The effects of Low α-Linolenic fatty acid Soybean Oil and Mid Oleic acid Soybean Oil on the growth of Her-2/*neu* and Fatty acid synthase over-expressing human breast cancer (SK-Br3) cells

Jee Hyun Bark

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Human Nutrition, Food and Exercise

Young Ju, Chair,

Eva M Schmelz,

Dongmin Liu

09-09-2010

Blacksburg, VA

Keywords: Human Breast Cancer, SK-Br3, Soybean Oil, Low linolenic Acid Soybean Oil, Epidermal Growth Factor Receptor, Fatty Acid Synthase

The effects of Low α-Linolenic fatty acid Soybean Oil and Mid Oleic acid Soybean Oil on the growth of Her-2/*neu* and Fatty acid synthase over-expressing human breast cancer (SK-Br3) cells

Jee Hyun Bark

ABSTRACT

A variety of soybean oils (SOs) were developed with improved functional properties. Some of the modified SOs contain altered fatty acid (FA) composition by selective breeding methods. Currently, low α- linolenic acid soybean oil (LLSO) and low α- linolenic acid and mid oleic acid soybean oil (LLMOSO) are available FA modified SOs in the market. The consumption of FA modified SOs has been increased because the United States Food and Drug Administration required listing *trans* fat content in food products sold in U.S. as an effort to reduce possible health risks caused by *trans* fat beginning 2006. However, the effects of these FA modified SOs on human chronic diseases including breast cancer (BC) have not been studied. BC has become the most frequently diagnosed cancer and is the second leading cause of cancer death among American women. The type of dietary fat, FA composition, and n-6/n-3 ratio are known to influence BC development. Therefore, it is possible that the changed FA composition and n-6/n-3 ratio in the FA modified SOs may affect BC progression, and its critical health concern needs to be investigated. Increased human epithelial growth factor receptor 2 (Her-2/*neu*) and fatty acid synthase (FAS) are associated with BC progression. In fact, FAS activity and expression are affected by dietary FA composition and FA metabolism. Hypothesis of this research is that LLSO and LLMOSO may affect Her-2/*neu* and FAS expressing human BC (SK-Br3) cell growth *in vitro* and *in vivo*. To test our hypothesis, we investigated the potential adverse or beneficial effects of LLSO and LLMOSO in comparison with conventional SO and lard on human BC cells and then examined the possible mechanisms of action by evaluating the expression level of genes markers involved in growth factor mediated signal transduction pathway, specifically Her-2/*ne*- PI 3-kinase (phophoinositide 3- kinase)-FAS signal transduction pathway. *In vitro* study demonstrated that all of the tested oils at 0-2 μl/ml had cytotoxic effects. LLMOSO showed less cytotoxic effects on the growth of SK-Br3 cells compared to SO. However, there was no difference in SK-Br3 cell growth between LLSO and SO. The apoptotic protein markers (mutant p53 and caspase-3) analysis revealed that the cell growth inhibition by oil treatments was cytotoxic by triggering apoptosis. Western blot analysis demonstrated that LLSO- and LLMOSO- induced changes on cell growth involve Her-2/*neu* and FAS signaling transduction pathway and sterol regulatory element binding protein-1 (SREBP-1), mitogen activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI 3-kinase) were possible down-stream effectors of Her-2/*neu* signaling pathway. We also evaluated the dietary effects of LLSO (20% fat of total calorie), SO (20%), and lard (20%) on the growth of SK-Br3 tumors implanted in athymic mice. Changes in tumor surface area, body weight, and food intake were monitored during the 6 months feeding study. After termination, tumor net weight, Her-2/*neu* and FAS mRNA expression in tumors, FAS protein expression in liver, lipid composition in diets, abdominal fat, and serum, as well as plasma total cholesterol and triglyceride levels were analyzed. *In vivo* study showed that there were no statistical differences in tumor size and tumor net weight among SO, LLSO, and lard groups. No differences in FAS mRNA and protein

expression levels between the LLSO and SO groups were observed. Tumors from the lard group expressed higher Her-2/*neu* and FAS mRNA than those from the LLSO and SO group. The lipid analysis demonstrated that LLSO was not significantly distinct from SO in *trans* fat concentration after metabolism. Serum cholesterol and triglyceride levels were unchanged in LLSO fed compared to SO fed mice. In summary, LLSO which contained modification in αLA concentration showed similar effects on SK-Br3 as SO in both *in vitro* and *in vivo*. However, LLMOSO which contained more drastic modifications on FA composition exhibited less cytotoxicity compared to SO *in vitro.*

Acknowledgement

I would like to express my deep gratitude and respect to my advisor, Dr. Young Ju for her ceaseless guidance, encouragement, patience and support throughout my graduate studies.

It is my great pleasure to acknowledge Dr. Eva Schmelz and Dr. Dongmin Liu for their willingness to serve on my committee as well as providing their words of support and encouragement.

I would also like to show my acknowledgement to Dr. Benjamin Corl for his valuable assistance on lipid analysis.

I would also thank the lab members, Mary Pat Meaney, Elaine McCall, and James Spence from whom I have shared enjoyable experience. I especially would like to thank JiEun Kwak for all her help and guidance.

Finally, I want to thank all my friends for their support and encouragement they have given me.

Table of Contents

1 Literature Review 1
1.1 Breast cancer (BC) and dietary fatty acids (FAs) 1
1.2 Possible Mechanisms of dietary fat on Her-2/*neu* and FAS overexpressing BC 2
1.3 FA modified SO oils, LLSO and LLMOSO 4
Chapter 1 5
The effects of LLSO and LLMOSO on the growth of Her-2/*neu* and FAS- overexpressing human BC (SK-Br3) cells, *in vitro* 5
1. Introduction 5
2 Rationale, Hypothesis, and Specific Aims 8
2.1 Justification of Research 8
2.2 Hypothesis 8
2.3 Aims 8
2.3.1 Aim 1. To determine the effect of LLSO, LLMOSO in comparison to SO and lard on cell viability, proliferation and apoptosis of SK-Br3 cells *in vitro* 8
2.3.2 Aim 2. To evaluate the protein expression levels of Her-2/*neu*, FAS, SREBP1, p-ERK, and p-Akt that are involved in Her-2/*neu* and FAS signal transduction pathway. 9
3 Material and Methods 10
3.1 Cell culture 10
3.2 Cell Viability Assay 10
3.3 Cellular Proliferation Assay 11
3.4 FA Composition analysis 12
3.4.1 Lipid Extraction and Methylation 12
3.4.2 GC 13
3.5 qRT-PCR Analysis of mRNA Expression in Cells 13
3.6 Western Blot Analysis 14
3.7 Statistical Analysis 15
4 Results 16
4.1 The effects of αLA, OA, and LA on the growth of SK-Br3 cells 16
4.2 The effects of SO, LLSO, LLMOSO, and lard on viability and proliferation of SK-Br3 cells 17
4.3 FA composition analysis of SK-Br3 cells after oil treatments. 18
4.4 Comparison of Her-2/*neu* and FAS protein expression levels in BC cell lines 18
4.5 The effects of SO, LLSO, lard and LLMOSO treatments on mutant p53, caspase-3, Her-2/*neu*, FAS, SREBP-1, p-Akt, and p-ERK-1 protein expression 19
4.6 The effects of SO, LLSO, Lard and LLMOSO treatments on Her-2/*neu* and FAS mRNA expression in SK-Br3 cells. 21
5 Discussion 22
Chapter 2 28
The effects of LLSO on the growth of Her-2/*neu* and FAS- overexpressing human BC (SK-Br3) cells, *in vivo* 28
1 Introduction 28
2 Rationale. Hypothesis and Specific Aims 29
2.1 Justification of Research 29

2.2 Hypothesis and Specific Aims ... 29
2.3 Specific Aims :Determine the effect of SO, LLSO, and lard on the growth of SK-Br3 implanted in athymic mice. ... 29

3 Materials and Methods ... 30
3.1 Feeding Study ... 30
3.2 qRT-PCR Analysis of mRNA Expression in Tumors ... 30
3.3 Total Protein Extraction from the Liver of Mice ... 31
3.4 FA Composition in Diets, Serum, and Abdominal Fat ... 31
3.4.1 Lipid Extraction and Methylation and GC ... 31
3.5 Measurement of total blood cholesterol and triglyceride level ... 32
3.6 Statistical Analysis ... 32

The results were expressed as mean ±SEM. One-way ANOVA with Tukey's post hoc test was used to calculate *P* values using SigmaStat 3.5 (Systat Software Inc, CA). A probability of $p<0.05$ was taken as denoting a significant difference between samples. ... 32

4 Results ... 33
4.1 The effects of dietary SO, LLSO, and lard on the growth of SK-Br3 implanted in athymic mice ... 33
4.2 The effects of dietary SO, LLSO, and lard on the expression of Her-2/*neu* and FAS mRNA and protein expression in tissues ... 33
4.3 The FA compositions in diets, plasma, and abdominal fat ... 34
4.4 The effects of SO, LLSO, and lard on total cholesterol and triglyceride in mice serum ... 35

5 Discussion ... 36

Reference ... 41

List of Table

Table 1: Analysis of FA composition in SO, LLSO, LLMOSO, and lard. 47
Table 2: Oligonucleotide sequences for qRT-PCR.. 48
Table 3: FA compositions of SK-Br3 cells after oil treatments.. 48
Table 4: Relative expression levels of Her-2/*neu* and FAS expression in human BC cells. 48
Table 5: Dietary treatment groups. 49
Table 6. FA compositions (g/100g FAs) of diets (A), abdominal fat (B), and plasma (C) after feeding study. 50
Table 7: The amounts of *trans* fat, total n-3 and n-6 in diets (A), abdominal fat (B), and plasma (C)... 53

List of Figures

Figure 1 Hormonal regulation of FAS gene expression in cancer cells. 54
Figure 2: The effect of αLA, OA, and LA on cell viability of SK-Br3. 55
Figure 3: The effects of SO, LLSO, LLMOSO and lard on cell viability of SK-Br3. 56
Figure 4: The effects of SO, LLSO, lard, or LLMOSO on cell proliferation of SK-Br3. 57
Figure 5: Her-2/*neu* and FAS expression in human BC cells. 58
Figure 6: Protein expression levels in SK-Br3 cells after oil treatments. 59
Figure 7: Her-2/*neu* and FAS mRNA expression levels in SK-Br3 cells treated with oils. 61
Figure 8: Tumor growth (surface area) of SK-Br3 fed with diets containing chow, SO, LLSO, and lard. 62
Figure 9: Her-2/*neu* and FAS mRNA expression levels in mice tumors fed with diets containing chow, SO, LLSO, or lard. 63
Figure 10: FAS expression in mice liver fed by diets containing chow, SO, LLSO, or lard. 64
Figure 11: Levels of total cholesterol and triglyceride in mice blood. 65

1 Literature Review

1.1 Breast cancer (BC) and dietary fatty acids (FAs)

BC has become the most frequently diagnosed cancer and is the second leading cause of cancer death among American women (1). In 2010, a prediction estimated 207,090 new cases of invasive BC among women and approximately 39,840 death from BC (2). BC prognosis and survival rate are closely related to cancer type and stage (3). Prognosis has strong influence in treatment decisions because patients with a good prognosis are more likely to receive less invasive treatments such as lumpectomy and sometimes radiation (4), while patients with poor prognosis may have to go through more aggressive treatment, such as more extensive mastectomy and possibly multiple chemotherapy drugs (3). For example, a type of BC cells that expresses estrogen receptor (ER) can be treated with drugs such as antiestrogens and aromatase inhibitors, to block ER-mediated BC cell functions, and generally have a better prognosis (3,5). In contrast to ER(+) BC, a type of BC that expresses human epithelial growth factor receptor 2 (Her-2/*neu*) had a worse prognosis (6) and they respond to drugs such as the monoclonal antibody, trastuzumab (in combination with conventional chemotherapy) (7). Epidemiological studies showed that lifestyle, including dietary patterns, may influence BC development (8-10). migrant studies supported a positive relationship between high-fat intake and an increased risk for BC due to patterns shift from a low-fat diet to a high-fat diet (11). In addition to its role as an energy source and membrane lipid composition, dietary fat has important effects on gene expression leading to changes in metabolism, growth, and cell differentiation (12). FAs are organic compounds consisting of a hydrocarbon chain and a terminal carboxyl group. They can be categorized into saturated fatty acid (SFA) or unsaturated fatty acid (USFA) which includes monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) depending on the number of double bond. Essential fatty acids are types of PUFAs that cannot be synthesized in the body and therefore must be obtained through food intake (9). Two families of essential fatty acids are n-6 (linoleic acid group) and n-3 (linolenic acid group). Animal studies have provided strong data supporting a relationship between dietary fats and BC incidences (13,14). N-6 PUFAs, especially linoleic acid (LA), have shown a stimulating effects (13,14). Diets with a high in SFAs have appeared to have a tumor-enhancing effect, although such effect is less severe than the effect exerted by LA (15). On the other hand, high levels of n-3 PUFA, including α-linolenic acid (αLA; C18:3), eicosapentaenoic acid (EPA; C20:5), or docosahexaenoic acid (DHA; C22:6) have shown inhibitory effects on BC incidence (16-19). The role of MUFA, such as oleic acid (OA) appears to be protective although some inconsistent results have been reported (20). Studies in rodents showed that not only quality (the type of fat) but also the quantity of fat is an important modulator of BC risk (15,18). The ratio of n-6/n-3 appears to be one of the important factors on BC development because excessive amounts of n-6 and a high n-6/n-3 ratio are linked with pathogenesis of many diseases including cardiovascular disease, cancer, and inflammatory diseases (21). The n-6/n-3 ratios of 2:1 to 4:1 have been associated with reduced mortality from cardiovascular disease and BC (21).

