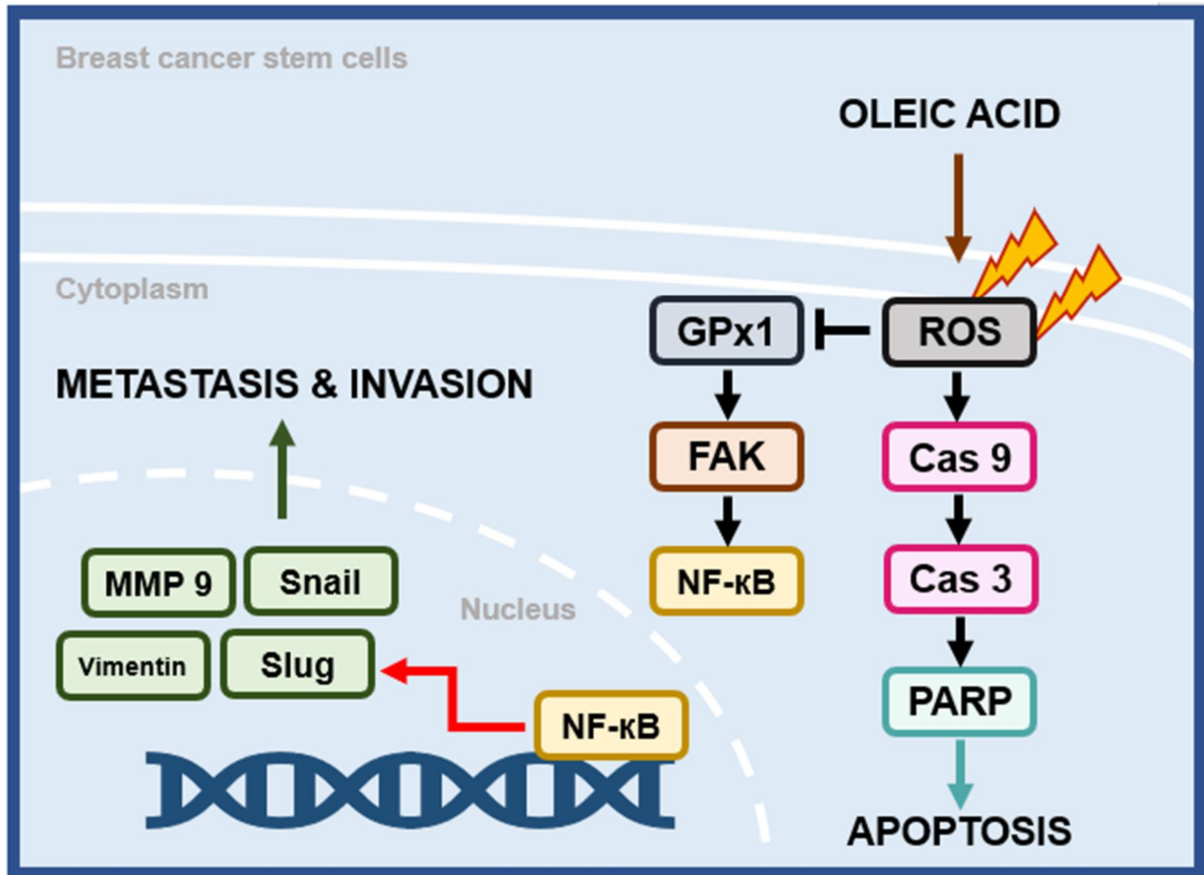

Figure 10. The schematic of the oleic acid-mediated mechanism on MCF-7/SCs and MDA-MB-231/IR cells, like a BCSCs. In BCSC, oleic acid-induced increase in ROS level and decrease in GPx1 decrease FAK phosphorylation, thereby inhibiting nuclear translocation and activity of NF-kB, resulting in down-regulate ability of metastasis and invasion. On the other hand, an increase in ROS level induces cell death through apoptosis.

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5. Discussion

As germinated vegetables grow, they produce many bioactive substances that can defend themselves to adapt to different environments (Simon Okomo Aloo et al., 2021). Broccoli (*Brassica oleracea L.*) sprouts, a member of the cruciferous vegetables, are well known for providing health benefits just as much as their mature broccolis (Jang HW et al., 2015; Simon Okomo Aloo et al., 2021) Especially, broccoli sprout is rich in metabolite glucosinolate, which produces a lot of isothiocyanate with excellent antioxidant and anti-cancer activities (TA Shapiro et al., 2001). As research on their activity through extracts of cruciferous vegetables, including broccoli sprouts, continues, expectations for the pharmacological functions of medicinal plants are also increasing. Extracting broccoli sprouts using polar and non-polar solvents is a fundamental method to isolate hydrophilic and hydrophobic bioactive compounds present in broccoli sprouts, and serves as a basis for evaluating the difference in activity of bioactive compounds (Abdullahi R. Abubakar et al., 2020). In this study, we confirmed the difference in antiproliferative properties according to the broccoli sprout fraction separated by extracting with various solvents. We obtained five fractions including HF, CF, EF, BF and WF from crude ME of broccoli sprout using stepwise fractionation, and the antiproliferative effect was most sensitive to the CF in MCF-7/SCs, like a BCSCs (Figure 1). MCF-7/SCs are cells that overexpress CD44, a cell surface glycoprotein that is also used as a marker for BCSCs. In addition, previous study has shown that MCF-7/SC exhibits characteristics of BCSCs (To NB et al., 2020). CF impaired the stemness of these MCF-7/SCs together with reducing migration and invasion, and induced apoptosis (Figure 2 and 3). GC-MS demonstrated that the CF contained various fatty acids, especially a lot of oleic acid (Table 2). According to reported studies, broccoli and broccoli sprout are high in oleic acid, an unsaturated fatty acid (P Pasko et al., ;2018 Adam M et al.,2022). Oleic acid is an