1.2 Possible Mechanisms of dietary fat on Her-2/*neu* and FAS overexpressing BC

The specific mechanisms of the effect of the dietary fat on BC have not been well established but alterations in hormonal status, structure and function of cell membranes, cell signal transduction pathways, oxidative stress, gene expression and the immune system have been reported (10,22,23). One potential mediator of the effect of specific FAs on mammary tumorigenesis is fatty acid synthase (FAS), a key anabolic enzyme that catalyzes the terminal steps in the *de novo* biosynthesis of FAs (24) Dietary fats have shown to regulate the FAS expression in animal studies (25,26). The expression of hepatic FAS in the high linolenic (LA) lard group (4.1% LA) was lower than in the control lard group (0.4% LA) indicating that the intake of high linolenic lard suppressed the FAS expression in rats (25). FAS provides proliferating tumor cells with endogenously synthesized FAs that can be incorporated into membrane lipids (27). In BC, elevated FAS expression is associated with more advanced disease and portends a poor diagnosis (28,29). Pharmacological inhibition of FAS is selectively cytotoxic to tumor cells in culture and *in vivo* (30,31), and thus represents an experimental strategy for cancer therapy. It was known that FAS regulation is closely related to Her-2/*neu* expression in BC cells (32-34). Her-2/*neu* overexpression stimulates the FAS promoter and ultimately mediates increased endogenous FA synthesis (35). Her-2/*neu* belongs to a family of cell surface tyrosine kinase receptors, the ErbB, also known as epidermal growth factor receptor (EGFR) family (36). In tumorigenesis, ErbB receptors are activated by mechanisms such as gene mutation, gene amplification and abnormal production of epidermal growth factor family ligands (36). The activation of tyrosine kinase domain activates intracellular signaling pathways and eventually regulates diverse biological responses including proliferation, differentiation, cell motility, and survival (36). Aberrant expression of Her-2/*neu*, in particular, is known to increase cell proliferation, resistance to apoptosis, and metastatic behavior through the PI 3-kinase and the mitogen-activated protein kinase (MAPK) pathways (36). The transduction cascades induced by Her-2/*neu* drive the constitutive activation of sterol regulatory element binding protein 1-c (SREBP-1c), resulting in the induction of FAS gene (32,33,37,38). Figure 1 illustrates possible FAS- involved signal transduction pathway. Dietary fats may regulate the expression levels of Her-2/*neu* as well as FAS through down-stream effectors (i.e. SREBP-1c, MAPK, and/or PI 3-kinase) of Her-2/*neu* signaling pathway therefore modulate the BC proliferation and survival.

1.3 FA modified SO oils, LLSO and LLMOSO

SO occupies 75-80% of vegetable oil consumed in the United States. Conventional SO is low in SFAs (less than 15%), high in UFAs (61% of PUFAs and 24% of MUFAs) (39,40). SO requires hydrogenation or partial hydrogenation process for longer shelf life and better texture. Hydrogenation oils are produced at high temperature with metal catalysts and pressurized hydrogen. In the process of hydrogenation, double bonds between the carbon atoms of the FA are broken and hydrogen is added. This process of hydrogenation acts to weaken the double bonds and causes a large percentage of the natural *cis* double bonds to change *trans* double bonds. In last few decades, a variety of SOs were developed with improved functional properties, such as physical characteristics and chemical stability, by selective breeding or genetic modification (41). These oils are low α- linolenic acid soybean oil (LLSO), low α- linolenic acid and mid oleic acid soybean oil (LLMOSO), and low α- linolenic acid and high oleic acid soybean oil (LLHOSO). The primary intent of FA modifications in SOs is to avoid *trans* fat generation during hydrogenation process of SO. Decreasing the relative proportion of *trans* FAs and SFAs may make oil more desirable from a heart disease prospective because t*rans* fats raise the level of low- density lipoproteins (LDL) increasing the risk of coronary heart diseases (42,43). Lichtenstein et al., demonstrated that all varieties of SOs including LLSO and LLHOSO, resulted in more favorable lipoprotein profiles than the partially hydrogenated conventional SOs therefore claimed the FA modified SOs as viable alternatives of conventional SO (43). However, the influences of the FA modified SOs have not been evaluated on BC. In this study, we investigated the effects of the FA modified SOs, LLSO and LLMOSO in particular, on a FAS and Her-2/*neu* over-expressing SK-Br3 cells.

Chapter 1

The effects of LLSO and LLMOSO on the growth of Her-2/*neu* and FAS- overexpressing human BC (SK-Br3) cells, *in vitro*

1. Introduction

In the United States, BC is the second leading cause of cancer health in women and has been increasing in men (44). Risk factors for BC incidence include age, country, reproductive status, exogenous hormones and lifestyle risk factors (alcohol, diet, obesity, and physical activity) (45). A number of studies have demonstrated that the influence of dietary fats on BC depends not only on the quantity but also on the type of lipids (15,18). SO is the most widely used oil in the U.S., accounting for 75-80% of vegetable oil consumed. However, SO has to undergo hydrogenation for a longer shelf life and a better texture and as a side effect of the hydrogenation process, a large percentage of the natural *cis* double bonds are changed to *trans* double bonds (*trans* fat). Consumption of the dietary *trans* FAs results in raised levels of LDL thus increasing the risk of heart diseases (42,43) and also related with increased BC risks (46). In 2006, Food and Drug Administration mandated that all food products containing *trans* fats greater than 0.5g per serving must be properly identified on the nutrition facts label. As a consequence, a variety of SOs were developed to reduce *trans* fat contents in the oil. LLSO and LLMOSO, in particular are developed to avoid *trans* fat generation during hydrogenation process of SO. According to gas chromatography analysis, LLSO and LLMOSO were distinguished from SO by the quantities of *a*LA, LA, and OA (Table 1A). The changes in the amount of *a*LA, LA, and OA affect to the concentrations of 1) total SFA, MUFA and/or PUFA and 2) the ratio of n-6/n-3 in LLSO and LLMOSO compared to SO (Table 1B).

Specifically, αLA content of the LLSO diet was 0. Due to the reduced amount of αLA in LLSO, the n-6/n-3 ratio for LLSO was 6.2-fold elevated compared to that in SO. The amount of other FA, for example palmitic acid (PA), stearic acid (SA), OA, and LA remained very close to the amount in SO. On the other hand, the LLMOSO contained about 2-fold increased the amount of OA compared to SO. LLMOSO also contained 3.6-fold lower amount of αLA compared to that in SO, making the ratio of n-6/n-3 approximately 6.7-fold higher than that of SO. The amount of total SFA, MUFA, and PUFA of LLSO was similar to that of the SO. In contrast to LLSO, LLMOSO had greater changes in total amount of SFA, MUFA, and PUFA. The inconsiderable modification in the amount of total SFA, MUFA, and PUFA found in LLSO was originated from the fact that the aLA content which was a main modification in LLSO was not significant in SO (5.81% of total lipids).

The modification of the total amount of SFA, MUFA, and PUFA and changes in n-6/n-3 ratio can lead to unexpected effects on BC biology because the influence of dietary fats on cancer depends on the quantity and the type of lipid; n-6 PUFA and n-3 PUFA differ in their ability to affect mammary tumor formation and growth (47). A high n-6/n-3 ratio are linked with pathogenesis of many diseases including cancer, and inflammatory diseases (21). For example, the substitution of canola oil for corn oil in the diet (8 % in diet) slowed the growth of low- FAS expressing human BC (MDA-MB-231) tumors in athymic mice (48). The substitution of canola oil for the corn oil reduced the amount of LA and decreased the n-6/n-3 ratio from about 341.70 to 146.20 (48). The n-6/n-3 ratio in SO, LLSO, and LLMOSO were 8.46, 52.36, and 54.70, respectively (Table 1B). Therefore, it is highly likely that the modifications of FA profiles in SO variants modulate the growth of BC cells. In our lab, LLSO has shown to stimulate the growth of ER(+) and ER(-) human BC cells *in vitro* (unpublished study by Ji Eun Kwak). LLSO treatments (≥ 1 μl) significantly increased the growth of human ER(-) BC (MDA-MB-231)cells compared to SO. LLSO (≥ 1 μl) also increased ER(+) human BC (MCF-7) cell growth. Whereas

SO, at the selected concentration range, did not alter the growth MDA-MB-231 and MCF-7 cells. The possible mechanism that LLSO stimulate BC cell growth is not clear at this point. In this chapter, the effects of FA modified SOs on FAS and Her-2/*neu* overexpressing BC cells will be examined and the possible mechanisms of their action will be investigated

2 Rationale, Hypothesis, and Specific Aims

2.1 Justification of Research

This study is designed to determine the effects of LLSO and LLMOSO on SK-Br3 cell lines *in vitro*. The preliminary studies conducted in our lab showed that LLSO stimulated the growth of BC cells (MDA-MB-231 and MCF-7) *in vitro*. However the mechanism by which LLSO acted on BC was unclear. By using SK-Br3 which overexpresses both Her-2/*neu* and FAS, the possible involvements of the FAS and Her-2/*neu* pathways as well as their main effectors (SREBP-1, MAPK/ERK, PI 3-kinase/Akt) can be investigated.

2.2 Hypothesis

My hypothesis is that LLSO and LLMOSO might have potential adverse effects on SK-Br3 cell growth due to its reduced content of αLA and elevated ratio of n-6/n-3 by altering the protein expressions involved in Her-2/neu-FAS signal transduction pathway. We hypothesized that FA modifications made in LLSO or LLMOSO might modulate the Her-2/*neu* expression and therefore regulate FAS which is a downstream effector of Her-2/*neu*. I plan to test this hypothesis by pursuing the following two specific aims:

2.3 Aims

2.3.1 Aim 1. To determine the effect of LLSO, LLMOSO in comparison to SO and lard on cell viability, proliferation and apoptosis of SK-Br3 cells *in vitro*

To test if LLSO and LLMOSO stimulate the SK-Br3 cell growth, we will test the effects of and oils (SO, LLSO, LLMOSO, and lard at 0-2 μl/ml) and FAs (at 0-0.1 μl/ml media) on SK-Br3 cell viability using (4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We will further evaluate whether the oil induced changes in SK-Br3 cell viability evolves cellular proliferation and apoptosis by 5-bromo2′-deoxyuridine (BrdU) assay and by western blot (mutant p53 and caspase 3).

2.3.2 Aim 2. To evaluate the protein expression levels of Her-2/*neu*, FAS, SREBP1, p-ERK, and p-Akt that are involved in Her-2/*neu* and FAS signal transduction pathway.

To test if the stimulatory effects by LLSO and LLMOSO involves Her-2/*neu* and FAS signal transduction pathway, we will examine the LLSO or LLMOSO induced changes on the expressions of Her-2/*neu* and FAS as well as the protein expressions of their downstream effectors (SREBP1, p-ERK, and p-Akt) in SK-Br3 cells at transcription and translation levels using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and western blot analyses.

3 Material and Methods

3.1 Cell culture

SK-Br3 BC cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). SK-Br3 cells were originally derived from 43-year-old Caucasian woman at Memorial Sloan-Kettering Cancer Center in 1970 (49). The cells were maintained in McCoy's 5A (Mediatech Inc, VA) supplemented with 10% (v/v) bovine calf serum and 1% penicillin (100 units/ml) and streptomycin (100 µg/ml) in an incubator at 37 °C with a constant supply of 5% CO_2.

3.2 Cell Viability Assay

The effects of individual FAs (αLA, LA, and OA) (Sigma, MO) and the FA modified SOs (LLSO and LLMOSO, Asoya, IA) on SK-Br3 cell viability were tested by using MTT assay. The results were expressed as an average of three independent experiments.

Individual FA: SK-Br3 cells (5×10^4 cell/ml) were seeded in 24-well culture plates. One day after seeding, cells were treated with various concentrations (0 - 0.1 µl/ml, equivalent to 0.3 - 300 µM) of FAs every 48 h for 96 h. These concentrations were in the relevant range of free FA in serum. In human serum, the circulating free FAs is ~500 µM (50) and the free FA concentration in human BC tissue is 150 - 400 nM (51). The FAs were dissolved in 100% ethanol and stored as stock solution (1 g/ml, and 3 M) at -20°C. For experimental use, all FAs were freshly prepared from stock solution and diluted with growth media. Media containing ethanol was used as a negative control. After 96 h treatment, medium was then removed and 50 µl of MTT solution (1 mg/ ml in phosphate buffered saline) in 450 µl of media was added to each well. The cells were additionally incubated at 37°C for 5 h. The blue formazan crystals were dissolved in 500 µl of l mg/ml SDS in 0.1 N HCl. After overnight incubation, the optical density (OD) was measured at 570 nm using a spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT). OD values were normalized to numbers of cells based on a standard growth curve.

Oils: SK-Br3 cells (5×10^4 cell/ml) were treated with 0-2 µl/ml of SO, LLSO, LLMOSO, and lard in 24-well culture plate every 48 h for 96 h. These concentrations were selected because the molarity of each FA in these oils concentration ranges was equivalent to the FA concentrations used in cell viability assay. For better delivery of oils to the cell, the oils were mixed with dimethylsulfoxide (DMSO) at a ratio of 1:1 (52). DMSO in media without oil was included as a negative control. The rest of the experimental processes were identical as described in the cell viability assay with individual FAs.

3.3 Cellular Proliferation Assay

To assess whether the oils- induced growth inhibition was resulted from the reduced cell proliferation, the immunodetection of BrdU incorporation by S-phase cells was employed. BrdU cell proliferation assay kit (Roche Applied Science, Mannheim, Germany) was used to measure cell proliferation by quantifying BrdU incorporated into the newly synthesized DNA of replicating cells. Briefly, SK-Br3 cells (1×10^4 cells/100 µl/well) were incubated for 24 h in 96-well flat bottom culture plates in CO_2 incubator at 37 °C. The cells were then treated with oils (0.2 µl SO, LLSO, LLMOSO, or lard in 100 µl media) and incubated for 96 h at 37°C. The oils were replaced with fresh oils/media after 48 h incubation. After addition of BrdU solution (10 µl/well), the culture was re-incubated for additional 24 h. After this period of BrdU incorporation, the medium was removed by aspiration. Cells were fixed and denatured by incubating FixDenat (for simultaneous fixation and denaturation of DNA) (200 µl/well) at room temperature for 30 min. After the incubation, BrdU incorporated cells were detected by anti-BrdU-POD (peroxidase-conjugated anti-BrdU antibody) (100 µl/well) and its substrate solutions. The color development was measured in an enzyme-linked immunosorbent assay (ELISA) reader at 370 nm (Bio-Tek Instrument, Inc., Winooski, VT). The results were expressed as an average of three independent experiments. The molarities of αLA, OA, and LA in 0.2µl oils/100 µl media were as follow; SO (OA:LA:αLA=15.75 µM:29.70 µM:0.64 µM) , LLSO (OA:LAαLA=12.89 µM:29.52 µM:0.08 µM), LLMOSO (OA:LAαLA=23.28 µM:18.54 µM:0.99 µM) lard (OA:LA:αLA=20.23 µM:11.88 µM:0.67 µM). The molarity of eachFA in oils was equivalent to the concentrations of 0.005 - 0.01 µl/ml in cell viability assay for individual FAs. The only exception was αLA; its quantities in the experimental oils were very low and this concentration was not included in cell viability assay with individual assay.

3.4 FA Composition analysis

3.4.1 Lipid Extraction and Methylation

In collaboration with Dr. Ben Corl (Dairy Science, Virginia Tech), FA composition of oil-treated SK-Br3 cells was determined by gas chromatography (GC). Triplicate samples of SK-Br3 cells (1×10^5 cell/ml) were treated with 2 µl/ml of SO, LLSO, LLMOSO, and lard every 48 h for 96 h. The concentration of 2 µl oils/ml was selected because the difference between oil treatments on SK-Br3 cell viability and proliferation was the most drastic at this concentration. The cells were harvested with Trypsin-EDTA incubation for 5 min followed by centrifugation at 800 rpm for 5 min. Total lipids in the cell pellet were extracted after homogenization in hexane-isopropanol (3:2) solution according to the procedure of Hara and Radin (53,54). FAs from cell pellets were methylated using the base-catalyzed transmethylation procedure for esterified FAs as described by Christie (54). The lipids were methylated using 1% methanolic sulfuric acid (50°C for 2 h) to methylate both the esterified and non-esterified FAs.

3.4.2 GC

FA composition was determined using a GC (Agilent 6890N, Santa Clara, CA) equipped with a 100 m CP-Sil 88 capillary column (i.d., 0.25 µm, film thickness, 0.20 µm; Varian, Palo Alto, CA) and a flame ionization detector. At the time of sample injection, the column temperature was 80°C and then the temperature was ramped at 2°C/min to 190°C and maintained for 13 min. Temperature was then ramped again at 2°C/min to 210°C and maintained for 20 min. Inlet and detector temperatures were 250°C. The split ratio was 100:1. The flow rate for H_2 carrier gas was 1 mL/min. Most FA peaks were identified and quantified using a quantitative mixture of pure methyl ester standards (Nu Chek Prep, Elysian, MN).

3.5 qRT-PCR Analysis of mRNA Expression in Cells

FA modified SO-induced changes in FAS and Her-2/*neu* mRNA expressions were analyzed by qRT-PCR using the iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA). Major changes in FAS and Her-2/*neu* were evaluated. Primer pairs were designed using Becon Designer (Premier biosoft international, CA) and the sequences are listed in Table 2. SK-Br3 cells (2×10^5 cells/ ml) were incubated with 2 µl oils/ml for 48 h. Total RNA was prepared from cell pellet (30 mg) using Qiagen reagents (Qiagen, CA) according to the manufacturers' protocol. RNA quality was assessed by GeneQuant II (Pharmacia Biotech, NJ). cDNA was prepared by reverse transcription of 10 ng of total RNA using iScript Reverse Transcription Reagents (Bio-Rad, CA). PCR reactions were amplified and analyzed using iCycler (Bio-Rad, CA). Data were analyzed using a comparative threshold cycle (Ct) method. Each sample was run as triplicates in separate tubes to permit quantification of target genes normalized to a control, glyceraldehyde 3-phosphate dehydrogenase (GADPH). At the end of the PCR, samples were subjected to a melting analysis to confirm the specificity of the amplicon.

3.6 Western Blot Analysis

Changes in the expression levels of proteins that are involved in Her-2/*neu*-FAS signaling pathway (Her-2/*neu*, FAS, SREBP-1, p-ERK-1, p-Akt) and apoptotic marker proteins (caspase-3 and mutant p53) were evaluated by western blot analysis. SK-Br3 cells (1×10^5/ml) were incubated with 2 µl oils/ml every 48 h for 96 h. Cells were lysed in RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 15 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 10 mM 2-mercaptoethanol, 10 µg/ml PMSF, 15 µl/ml Triton X-100, and protease inhibitor cocktail and the protein concentration was determined by Bradford assay (Bio-Rad, CA) . Protein extracts (10 µg/well) were separated by 7.5% SDS/PAGE and electrophoretically transferred onto nitrocellulose membrane. Blocking was carried out in 5% non-fat milk followed by incubating the membrane with primary antibody [rabbit anti-Her-2/*neu*; 1:4,000, rabbit anti-FAS; 1:20,000, mouse anti-p53; 1:5,000, rabbit anti-SREBP-1; 1:5.000, mouse anti-caspase-3; 1:5,000, mouse anti-p-ERK-1; 1:1,000, rabbit anti-p-Akt; 1:1,000, or goat anti-actin; 1:20,000] overnight at 4 °C. Excess antibodies were removed by washing with TBS–Tween 20 (TBS-T). Incubation with secondary antibody conjugated to horseradish peroxidase [anti-mouse IgG, anti-goat IgG or anti-rabbit IgG, diluted 1:200,000 in 1× TBS-T] was performed for 1 h at room temperature. After three washes, the reaction was developed by the addition of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL). The emitted light was captured on X-ray film. Beta-actin was used as a loading control. The protein expression levels were quantified using a densitometer with Image-Pro Plus software (Bethesda, MD). The relative level was calculated as the ratio of each protein expression to beta-actin level. The results were expressed as an average of three independent experiments.

3.7 Statistical Analysis

All experiments were done in triplicates. The results were represented as mean ±SEM. One-way ANOVA (analysis of variance) with Tukey's post hoc test was used to calculate *P* values using SigmaStat 3.5 (Systat Software Inc, CA). A probability of $p<0.05$ was taken as denoting a significant difference between samples.

4 Results

4.1 The effects of αLA, OA, and LA on the growth of SK-Br3 cells

Cell viability assays indicated that all three FAs (0.001 μl – 0.1 μl/ml media) induced significant dose- dependent growth inhibitory effects on SK-Br3 cells (Figure 2).

αLA inhibited SK-Br3 cell viability by 34.60 % at 0.0001 μl/ml (0.33 μM), 67.70% at 0.001 μl/ml (3.29 μM), 72.90% at 0.005 μl/ml (16.50 μM), 76.00% at 0.01 μl/ml (32.90 μM), 87.70% at 0.05 μl/ml (164.50 μM), and 100% at 0.1 μl/ml (329 μM) compared to the vehicle control ($p<0.05$) (Figure 2A).

LA inhibited SK-Br3 cell viability by 8.30% at 0.0001 μl/ml (0.32 μM),14.60% at 0.001 μl/ml (3.21 μM), 49.60% at 0.005 μl/ml (16.05 μM), 72.40% at 0.01 μl/ml (32.10 μM), 71.10% at 0.05 μl/ml (160.50 μM), and 74.50% at 0.1 μl/ml (321 μM), compared to the vehicle control ($p<0.05$) (Figure 2B).

OA inhibited SK-Br3 cell viability by 15.80% at 0.0001 μl/ml (0.28 μM), 44.80% at 0.001 μl/ml (2.77 μM),, 62.70% at 0.005 μl/ml (13.85 μM), 69.40% at 0.01 μl/ml (27.70 μM), 70.00% at 0.05 μl/ml (138.50 μM), and 75.30% at 0.1 μl/ml (277 μM), compared to the vehicle control ($p<0.05$) (Figure1) (Figure 2C).

At 0.01 μl/ml media, the cell viability following αLA treatment was significantly lower than that of LA treatment ($p<0.05$) (Figure 2D). At 0.05 μl/ml and 0.1 μl/ml, the cell viability after αLA treatment was significantly lower than that of OA and LA ($p<0.05$) (Figure 2D). Although all three FAs inhibited the growth of cells, αLA showed the strongest inhibitory effect among the tested FAs ($p<0.05$).

4.2 The effects of SO, LLSO, LLMOSO, and lard on viability and proliferation of SK-Br3 cells

Cell viability: Dose-response studies of SO, LLSO, LLMOSO, and lard (0-2 µl/ml) were conducted to evaluate their effects on cell viability of SK-Br3 cells. The magnitudes of growth inhibitory effects of oils were found increase with higher concentrations (0.5- 2µl/ml) (Figure 3). SO inhibited cell viability by 49.20% at 0.5 µl/ml, 55.10% at 1 µl/ml, and 56.10% at 2 µl/ml compared to the control (p<0.05) (Figure 3A). LLSO inhibited cell viability by 13.80% at 0.5 µl/ml, 39.70% at 1 µl/ml, and 50% at 2 µl/ml compared to the control (p<0.05) (Figure 3B). Lard inhibited cell viability by 17.10% at 0.5 µl/ml, 28.40% at 1 µl/ml, and 44.00% at 2 µl/ml compared to the control (p<0.05) (Figure 3C). LLMOSO inhibited cell viability by 15.50% at 0.5 µl/ml, 27.90% at 1 µl/ml, and 31% at 2 µl/ml compared to the control (p<0.05) (Figure 3D). According to the cell viability assay, SO treatment induced the greater growth inhibition effect than LLSO, lard, and LLMOSO (p<0.05) at concentration of 0.5-2 µl/ml (Figure 3E).