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unsaturated fatty acid often found in olives, sunflower seeds, almonds (M. L Hernandez et al., 2021; M R Akkaya, 2018; S Sathe et al., 2008). In addition to its ability to promote wound healing, oleic acid is suggested to have beneficial effects on autoimmune and inflammatory diseases and contributes to its impact on human healths (H Sales-Campos et al., 2013). According to a previously studies, oleic acid suppresses breast cancer by decreasing cell viability and inducing apoptosis in MCF-7 (TH Kim et al., 2020), or inhibiting the Her2/neu expression in Her2 positive cells (J A Menedez et al., 2005). Moreover, oleic acid induced autophagy in hepatocellular carcinoma (Giulitti F et al., 2021) and reduced store-operated calcium entry at low concentration leading to affecting growth signaling in colon carcinoma cells (C Carrillo et al., 2012). On the other hands, oleic acid induced metastasis through AKT-dependent signaling (Cleofas M-M et al., 2019), or increased invasion by promoting EGFR activation in breast cancer cells (A S-Guzman et al., 2010). The effects of oleic acid on breast cancer have shown conflicting results according to the studies performed so far, and the role of oleic acid on cancer stem cells is still unknown. Therefore, an in-depth study on oleic acid related to breast cancer stem cells is needed. In this study, oleic acid had specific cytotoxic effect on MCF-7/SCs and MDA-MB-231/IR cells (Figure 4). This inhibited cell growth of MCF-7/SCs and MDA-MB-231/IR cells by colony formation, and increased cell death via apoptosis (Figure 5). In addition, it impaired the characteristics of stem cells and lowered the ability of migration and invasion (Figure 6 and 7). Therefore, we investigated the mechanisms by which oleic acid affects MCF-7/SCs and MDA-MB-231 cells, stem-like cells. Cancer stem cells have a lower ROS level than normal cancer cells, but the presence of intrinsic ROS can induce stem cells by improving cell differentiation and regeneration while maintaining homeostasis (X Shi et al., 2012). However, high ROS levels promotes to broken cell function, leading to cell exhaustion or death (A Nugud et al., 2018; Y Ikeda et al., 2021). Several previous studies demonstrated that oleic acid increases ROS levels in vascular

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smooth muscle cell, fibroblast and neuronal tissues (G Lu et al., 1998; E Hatanaka et al., 2013; J.L Alves et al., 2020). Surprisingly, oleic acid enhanced the ROS levels of MCF-7/SCs and MDA-MB-231/IR cells, which was low levels compared to parental cells (Figure 8). Oleic acid decreased only GPx1 among the GPx family that hydrolyze active oxygen. GPx1, which showed a particularly high expression level in MCF-7/SCs and MDA-MB-231/IR cells among the GPx families opposite to ROS was reduced by oleic acid treatment. Previous studies have described the interaction between GPx1 and FAK, showing that FAK activity is inhibited when expression of GPx1 is silenced (EK Lee et al., 2020). In MCF-7/SC and MDA-MB-231/IR cells, FAK phosphorylation was attenuated by oleic acid, which may be due to GPx1 reduction (Figure 9), but it is necessary to study the interaction between GPx1 and FAK in our cells. In addition, NF-kB, a transcription factor as a sub-regulator of FAK, was also reduced by oleic acid. NF-kB regulates the transcription level of EMT-related markers (BRB Pires et al., 2017). Due to this, it is possible that the reduction of EMT-related markers regulated by NF-kB may inhibit breast cancer stem cell metastasis and invasion.

6. Conclusions

Broccoli sprouts are cruciferous vegetables that are functional plants with a variety of benefits. The broccoli sprout extract showed differences in antiproliferative activities according to stepwise fractions, and CF among the fractions directly inhibited BCSCs. This study suggests that broccoli sprout have beneficial effect as various functional foods. Oleic acid, a major component of CF, induced cell growth inhibition and apoptosis in MCF-7/SCs and MDA-MB-231/IR cells. In addition, oleic acid attenuated FAK/NF-kB signaling by increasing ROS levels, and decreased metastasis and invasion of breast cancer with stem characteristics (Figure 10). Our study suggests that broccoli sprouts may be a functional food

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contributing to their anticancer effects on BCSCs, and may provide sufficient evidence for a hitherto unexplained role of oleic acid on BCSCs.

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