Cellular Proliferation: The effects of oil treatments on SK-Br3 cellular proliferation were examined using BrdU incorporation method (Figure 4). Cells treated with oils (2 µl/ml) showed drastic reduction of proliferating cells. The BrdU positive cells after SO treatment were 54.30% and the BrdU positive cells after LLSO, lard, and LLMOSO treatments were 65.20%, 68.10%, and 77.10%, respectively. There was a statistical significance between SO and LLMOSO treatments (p<0.05). However, no statistically significant differences were found among the SO, LLSO, and lard groups.

4.3 FA composition analysis of SK-Br3 cells after oil treatments.

The FA composition analysis was performed to determine the uptake of lipid by SK-Br3 cells. The FA compositions of SK-Br3 cells after oil treatments were presented in Table 3.

The contents of C18:1 n-9 in oil itself were 23.51% in SO, 25.56% in LLSO, 35.73% in lard, and 46.80% in LLMOSO (Table 1A) and the values in SK-Br3 after oil treatments were 23.97% in SO, 25.62% in LLSO, 33.66% in lard, and 41.67% in LLMOSO. The value of C18:1 n-9 in the vehicle control was 29.16%. The C18:1 n-9 value of the control was not significantly different from the oil-treated groups. However the C18:1 n-9 value of LLMOSO was significantly different from the control (p<0.05).

C18:2 n-6 values in oil itself were 49.57% in SO, 52.36% in LLSO, 12.15% in lard, and 31.82% in LLMOSO (Table 1A), respectively. C18:2 n-6 values in the oil treated SK-Br3 cells were 5.71% for the control, 40.11% in SO, 43.64% in LLSO, 11.09% in lard, and 27.92% in LLMOSO, respectively. The C18:2 n-6 value of the control was significantly different from the oil-treated groups (p<0.05).

C18:3 n-3 values in SO, LLSO, lard, and LLMOSO in oil itself were 5.81% in SO, 0% in LLSO, 0.6% in lard, and 1.62% in LLMOSO (Table 1A), and their values after oil uptake by SK-Br3 were 4.49% in SO, 0.87% in LLSO, 0.52% in lard, and 1.46% in LLMOSO. The C18:3 n-3 value for control was 0.16% and the C18:2 n-6 values of SO, LLSO, and lard were significantly differently from the control (p<0.05). However the C18:3 n-3 value of LLMOSO was not significantly different from the control.

4.4 Comparison of Her-2/*neu* and FAS protein expression levels in BC cell lines

The expression levels of Her-2/*neu* and FAS protein in a panel of human BC cells, including SK-Br3, MCF10A, MCF10AT1, MCF-7, T47-D, MDA-MB-231, and MDA-MB-468 were analyzed using Western blot analysis (Figure 5). The expression levels of Her-2/*neu* and FAS protein were graded from ++++ (overexpression) to -/+ (low expression) based on relative density (Table 4). Table 4 shows that SK-Br3 is the only cell line that overexpresses both Her-2/*neu* and FAS among the BC cell lines analyzed. Other cell lines have low (MDA-MB-231) to moderate (MCF10A, MCF10AT1, MCF-7, and T47-D) expression of FAS but they barely express Her-2/*neu*.

4.5 The effects of SO, LLSO, lard and LLMOSO treatments on mutant p53, caspase-3, Her-2/*neu*, FAS, SREBP-1, p-Akt, and p-ERK-1 protein expression

To investigate the mechanism of SO-, LLSO-, lard-, and LLMOSO- induced cytotoxic effect on SK-Br3 cells, the expression of apoptotic markers, mutant p53 and caspase-3 were measured. The effects of oils on protein expression of Her-2/*neu*, FAS, and downstream affecters of FAS signaling pathway (SREBP-1, p-Akt, and p-ERK-1) were examined (Figure 6).

Mutant p53: As shown in Figure 6A, the relative mutant p53 protein expression was 0.91 ± 0.10, 1.01 ± 0.09, 0.80 ± 0.15, 0.80± 0.21 for SO, LLSO, lard, and LLMOSO, respectively. SO, LLSO, lard, and LLMOSO treatments reduced mutant p53 protein expression by 50.70 ± 0.03%, 46 ± 0.03%, 57.10 ± 0.04%, 56.80 ± 0.06%, respectively, compared to the vehicle control (Figure 6A). There was no statistical difference among the oil treatment groups.

Caspase-3: The caspase-3 protein expression was increased by the oil treatments (Figure 6B). The relative caspase-3 protein expression was 2.10 ± 0.21, 2.44 ± 0.33, 1.62 ± 0.13, 2.12 ± 0.16 for SO, LLSO, lard, and LLMOSO, respectively. SO, LLSO, lard, and LLMOSO treatments increased caspase-3 protein expression by 250 ± 0.40%, 290 ± 0.60%, 190 ± 0.20%, 250 ± 0.20% compared to the control (p<0.05). There was no statistical difference among SO, LLSO, and LLMOSO treatments (Figure 6B).

Her-2/*neu*: As shown in Figure 6C, the relative Her-2/*neu* protein expression was 0.63 ±

0.10, 0.84 ± 0.10, 0.81 ± 0.10, 0.76 ± 0.20 for SO, LLSO, lard, and LLMOSO, respectively. SO significantly down-regulated Her-2/*neu* protein expression by 34.70 ± 0.02% compared to the control (p<0.05). However, the LLSO, lard, and LLMOSO did not change Her-2/*neu* protein expression.

FAS: SO, LLSO, lard, and LLMOSO treatments significantly down-regulated FAS protein expression in SK-Br3 cells compared to the control (p<0.05) (Figure 6D). The relative FAS protein expression was, 0.95 ± 0.10, 0.92 ± 0.10, 1.25 ± 0.20, 1.33 ± 0.10 for SO, LLSO, lard, and LLMOSO, respectively. SO treatment reduced FAS protein expression by 51.00 ± 0.01%, LLSO by 52 ± 0.01%, lard by 19.60 ± 0.02%, and LLMOSO by 24.50 ± 0.02%, compared to the control. A statistical difference between SO and LLMOSO treatments was found (p<0.05). There was no statistical significance among SO, LLSO, and lard.

SREBP-1: The relative SREBP-1 protein expression was 1.06± 0.15, 0.94 ± 0.12, 1.41 ± 0.10, 0.33 ± 0.20 for SO, LLSO, lard, and LLMOSO, respectively (Figure 6E). SO, LLSO, lard, and LLMOSO significantly inhibited the SREBP-1 protein expression by 63 ± 0.01%, 58.10 ± 0.01%, 37 ± 0.01%, and 85.30 ± 0.02%, respectively compared to the control (p<0.05). There were statistical differences in SREBP-1 protein expression between SO and lard, and between SO and LLMOSO (p<0.05). However, no difference SO and LLSO was observed.

p-ERK-1: As shown in Figure 6F, the relative p-ERK-1 protein expression was 0.51 ± 0.11, 0.87 ± 0.15, 1.32 ± 0.12, 0.78 ± 0.19 for SO, LLSO, lard, and LLMOSO, respectively. LLSO and lard treatments significantly increased the expression of p-ERK by 158 ± 0.20% and 240 ± 0.20%, respectively (p<0.05). SO inhibited p-ERK-1 expression by 92.50 ± 0.03% and LLMOSO stimulated p-ERK-1 expression by 150 ± 0.40% compared to the control however their expressions were not significantly different from the control. A statistical significance was found between SO and LLSO (p<0.05). There was no statistical difference between SO and LLMOSO.

p-Akt: All oil treatments significantly suppressed p-Akt expression compared to the control (p<0.05) (Figure 6G). The relative p-Akt protein expression was 2.11± 0.10, 1.36 ± 0.20, 1.85 ± 0.24, 2.10 ± 0.12 for SO, LLSO, lard, and LLMOSO, respectively. The p-Akt expression after SO was 30.60 ± 0.03%, after LLSO was 55.10 ± 0.05%, after lard was 39.10 ± 0.05%, and after LLMOSO was 30.80 ± 0.04% of the control. However, there was no significant difference between SO, lard, and LLMOSO on p-Akt protein expression. A statistical difference was found between SO/lard/LLMOSO and LLSO (p<0.05).

4.6 The effects of SO, LLSO, Lard and LLMOSO treatments on Her-2/*neu* and FAS mRNA expression in SK-Br3 cells.

Relative Her-2/*neu* mRNA expression were 0.71 ± 0.10 for SO, 1.33 ± 0.10 for LLSO, 1.15 ± 0.07 for lard, and 0.86 ± 0.10 for LLMOSO (Figure 7A). None of the oil treatments altered Her-2/*neu* mRNA expression significantly. Relative FAS mRNA expressions were observed to be 0.95 ± 0.17 for SO, 1.15 ± 0.08 for LLSO, 0.90 ± 0.10 for lard, and 0.95 ± 0.16 for LLMOSO (Figure 7B). SO treatment significantly inhibited FAS mRNA expression whereas LLSO treatment significantly stimulated FAS mRNA expression (p<0.05) (Figure 7B).

5 Discussion

Experimental studies showed that dietary effects not only depend on the quantity but also quantities of fat (15,18). In this chapter, we evaluated the effects of the modified FAs compositions in LLSO and LLMOSO on SK-Br3 human BC by evaluating their effects on SK-Br3 cell viability, proliferation, and apoptosis. The effects of *a*LA, LA, and OA on SK-Br3 cell viability were tested first because SO mainly differs from the LLSO and LLMOSO in these three FAs (Table 1). Our cell viability assay with individual FAs (*a*LA, LA, and OA) demonstrated that they had different magnitudes of inhibitory effects on SK-Br3 cell growth (Figure 2). Even though all of these FAs significantly inhibited SK-Br3 cell growth, *a*LA (0.33-329 μM) showed the stronger inhibitory effect than OA (0.28-277 μM) or LA (0.32-321 μM) (Figure 2). The greatest inhibitory effect of *a*LA can be partially due to its role in the FA metabolism. αLA is a substrate for synthesis of DHA and EPA which have been shown to suppress growth of most cancer cells (55). Consistent with our findings, OA at 10 μM induced cell growth inhibition in FAS overexpressing SK-Br3 (33). Interestingly, OA and LA showed the stimulatory effects on other BC cell lines; OA slightly stimulated the cell growth at high concentration (210.70-1686.30 μM) in low-FAS expressing MDA-MB-231 cell (56) and LA dose-dependently (0-3.57 μM) stimulated the growth of moderately-FAS expressing T47D cells (13,33). These conflicting results on different cell lines might be explained in part by the Her-2/*neu* and/or FAS expression status in various cell lines. Among the human BC cells tested, SK-Br3 was the only cell line that overexpresses both Her-2/*neu* and FAS (Figure 5). MDA-MB-231 expresses low level of FAS and no Her-2/*neu* whereas T47D expresses moderate amount of FAS with low level of Her-2/*neu*. (Figure 5). A previous study demonstrated that cerulenin, a component of fungi (*Cephalosporium caerulens)*, induced cytotoxicity, and irreversibly inhibited FAS expression in SK-Br3 cells (57); cerulenin-induced cytotoxicity in SK-Br3 cells was more significant than

MCF-7 cells and MDA-MB-231 cells suggesting that the levels of FAS expression in BC cell lines can be used as an indicator of cytotoxic effects of a drug (33). Based on the FA composition analysis of LLSO and LLMOSO and the SK-Br3 cell viability assays with individual FAs (OA, LA, and αLA), we hypothesized that LLSO and LLMOSO might have potential adverse effects on SK-Br3 cells because of their high n-6/n-3 ratio and the reduced amount of αLA and the cell growth regulations by LLSO and LLMOSO might be mediated by FAS expression.

To test our hypothesis that LLSO and LLMOSO differentially modulate FAS-overexpressing SK-Br3 cell growth, we evaluated the effects of SO, LLSO, LLMOSO with reference to lard on SK-Br3 cell viability. The concentrations of oils (0-2 µl/ml), which were used for cell viability assay, did not exceed the circulating free FAs in human serum (50). The molarity of total FAs in 2 µl/ml SO, LLSO, lard, and LLMOSO was approximately 50 µM. In human serum, the circulating free FAs is ~500 µM (50). SO reduced SK-Br3 cell viability (Figure 3). Unlike our hypothesis, both LLSO and LLMOSO also showed inhibitory effects on SK-Br3 cells (Figure 3). However, LLSO and LLMOSO showed less growth inhibitory effects compared to SO demonstrating that these FA modified SOs were less cytotoxic to SK-Br3 cells than the conventional SO. It was interesting that lard, which contains high SFA and therefore implicated in higher BC risk (15,58), also exhibited SK-Br3 cell growth inhibitory effects (Figure 3). The growth inhibition by lard could be explained partially by the findings from the European Prospective Investigation on dietary fat and BC (59). Sieri et al found that saturated fat was greatly associated with BC risk but total fat was not associated with the risk. They concluded that when the subtypes of fat were mutually adjusted, SFA and MUFA consumption was not significantly associated with risk (59). FA compositions of SK-Br3 cell pellet after oil treatments appropriately reflected FA compositions of oil samples indicating a selective incorporation of FA by SK-Br3 cells (Table 3). We further evaluated whether the FA modified SOs induced inhibitory effects on cell viability involves anti-proliferation and apoptosis by evaluating cellular proliferation (Figure 4) and expression of apoptotic protein markers (mutant p53 and caspase-3) at 2 μl /ml concentration (Figure 6). The treatments of SK-Br3 cells with oils were associated with a decrease in cell proliferation reflected by reduced BrdU incorporation in all treatments (Figure 4). Consistent with the patterns found in cell viability assay at 2 μl /ml concentration (Figure 3), SO exhibited the greatest anti-proliferation effect and LLMOSO had the least anti-proliferative effect. The oil treatments suppressed the mutant p53 protein expression compared to the control indicating that the oils can suppress cancer cell growth by down-regulating pro-tumor protein, however, there was no statistical difference among oil treatments (Figure 6A). It was previously reported that OA at 1.75 μM, which was about 10 folds lower concentration than that was used in our oil treatments, induced apoptosis by down-regulating mutant p53 protein in SK-Br3 cells (33). Previous studies showed that SK-Br3 cell growth inhibition as represented by down-regulation of mutant p53 might be mediated by FAS inhibition (60-62). Therefore, the SK-Br3 apoptosis induced by the oil treatments might be due to FAS inhibition. To obtain further evidence that the oil treatments induce apoptosis, we examined the caspase-3 protein expression level. The caspase-3 protein was significantly induced by SO, LLSO, and LLMOSO, but there was no statistical difference among these three oil treatments. (Figure 6B). The cell proliferation assay taken together with apoptosis analysis demonstrated that the reduced SK-Br3 cell viability induced by oil-treatments were associated with reduced cell proliferation, and were mediated by promoting apoptosis. Since there was no statistical difference between SO and LLSO treatments on SK-Br3 cell proliferation and apoptosis, it can be concluded that n-6/n-3 ratio was not an important factor in modulating SK-Br3 cell growth. However, the reduced cytotoxic effects by LLMOSO implied that reduced n-6/n-3 ratio together with modified total amounts of MUFA and

PUFA were possible factors that altered SK-Br3 cell growth.

The possible cell signaling pathway involved in SK-Br3 cell growth inhibition by oil treatments was speculated to be Her-2/*neu* and FAS. From the cell viability, proliferation, and apoptosis, it was hypothesized that SO and FA modified SOs down-regulate expressions of Her-2/*neu* and FAS and as well as downstream effectors (SREBP-1, MAPK/ERK, and PI 3-kinase/Akt) of Her-2/*neu*. As we hypothesized, the FAS protein expression was down-regulated by all oils treatments (Figure 6C). FAS protein suppressions by SO and LLSO were greater than that of lard and LLMOSO. The pattern of FAS suppression was somewhat similar to the pattern of cell viability and proliferation assay results (Figure 3 and Figure 4). LLMOSO showed the least growth inhibition and its effect on FAS suppression was the minimum among the oils tested. The effects of oils on Her-2/*neu* were less drastic than their effects on FAS; only SO down-regulated Her-2/*neu* and LLSO and LLMOSO did not affect Her-2/*neu* protein expression when compared to the control (Figure 6D). However, Her-2/*neu* mRNA expression levels were not changed by any of the oil treatments (Figure 7A). Interestingly, FAS mRNA expression was reduced by SO and increased by LLSO indicating that there was no relationship between FAS mRNA and protein expression by oil treatments (Figure 6C and Figure 7B). It has been previously reported that dietary FAs, especially OA (1.75 μM) exposure significantly reduced both FAS and Her-2/*neu* protein expressions in SK-Br3 cells (32,33,63). αLA treatment (20 μM) also suppressed the Her-2/*neu* oncoprotein expression (64). Our findings suggest the possible involvement of Her-2/*neu* in the FAS signaling pathway in oil- induced cytotoxic effects. To examine the involvement of the SREBP-1, MAPK/ERK, and PI 3-kinase/Akt signaling pathways in oil-induced FAS down-regulation, we tested the effects of the oils on the expression of SREBP-1, p-Akt (PI 3-kinase/Akt signaling pathway), and p-ERK-1 (MAPK/ERK signaling pathway) proteins. We found that the all oil treatments inhibited SREBP-1 protein expression (Figure 6E).

The pattern of SREBP-1 down-regulation was similar to that of FAS down-regulation (Figure 6C) except LLMOSO suggesting the involvement of SREBP-1 in the oil-mediated FAS signaling pathway. Similarly, dietary supplementations of PUFA inhibited hepatic SREBP-1 mRNA expression in rats (65). The p-Akt protein expression was also suppressed by SO, LLSO, lard, and LLMOSO (Figure 6G). This result was consistent with the previous finding that OA (5 and 10 μM) treatment-mediated Her-2/*neu* repression involved p-Akt protein inhibition in SK-Br3 (32). Unexpectedly, LLSO and lard increased p-ERK-1 expression by 150% and 240% compared to the control, respectively, whereas SO and LLMOSO had no effect on the expression of p-ERK-1 (Figure 6F). The p-ERK-1 up-regulation by LLSO and lard seemed to contradictory to their down-regulatory effects on p-Akt protein since both p-ERK-1 and p-Akt provide cell survival and proliferation signals. Previous studies showed that exogenous OA supplementation inhibited Her-2/*neu* expression through MAPK/ERK and PI 3-kinase/Akt in SK-Br3 by inhibiting both p-MAPK and p-Akt protein (32,33). However, recent studies have revealed that many chemotherapeutic agents also triggered cell survival pathways, potentially limiting the effectiveness of the chemotherapy. Aberrantly activated MAPK/ERK signaling pathway was being increasingly observed in response to chemotherapy (66). A variety of anticancer drugs which stimulated apoptosis had shown to down-regulate the Akt pathway and up-regulate ERK pathway (67,68). Similarly, exposure to cerulenin increased p-ERK-1 expression level in SK-Br3 cells (33). A possible explanation for the up-regulation of p-ERK-1 by cerulenin was that perturbation of FAS was one of the cellular injury that can induce aberrant expression of MAPK/ERK signaling (33). Although the SREBP-1, p-ERK-1, and p-Akt expression levels were not exactly matched with the expression levels of FAS and Her-2/*neu*, our data supported the involvements of SREBP-1, MAPK/ERK, and PI 3-kinase/Akt in FAS signaling pathway. The differences in expression patterns of the FAS and Her-2/*neu* pathway downstream effectors might implicate that oils exert their modulatory effects on SK-Br3 cells through a different effectors combination of FAS and Her-2/*neu* pathways.

In summary, our findings provided a scientific evaluation of newly developed FA modified SO products (LLSO and LLMOSO) in comparison to the conventional SO on the growth of FAS-overexpressing BC (SK-Br3) cells. SO and FA modified SOs inhibited FAS overexpressing SK-Br3 cell growth altering proliferation and apoptosis. Unlike our hypothesis, the altered n-3 and n-6/n-3 ratio in the LLSO did not change SK-Br3 BC cell growth compared to SO. However LLMOSO, which had modification on n-6/n-3 ratio as well as total MUFA and PUFA compared to SO, showed less growth inhibitory effects than SO. Her-2/*neu* and FAS protein expressions were down-regulated by SO, LLSO, or LLMOSO indicating Her-2/*neu* and FAS signaling pathway as a possible mechanism of action. Our data also revealed that SREBP-1, MAPK/ERK, and PI 3-kinase/Akt were possible intermediates in the oil-regulated Her-2/*neu* signaling pathway.

Chapter 2

The effects of LLSO on the growth of Her-2/*neu* and FAS- overexpressing human BC (SK-Br3) cells, *in vivo*

1 Introduction

The consumption of dietary FAs or oils in relation to BC risk has been examined extensively (23,25,48,52,69). Animal studies provided strong evidence supporting a positive relationship between dietary fat consumption and incidence of BC (15,25,48,70). In chapter 1, we demonstrated no significant difference between SO and LLSO on FAS and Her-2/*neu* expressing BC cell growth *in vitro*. Previously in our lab, however, dietary LLSO stimulated both ER(-) MDA-MB-231 tumors (unpublished study by JeeEun Kwak) and human pre-malignant BC (MCF10AT1) tumor growth (manuscript by Elaine McCall under revision) compared to SO in athymic mice. LLSO (20% total energy intake) treatment for 10 weeks stimulated the MDA-MB-231 tumor growth by 1.5 folds compared to SO. Dietary LLSO (20%) treatment for 24 weeks accelerated the malignant transformation of MCF-10AT1 tumors compared to SO. To further evaluate the effects of LLSO and to extend our *in vitro* observations into *in vivo* study, a feeding study of oils will be conducted using human BC xenograft in athymic mice. We will evaluate the biological effects of dietary intake of LLSO on the growth of SK-Br3 tumors and elucidate the mechanism of LLSO on FAS expressing tumor growth.

2 Rationale. Hypothesis and Specific Aims

2.1 Justification of Research

Even though testing of LLSO in cell culture indicated that the effect of LLSO on the growth of SK-Br3 were not significantly different from that of the conventional SO (chapter 1), it has to be examined on animal model since mice are more relevant to human than cell culture model in the fact that mice are capable of desaturating and elongating 18 carbon FAs to 20 or 22 carbon FAs like human (48). We selected 20% oil diet which was within the context of typical Western diet and as used in many previous experiments (48). By using animal model, we can evaluate how biological effects of dietary intake of LLSO is different from SO in BC growth, lipid metabolism, and the levels of cholesterol and triglyceride.

2.2 Hypothesis and Specific Aims

We hypothesize that LLSO had no stimulatory effect on the growth of SK-Br3 cells implanted in athymic mice, and did not alter Her-2/*neu* and FAS signaling pathways as observed *in vitro* study.

2.3 Specific Aims :Determine the effect of SO, LLSO, and lard on the growth of SK-Br3 implanted in athymic mice.

To test our hypothesis, a feeding study of LLSO (20% of calories), SO (20%), and lard (20%) in athymic mice implanted with SK-Br3 cells will be conducted and LLSO-induced changes in tumor surface area, tumor weight, and mammary glands will be investigated. Also, the effects of LLSO on FAS and Her-2/*neu* mRNA expression in tumors and FAS protein expression in livers; on FA compositions in abdominal fat, and serum; and on triglyceride and total cholesterol levels in serum will be evaluated.

3 Materials and Methods

3.1 Feeding Study

Dietary effects of SO, LLSO, and lard were evaluated by conducting a feeding study. Female athymic mice (5 weeks old, n=36) were purchased from Harlan (Indianapolis, IN). Mice were kept in a ventilated rack with controlled humidity, 12 h day/night cycles at 22°C. We used American Institute of Nutrition 93 growth semi-purified diet (AIN93G, Dyets Inc, PA) as a base diet. AIN93G has been established as meeting the nutritional requirement of mice (71). After 1 week acclimation, all mice received SK-Br3 cells (1×10^5/40 µl Matrigel/site, 4 sites/animal) subcutaneously. Right after the cell injection, the mice were divided into four treatment groups; SO (n=10), LLSO (n=10), or lard (n=10), chow (n=6), and the dietary treatment was begun. The treatment diets contained substituted fat (20% of total calories; 85.4g/kg diet) with SO, LLSO, or lard. A feeding study of regular chow diet (diet 2018, Harlan Laboratories, WI) was carried out after initial oil diet study to see whether SK-Br3 is tumorigenic. Chow diet contained less fat (17% of total calories, 62.5g/kg diet) than the other oils (Table 4) Tumor growth and body weight were measured weekly for 6 months, and tumor cross-sectional area was determined using the formula [length (mm)/2×width (mm)/2×π] (72).. At the end of study, blood was collected by cardiac puncture under isoflurane anesthetization, after blood collection mice were sacrificed by cervical dislocation. Blood and tissues including tumors, liver, mammary gland, uterine, and abdominal fat were collected, weighed, and stored in appropriate reagent for later analysis.

3.2 qRT-PCR Analysis of mRNA Expression in Tumors

The tumors were collected and stored in RNA later (Qiagen, CA) for Her-2/*neu* and FAS mRNA analysis. Since the tumor size was too small to analyze individual tumors, tumors from same treatment group (SO [n=10], LLSO [n=10], lard [n=10], and chow diet [n=6]) were pooled prior to total RNA extraction. Total RNA was prepared from the pooled tumors (100 mg) using Qiagen reagents (Valencia, CA). cDNA preparation and qRT-PCR analysis were performed as described in chapter 1. Each sample was run in triplicates for qRT-PCR analysis.

3.3 Total Protein Extraction from the Liver of Mice

Protein was extracted from mouse liver using the modified method of Menendez et al. (33). Liver was used because FA synthesis by FAS occurs in liver (24). Briefly, a mouse was sacrificed and the liver (0.7-1 g) was collected, SO (n=10), LLSO (n=10), or lard (n=10) chow diet (n=6). The liver was kept in 2 ml of homogenized buffer (154 mmol/L KCl, 50 mmol/L Tris-HCl, and 1 mmol/L EDTA buffer, pH 7.4) and homogenized individually by PowerGen (Fisher Scientific, NJ). The homogenized suspension was centrifuged at 10,000 ×*g* for 20 min, followed by re-centrifugation of the supernatant at 105,000 ×*g* for 60 min using Beckman ultracentrifuge (Beckman Coulter, Inc., CA). The supernatant was considered as the cytosol and used for Western Blot (see general procedures of western blot analysis written above for protein markers and dilutions). The aliquots of cytosol fraction were stored at -80°C until the analysis was performed.

3.4 FA Composition in Diets, Serum, and Abdominal Fat

3.4.1 Lipid Extraction and Methylation and GC

FA composition of diets (300mg), abdominal fat (300mg) and total plasma (100 µl) lipids was determined by GC. Pooled samples of blood and abdominal fats from the same treatment groups, SO (n=10), LLSO (n=10), or lard (n=10), and chow diet (n=6) were employed for the analysis. Total lipids in diets, abdominal fat, and plasma were extracted after homogenization in hexane-isopropanol (3:2) solution according to the procedure of Hara and Radin (53,54). Lipid methylation and GC analysis were performed as described under FA composition analysis in chapter 1.

3.5 Measurement of total blood cholesterol and triglyceride level

Blood from each mouse in treatment groups (SO; n=10, LLSO; n=10, lard; n=10, and chow; n=6) was collected by cardiac puncture upon termination of study. The plasma was prepared by centrifuge at 3000 rpm for 20 min at 4°C. Then the plasma was stored at -20°C for later use. Total cholesterol and triglyceride were measured using enzymatic reagents from Stanbio Laboratories (Boerne, TX). One hundred µl of each mouse plasma was used for the assay.

3.6 Statistical Analysis

The results were expressed as mean ±SEM. One-way ANOVA with Tukey's post hoc test was used to calculate *P* values using SigmaStat 3.5 (Systat Software Inc, CA). A probability of $p<0.05$ was taken as denoting a significant difference between samples.

4 Results

4.1 The effects of dietary SO, LLSO, and lard on the growth of SK-Br3 implanted in athymic mice.

Figure 8 showed the tumor growth (surface area) during 25-week dietary treatment. At week 10, the tumor size reached about 20 mm^2 in SO, LLSO, and lard groups. The average tumor size got smaller from week 12 and reduced in size to 10-11 mm^2 by the time of termination (chow group; 10.91 ± 0.85 mm^2, SO group; 10.88 ± 0.71 mm^2, LLSO group; 10.37 ± 0.84 mm^2, lard group; 11.18 ± 0.44 mm^2) (Figure 8). Tumor incidences were 85%, 90%, 97.5%, and 91.7% for the LLSO, SO, lard, and chow groups, respectively. At the time of termination, there was no significant difference in average tumor surface area, body weight, tumor weight, uterine gland weight, mammary gland weight, and abdominal fat weight among treatment groups (data not shown). During the feeding study, the average food intake was also monitored every month and there were no significant differences on food intake among groups (data not shown).

4.2 The effects of dietary SO, LLSO, and lard on the expression of Her-2/*neu* and FAS mRNA and protein expression in tissues.

Her-2/*neu* and FAS mRNA Expressions in Tumors: Relative Her-2/*neu* mRNA expression over the SO was observed to be 0.19 ± 0.04 for LLSO, and 3.02 ± 0.61 for lard (Figure 9A). . Relative FAS mRNA expression over the SO was observed to be 0.13 ± 0.03 for LLSO, and 1.87 ± 0.14 for lard (Figure 9B).

FAS Protein Expression in the Liver: We investigated the effects of SO, LLSO, and lard diets on the expression of FAS protein in mouse liver. Relative expression of FAS protein in SO group was 1.25 ± 0.12, LLSO group was 1.70 ± 0.16, lard group was 2.47 ± 0.18 (Figure 10). There was no statistical difference in FAS expression levels among SO, and LLSO diets. However FAS level in the SO group was significantly lower than that in the lard group. (p<0.05)

4.3 The FA compositions in diets, plasma, and abdominal fat

The FA composition of diets was analyzed (Table 6A). Directly comparing the values for SO, LLSO, and lard was confounded by differences in dietary fat content.

αLA (C18:3 n-3): As expected, LLSO diet contained about 82% less αLA than SO diet (Table 6A). This relative composition difference was reflected in the FA compositions of abdominal fat and plasma (Table 6B and 6C). After the mice consumed the LLSO diet, the amount of αLA in abdominal fat was 84% lower than mice that consumed the SO diet. After the mice consumed the LLSO diet, the amount of αLA in plasma was 81% lower than after the mice that consumed the SO diet.

LA (C18:2 n-6): There was no difference in LA between LLSO and SO in diet, abdominal fat, and plasma (Table 6).

OA (C18:1 n-9): The amount of OA was slightly higher in SO diet than LLSO. Even though the OA intake was higher with SO diet, after metabolizing, the resulting concentration of OA in abdominal fat and plasma was similar (Table 6).

Other FAs: Little effect of the diets was observed in abdominal fat and plasma of each diet group on the relative proportion of C16:0 (PA, palmitic acid) and C18:0 (SA, stearic acid), possibly attributable to endogenous synthesis (Table 6B and 6C).

***Trans* Fat:** We also analyzed the amounts of *trans* fat in diets, abdominal fat, and plasma because LLSO was developed to avoid *trans* fat generation. The *trans* fat content was 3-fold higher in SO diet than in LLSO diet (LLSO 0.02% vs. SO 0.06%) (Table 7A), but these were minor amounts. However, the *trans* fat contents in abdominal fat and plasma from LLSO and SO group were similar (Table 7B and 7C). Lard diet contained about 11-fold higher *trans* fat than SO diet. Adipose tissue and plasma *trans* fat levels in lard fed mice were 6.4-fold and 2-fold higher than SO fed mice, respectively (Table 7B).

n-6/n-3: By reducing the amount of αLA, the total amount of n-3 FAs were decreased resulting in increased ratio of n-6/n-3 in the diet (Table 7A). The ratio of n-6/n-3 in LLSO diet was 396 and in SO diet was 48.04. The ratio of n-6/n-3 for LLSO remained high in both abdominal fat and plasma. In abdominal fat, n-6/n-3 ratio for LLSO was 80.82 and for SO was 14.64. In plasma, the ratio of n-6/n-3 for LLSO was 22.17 and for SO was 9.11 (Table 7B and 7C).

4.4 The effects of SO, LLSO, and lard on total cholesterol and triglyceride in mice serum

Total cholesterol: The total serum cholesterol is shown in Figure 11A. The average amount of cholesterol was 51.29 ± 6.90 mg/dL for SO, 62.02 ± 7.37 mg/dL for LLSO, and 65.08 ± 5.09 mg/dL for lard group, respectively (Figure 11A). Total cholesterol levels in SO and LLSO groups were significantly lower than the level in lard group, however there was no difference between SO and LLSO groups.

Triglyceride: As shown in Figure 11B, the average serum triglyceride levels are 107 ± 5.38 mg/dL for the SO, 123.17 ± 6.20 mg/dL for the LLSO, and 123.92 ± 7.94 mg/dL for lard group, respectively. There were no significant differences among SO, LLSO, and lard diets.

5 Discussion

FA modified oils with altered functional or nutritional characteristics are being introduced into the marketplace (41). The high OA and low αLA type oils were developed to enhance oxidative stability and these oils are targeted mainly to salad dressing, frying, and food coating applications (41). Concomitant with the development of new varieties of oil, there is a dearth of information about the effect of these oils on BC. We tested two of the FA modified SOs, LLSO and LLMOSO on human BC (SK-Br3) cells *in vitro* (chapter1). We found that SO and FA modified SOs exhibited cytotoxic effects on the growth of SK-Br3 cells by reducing FAS and Her-2/*neu* protein expression in the cells. However, there was no difference in cytotoxic effect between SO and LLSO. LLMOSO was less cytotoxic than SO, but it was not different from LLSO. We further tested weather dietary intake of LLSO modulated the growth of SK-Br3 tumors using a mouse xenograft model in chapter 2. LLSO induced changes in comparison with SO-, lard- induced effects were also evaluated. LLMOSO was not included in animal study since it was not commercially available by the time of experiment. The six months feeding study showed that dietary SO, LLSO, and lard had no effect on SK-Br3 tumor growth (Figure 8). The tumor growth was very slow and the tumors actually stopped growing after 12 weeks of diet treatment. A feeding study of chow diet was conducted later to see whether SK-Br3 is tumorigenic (Figure 8). SK-Br3 cells were predicted as tumorigenic in athymic mice by matrigel outgrowth assay (73) and 3D culture model (74). Whereas, some studies showed that SK-Br3 was not tumorigenic in athymic mice (75-78). According to Honjo et al., SK-Br3 was not tumorigenic in nude mice because it did not express galectin-3, which has been associated with alterations in cell growth, transformation, and metastasis (74,79). Therefore no tumor growth we observed might not be due to oil treatment, but due to the non-tumorigenic character of SK-Br3. Although LLSO, SO, lard and chow diet did not affect SK-Br3 tumor growth, the FAS and Her-

2/*neu* mRNA expressions seemed to be regulated by the diet treatments. LLSO and SO inhibited both Her-2/*neu* and FAS mRNA expression in tumors compared to lard (Figure 9). The reduced FAS and Her-2/*neu* mRNA expression by SO and LLSO suggested that SO and LLSO might be able to inhibit the tumor growth of other tumorigenic FAS expressing BC because FAS and Her-2/*neu* inhibition by these oils have shown to induce apoptosis in chapter 1. FAS protein expression levels in mice liver were lower in SO and LLSO groups than lard group indicating possible FAS protein inhibition by SO and LLSO (Figure 10). Less inhibition of FAS gene and protein by lard group might be due to its high content of saturated fat because induction of lipogenic gene expression by high fat diet and high in saturated fat diet was reported in rodent liver (26). Expression of FAS gene was induced in mice fed a diet high in saturated fat diet (17.1 kcal/day) compared to standard chow (12.3 kcal/day) fed mice and partial replacement of the saturated fat within the high fat diet by fish oil (16.8 kcal/day) strongly suppressed hepatic FAS gene expression (26). The dietary LLSO induced changes in FAS and Her-2/*neu* in SK-Br3 tumors were different from those in pre-malignant human BC (MCF10AT1) tumors. LLSO accelerated the transformation of MCF-10AT1, and increased Her-2/*neu* and FAS mRNA expression in tumors compared to SO. The difference on regulation of Her-2/*neu* and FAS mRNA expression by SO and LLSO between MCF10AT1 and SK-Br3 was possibly due to the fact that the effects of the lipid on BC depends on the types of lipid in addition to the BC stage (23). Our tumor growth analysis and mRNA and protein analysis demonstrated that LLSO did not alter the growth of SK-Br3 tumors. There was no significant difference between SO group and LLSO group on SK-Br3 tumor growth rate and mRNA and protein expression of FAS in SK-Br3 supporting our cell culture analysis in chapter 1.

The effects of dietary LLSO intakes on BC risk were further evaluated by the FA composition of plasma and adipose tissue (abdominal fat), and diet (Table 6). By analyzing the FA compositions in these samples, we could evaluate the influence of LLSO on the *trans* fat content, and the ratio of n-6/n-3 in abdominal fat and plasma after feeding study. The lipid composition analysis showed that LLSO diet contained about 82% less αLA than SO diet (Table 6A). The reduction of αLA in LLSO in the diet was well- reflected in FA composition of abdominal fat and plasma (Table 6B and 6C). After the mice consumed the LLSO diet, the amounts of αLA in abdominal fat and plasma were 84% and 81%, respectively (Table 6B and 6C). This indicated that the FA compositions of lipids in abdominal fat and plasma were influenced by dietary fat. SO was 3-fold greater in *trans* fat content compared to SO (Table 7A). *Trans* FAs have adverse physiologic effects because *trans* fat impairs PUFA metabolism. More specifically, *trans* fat inhibits the enzymatic desaturation of linoleic and linolenic acid therefore reduce production of the 3-series eicosanoids (80) and the perturbation of eicosanoid production affects carcinogenesis (46). Kohlmeier et al. demonstrated a positive association of the adipose concentration of *trans* FAs with BC (46). However, adipose and plasma percentage of total *trans* FA in LLSO fed mice were not significantly different from that of SO fed mice even though *trans* FA concentration in SO diet was 3-fold greater than that in LLSO, (Table 7B and 7C). The n-6/n-3 ratios in diet, abdominal fat, and plasma of LLSO consumed mice were higher than SO consumed mice. In McCall study (MS under revision), a positive association between n-6/n3 ratio and MCF-10AT1 tumor growth was observed among SO, LLSO, and lard diets and plasma. A European multicenter study examined FAs in adipose tissue found the direct association between the n-6/n-3 ratios and BC risk (46). However, there was also a report showing no evidence of an association between the ratio of n-6/n-3 and BC risk (81). The possible explanations for the discrepancy between case-control and cohort studies included different biomarkers with different exposure time to dietary fats (18). The FA composition analysis showed that adipose tissue and plasma percentage of total *trans* FA in LLSO fed mice were not significantly different from that of SO fed mice. The results also showed that the n-6/n-3 ratio was not an important factor to regulate SK-Br3 tumor growth and it could not regulate FAS and Her-2/*neu* protein expression.

In addition to enhancing oxidative stability, the main reason to modify the FA profile of SOs was to lower *trans* fat generation during hydrogenation process. *Trans* fats raise the level of LDL and therefore increase the risk of coronary heart diseases because *trans* fats reduce HDL, and raise levels of triglyceride in the blood (82). The high cholesterol and high serum triglycerides are also associated with BC risk (83,84). The consumption of LLSO has been hypothesized to lower the total cholesterol and triglyceride levels in serum due to its low *trans* fat content. This study demonstrated that LLSO did not alter plasma cholesterol and triglyceride concentrations compared to SO (Figure 11A). Our lipid analysis explained the cause; the lipid analysis showed that LLSO generated the same amount of *trans* fat as SO after metabolism (Table 7). In a direct comparison of SO and LLSO on serum lipid concentrations, Lu et al (85) reported similar effects of SO and LLSO on total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels. Consist with Lu et al (85), levels of total cholesterol and triglyceride in mice serum were not changed in LLSO group compared to SO group (Figure 11). Therefore, it can be concluded that there was no difference between LLSO and SO on the plasma cholesterol and triglyceride concentrations.

In summary, we found no distinct difference between dietary intake of SO and LLSO on SK-Br3 tumor growth, expression levels of Her-2/*neu* and FAS mRNA in tumor, FAS protein in livers, *trans* fat concentration after metabolism, and cholesterol and triglyceride concentrations in animal study. These results supported our *in vitro* study (chapter 1) which suggested that the effects of LLSO on SK-Br3 cell viability and proliferation were similar to that of SO. Our findings from both *in vitro* and *in vivo* study implicated that the FA modifications made on

LLSO were too trivial to lead changes and n-6/n-3 ratio is not an important factor in Her-2/*neu* and FAS expressing human BC cell growth.

Reference

1. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C. & Thun, M. J. (2006) Cancer statistics, 2006. CA Cancer J Clin 56: 106-130.

2. Cancer.org (2009) Breast Cancer Facts & Figures 2009-2010. American Cancer Society.

3. Kesisis, G., Kontovinis, L. F., Gennatas, K. & Kortsaris, A. H. Biological markers in breast cancer prognosis and treatment. J Buon 15: 447-454.

4. Families., N. C. I. a. t. N. R. C. f. W. (2004) Surgery choices for women with earlly stage breast cancer.

5. Thiantanawat, A., Long, B. J. & Brodie, A. M. (2003) Signaling pathways of apoptosis activated by aromatase inhibitors and antiestrogens. Cancer Res 63: 8037-8050.

6. Sotiriou, C. & Pusztai, L. (2009) Gene-expression signatures in breast cancer. N Engl J Med 360: 790-800.

7. Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Jr., Davidson, N. E., Tan-Chiu, E., Martino, S., Paik, S. et al. (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 353: 1673-1684.

8. Yonemoto, R. H. (1980) Breast cancer in Japan and United States: epidemiology, hormone receptors, pathology, and survival. Arch Surg 115: 1056-1062.

9. Jiang, W. G., Bryce, R. P. & Horrobin, D. F. (1998) Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. Crit Rev Oncol Hematol 27: 179-209.

10. Lee, M. M. & Lin, S. S. (2000) Dietary fat and breast cancer. Annu Rev Nutr 20: 221-248.

11. Ziegler, R. G., Hoover, R. N., Pike, M. C., Hildesheim, A., Nomura, A. M., West, D. W., Wu-Williams, A. H., Kolonel, L. N., Horn-Ross, P. L. et al. (1993) Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 85: 1819-1827.

12. Jump, D. B. & Clarke, S. D. (1999) Regulation of gene expression by dietary fat. Annu Rev Nutr 19: 63-90.

13. Reyes, N., Reyes, I., Tiwari, R. & Geliebter, J. (2004) Effect of linoleic acid on proliferation and gene expression in the breast cancer cell line T47D. Cancer Lett 209: 25-35.

14. Rose, D. P., Connolly, J. M., Rayburn, J. & Coleman, M. (1995) Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. J Natl Cancer Inst 87: 587-592.

15. Fay, M. P., Freedman, L. S., Clifford, C. K. & Midthune, D. N. (1997) Effect of different types and amounts of fat on the development of mammary tumors in rodents: a review. Cancer Res 57: 3979-3988.

16. Minami, M. & Noguchi, M. (1996) Effects of low-dose eicosapentaenoic acid, docosahexaenoic acid and dietary fat on the incidence, growth and cell kinetics of mammary carcinomas in rats. Oncology 53: 398-405.

17. Rose, D. P. & Connolly, J. M. (1999) Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol Ther 83: 217-244.

18. Saadatian-Elahi, M., Norat, T., Goudable, J. & Riboli, E. (2004) Biomarkers of dietary fatty acid intake

and the risk of breast cancer: a meta-analysis. Int J Cancer 111: 584-591.

19. Chajes, V., Hulten, K., Van Kappel, A. L., Winkvist, A., Kaaks, R., Hallmans, G., Lenner, P. & Riboli, E. (1999) Fatty-acid composition in serum phospholipids and risk of breast cancer: an incident case-control study in Sweden. Int J Cancer 83: 585-590.

20. Escrich, E., Solanas, M., Moral, R., Costa, I. & Grau, L. (2006) Are the olive oil and other dietary lipids related to cancer? Experimental evidence. Clin Transl Oncol 8: 868-883.

21. Simopoulos, A. P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother 56: 365-379.

22. Weng, M. S., Ho, C. T., Ho, Y. S. & Lin, J. K. (2007) Theanaphthoquinone inhibits fatty acid synthase expression in EGF-stimulated human breast cancer cells via the regulation of EGFR/ErbB-2 signaling. Toxicol Appl Pharmacol 218: 107-118.

23. Escrich, E., Moral, R., Grau, L., Costa, I. & Solanas, M. (2007) Molecular mechanisms of the effects of olive oil and other dietary lipids on cancer. Mol Nutr Food Res 51: 1279-1292.

24. Kuhajda, F. P. (2006) Fatty acid synthase and cancer: new application of an old pathway. Cancer Res 66: 5977-5980.

25. Murano, Y., Funabashi, T., Sekine, S., Aoyama, T. & Takeuchi, H. (2007) Effect of dietary lard containing higher alpha-linolenic acid on plasma triacylglycerol in rats. J Oleo Sci 56: 361-367.

26. Oosterveer, M. H., van Dijk, T. H., Tietge, U. J., Boer, T., Havinga, R., Stellaard, F., Groen, A. K., Kuipers, F. & Reijngoud, D. J. (2009) High fat feeding induces hepatic fatty acid elongation in mice. PLoS One 4: e6066.

27. Pizer, E. S., Lax, S. F., Kuhajda, F. P., Pasternack, G. R. & Kurman, R. J. (1998) Fatty acid synthase expression in endometrial carcinoma: correlation with cell proliferation and hormone receptors. Cancer 83: 528-537.

28. Kuhajda, F. P. (2000) Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. Nutrition 16: 202-208.

29. Swinnen, J. V., Heemers, H., Deboel, L., Foufelle, F., Heyns, W. & Verhoeven, G. (2000) Stimulation of tumor-associated fatty acid synthase expression by growth factor activation of the sterol regulatory element-binding protein pathway. Oncogene 19: 5173-5181.

30. Pizer, E. S., Wood, F. D., Heine, H. S., Romantsev, F. E., Pasternack, G. R. & Kuhajda, F. P. (1996) Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res 56: 1189-1193.

31. Pizer, E. S., Jackisch, C., Wood, F. D., Pasternack, G. R., Davidson, N. E. & Kuhajda, F. P. (1996) Inhibition of fatty acid synthesis induces programmed cell death in human breast cancer cells. Cancer Res 56: 2745-2747.

32. Menendez, J. A., Vellon, L., Colomer, R. & Lupu, R. (2005) Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu oncogene amplification. Ann Oncol 16: 359-371.

33. Menendez, J. A., Mehmi, I., Atlas, E., Colomer, R. & Lupu, R. (2004) Novel signaling molecules implicated in tumor-associated fatty acid synthase-dependent breast cancer cell proliferation and survival: Role of exogenous dietary fatty acids, p53-p21WAF1/CIP1, ERK1/2 MAPK, p27KIP1, BRCA1, and NF-

kappaB. Int J Oncol 24: 591-608.

34. Kumar-Sinha, C., Ignatoski, K. W., Lippman, M. E., Ethier, S. P. & Chinnaiyan, A. M. (2003) Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. Cancer Res 63: 132-139.

35. Menendez, J. A., Vellon, L. & Lupu, R. (2005) Targeting fatty acid synthase-driven lipid rafts: a novel strategy to overcome trastuzumab resistance in breast cancer cells. Med Hypotheses 64: 997-1001.

36. Ignatiadis, M., Desmedt, C., Sotiriou, C., de Azambuja, E. & Piccart, M. (2009) HER-2 as a target for breast cancer therapy. Clin Cancer Res 15: 1848-1852.

37. Yang, Y. A., Han, W. F., Morin, P. J., Chrest, F. J. & Pizer, E. S. (2002) Activation of fatty acid synthesis during neoplastic transformation: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. Exp Cell Res 279: 80-90.

38. Jeong, J. H., An, J. Y., Kwon, Y. T., Li, L. Y. & Lee, Y. J. (2008) Quercetin-induced ubiquitination and down-regulation of Her-2/neu. J Cell Biochem 105: 585-595.

39. Actis, A. B., Cremonezzi, D. C., King, I. B., Joekes, S., Eynard, A. R. & Valentich, M. A. (2005) Effects of soy oil on murine salivary tumorigenesis. Prostaglandins Leukot Essent Fatty Acids 72: 187-194.

40. Meydani, S. N., Lichtenstein, A. H., White, P. J., Goodnight, S. H., Elson, C. E., Woods, M., Gorbach, S. L. & Schaefer, E. J. (1991) Food use and health effects of soybean and sunflower oils. J Am Coll Nutr 10: 406-428.

41. Hazebroek, J. P. (2000) Analysis of genetically modified oils. Prog Lipid Res 39: 477-506.

42. Pala, V., Krogh, V., Muti, P., Chajes, V., Riboli, E., Micheli, A., Saadatian, M., Sieri, S. & Berrino, F. (2001) Erythrocyte membrane fatty acids and subsequent breast cancer: a prospective Italian study. J Natl Cancer Inst 93: 1088-1095.

43. Lichtenstein, A. H., Matthan, N. R., Jalbert, S. M., Resteghini, N. A., Schaefer, E. J. & Ausman, L. M. (2006) Novel soybean oils with different fatty acid profiles alter cardiovascular disease risk factors in moderately hyperlipidemic subjects. Am J Clin Nutr 84: 497-504.

44. Giordano, S. H., Cohen, D. S., Buzdar, A. U., Perkins, G. & Hortobagyi, G. N. (2004) Breast carcinoma in men: a population-based study. Cancer 101: 51-57.

45. Dumitrescu, R. G. & Cotarla, I. (2005) Understanding breast cancer risk -- where do we stand in 2005? J Cell Mol Med 9: 208-221.

46. Kohlmeier, L., Simonsen, N., van 't Veer, P., Strain, J. J., Martin-Moreno, J. M., Margolin, B., Huttunen, J. K., Fernandez-Crehuet Navajas, J., Martin, B. C. et al. (1997) Adipose tissue trans fatty acids and breast cancer in the European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Breast Cancer. Cancer Epidemiol Biomarkers Prev 6: 705-710.

47. Bagga, D., Capone, S., Wang, H. J., Heber, D., Lill, M., Chap, L. & Glaspy, J. A. (1997) Dietary modulation of omega-3/omega-6 polyunsaturated fatty acid ratios in patients with breast cancer. J Natl Cancer Inst 89: 1123-1131.

48. Hardman, W. E. (2007) Dietary canola oil suppressed growth of implanted MDA-MB 231 human breast tumors in nude mice. Nutr Cancer 57: 177-183.

49. Trempe, G. L. (1976) Human breast cancer in culture. Recent Results Cancer Res: 33-41.

50. Klein, S. & Wolfe, R. R. (1992) Carbohydrate restriction regulates the adaptive response to fasting. Am J Physiol 262: E631-636.

51. Kleinfeld, A. M. & Okada, C. (2005) Free fatty acid release from human breast cancer tissue inhibits cytotoxic T-lymphocyte-mediated killing. J Lipid Res 46: 1983-1990.

52. Chen, D. & Auborn, K. (1999) Fish oil constituent docosahexa-enoic acid selectively inhibits growth of human papillomavirus immortalized keratinocytes. Carcinogenesis 20: 249-254.

53. Hara, A. & Radin, N. S. (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90: 420-426.

54. Christie, W. W. (1982) A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J Lipid Res 23: 1072-1075.

55. Burdge, G. (2004) Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. Curr Opin Clin Nutr Metab Care 7: 137-144.

56. Menendez, J. A., del Mar Barbacid, M., Montero, S., Sevilla, E., Escrich, E., Solanas, M., Cortes-Funes, H. & Colomer, R. (2001) Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. Eur J Cancer 37: 402-413.

57. Omura, S. (1976) The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol Rev 40: 681-697.

58. Boyd, N. F., Stone, J., Vogt, K. N., Connelly, B. S., Martin, L. J. & Minkin, S. (2003) Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. Br J Cancer 89: 1672-1685.

59. Sieri, S., Krogh, V., Ferrari, P., Berrino, F., Pala, V., Thiebaut, A. C., Tjonneland, A., Olsen, A., Overvad, K. et al. (2008) Dietary fat and breast cancer risk in the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr 88: 1304-1312.

60. Bandyopadhyay, S., Zhan, R., Wang, Y., Pai, S. K., Hirota, S., Hosobe, S., Takano, Y., Saito, K., Furuta, E. et al. (2006) Mechanism of apoptosis induced by the inhibition of fatty acid synthase in breast cancer cells. Cancer Res 66: 5934-5940.

61. Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F. & Pizer, E. S. (2001) Pharmacological inhibition of fatty acid synthase activity produces both cytostatic and cytotoxic effects modulated by p53. Cancer Res 61: 1493-1499.

62. Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A. & Han, W. F. (1998) Pharmacological inhibitors of mammalian fatty acid synthase suppress DNA replication and induce apoptosis in tumor cell lines. Cancer Res 58: 4611-4615.

63. Menendez, J. A., Papadimitropoulou, A., Vellon, L. & Lupu, R. (2006) A genomic explanation connecting "Mediterranean diet", olive oil and cancer: oleic acid, the main monounsaturated fatty acid of olive oil, induces formation of inhibitory "PEA3 transcription factor-PEA3 DNA binding site" complexes at the Her-2/neu (erbB-2) oncogene promoter in breast, ovarian and stomach cancer cells. Eur J Cancer 42: 2425-2432.

64. Menendez, J. A., Vazquez-Martin, A., Ropero, S., Colomer, R. & Lupu, R. (2006) HER2 (erbB-2)-targeted effects of the omega-3 polyunsaturated fatty acid, alpha-linolenic acid (ALA; 18:3n-3), in breast cancer cells: the "fat features" of the "Mediterranean diet" as an "anti-HER2 cocktail". Clin Transl Oncol 8: 812-820.

65. Mater, M. K., Thelen, A. P., Pan, D. A. & Jump, D. B. (1999) Sterol response element-binding protein

1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. J Biol Chem 274: 32725-32732.

66. McDaid, H. M. & Horwitz, S. B. (2001) Selective potentiation of paclitaxel (taxol)-induced cell death by mitogen-activated protein kinase kinase inhibition in human cancer cell lines. Mol Pharmacol 60: 290-301.

67. Chang, G. C., Hsu, S. L., Tsai, J. R., Wu, W. J., Chen, C. Y. & Sheu, G. T. (2004) Extracellular signal-regulated kinase activation and Bcl-2 downregulation mediate apoptosis after gemcitabine treatment partly via a p53-independent pathway. Eur J Pharmacol 502: 169-183.

68. Karmakar, S., Banik, N. L. & Ray, S. K. (2008) Combination of all-trans retinoic acid and paclitaxel-induced differentiation and apoptosis in human glioblastoma U87MG xenografts in nude mice. Cancer 112: 596-607.

69. Thompson, L. U., Chen, J. M., Li, T., Strasser-Weippl, K. & Goss, P. E. (2005) Dietary flaxseed alters tumor biological markers in postmenopausal breast cancer. Clin Cancer Res 11: 3828-3835.

70. Gonzalez, M. J., Schemmel, R. A., Gray, J. I., Dugan, L., Jr., Sheffield, L. G. & Welsch, C. W. (1991) Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis 12: 1231-1235.

71. Reeves, P. G. (1997) Components of the AIN-93 diets as improvements in the AIN-76A diet. J Nutr 127: 838S-841S.

72. Gottardis, M. M., Wagner, R. J., Borden, E. C. & Jordan, V. C. (1989) Differential ability of antiestrogens to stimulate breast cancer cell (MCF-7) growth in vivo and in vitro. Cancer Res 49: 4765-4769.

73. Thompson, E. W., Paik, S., Brunner, N., Sommers, C. L., Zugmaier, G., Clarke, R., Shima, T. B., Torri, J., Donahue, S. et al. (1992) Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 150: 534-544.

74. Honjo, Y., Nangia-Makker, P., Inohara, H. & Raz, A. (2001) Down-regulation of galectin-3 suppresses tumorigenicity of human breast carcinoma cells. Clin Cancer Res 7: 661-668.

75. Tang, Y., Scollard, D., Chen, P., Wang, J., Holloway, C. & Reilly, R. M. (2005) Imaging of HER2/neu expression in BT-474 human breast cancer xenografts in athymic mice using [(99m)Tc]-HYNIC-trastuzumab (Herceptin) Fab fragments. Nucl Med Commun 26: 427-432.

76. Xu, Y. M., Wang, L. F., Jia, L. T., Qiu, X. C., Zhao, J., Yu, C. J., Zhang, R., Zhu, F., Wang, C. J. et al. (2004) A caspase-6 and anti-human epidermal growth factor receptor-2 (HER2) antibody chimeric molecule suppresses the growth of HER2-overexpressing tumors. J Immunol 173: 61-67.

77. Bossi, G., Lapi, E., Strano, S., Rinaldo, C., Blandino, G. & Sacchi, A. (2006) Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene 25: 304-309.

78. van Slooten, H. J., Bonsing, B. A., Hiller, A. J., Colbern, G. T., van Dierendonck, J. H., Cornelisse, C. J. & Smith, H. S. (1995) Outgrowth of BT-474 human breast cancer cells in immune-deficient mice: a new in vivo model for hormone-dependent breast cancer. Br J Cancer 72: 22-30.

79. Perillo, N. L., Marcus, M. E. & Baum, L. G. (1998) Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. J Mol Med 76: 402-412.

80. De Schrijver, R. & Privett, O. S. (1982) Interrelationship between dietary trans Fatty acids and the 6- and 9-desaturases in the rat. Lipids 17: 27-34.

81. Thiebaut, A. C., Chajes, V., Gerber, M., Boutron-Ruault, M. C., Joulin, V., Lenoir, G., Berrino, F., Riboli, E., Benichou, J. & Clavel-Chapelon, F. (2009) Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. Int J Cancer 124: 924-931.

82. Mensink, R. P. & Katan, M. B. (1990) Effect of dietary trans fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects. N Engl J Med 323: 439-445.

83. Chang, N. W., Chen, F. N., Wu, C. T., Lin, C. F. & Chen, D. R. (2009) Apolipoprotein E4 allele influences the response of plasma triglyceride levels to tamoxifen in breast cancer patients. Clin Chim Acta 401: 144-147.

84. (2009) [Lipid metabolism disorders at the breast cancer patients receiving hormonotherapy]. Georgian Med News: 44-47.

85. Lu, Z., Hendrich, S., Shen, N., White, P. J. & Cook, L. R. (1997) Low linolenate and commercial soybean oils diminish serum HDL cholesterol in young free-living adult females. J Am Coll Nutr 16: 562-569.

86. Yan, C., Wen-Chao, L., Hong-Yan, Q., Rui, Z., Wei-Lin, J. & Hua, H. (2007) A new targeting approach for breast cancer gene therapy using the human fatty acid synthase promoter. Acta Oncol 46: 773-781.

Tables

Table 1: Analysis of FA composition in SO, LLSO, LLMOSO, and lard.

A. Percentages of FA compositions in SO, LLSO, LLMOSO and lard.

	SO	LLSO	LLMOSO	Lard
C14:0	0.00	0.00	0.00	1.21
C16:0	11.33	10.24	9.30	21.33
C16:1 n-7	0.00	0.00	0.00	1.86
C17:0	0.00	0.00	0.00	0.47
C18:0	5.14	5.10	4.97	13.10
C18:1 n-9	**23.51**	**25.56**	**46.80**	**35.73**
C18:1 n-7	1.43	1.41	1.41	2.39
C18:2 n-6	**49.57**	**52.36**	**31.82**	**12.15**
C20:0	0.41	0.39	0.45	0.23
C18:3 n-6	0.18	0.00	0.21	0.00
C18:3 n-3	**5.81**	**0.00**	**1.62**	**0.60**
C20:1 n-9	0.00	0.00	0.26	0.71
C20:2 n-6	0.00	0.00	0.00	0.56
C20:3 n-6	0.00	0.00	0.00	0.00
C20:3 n-3	0.00	0.00	0.00	0.00
C20:4 n-6	0.00	0.00	0.00	0.19
C20:5 n-3	0.00	0.00	0.00	0.00
C22:6 n-3	0.00	0.00	0.00	0.00
Others	2.62	4.94	3.16	9.45

LLSO and LLMOSO had changes in unsaturated FA compositions, mainly OA (C18:1 n-9), LA (C18:2 n-6) and αLA (C18:3 n-3) compared to SO. Lard was included as a comparison. Lipid compositions of individual oil were analyzed by GC. g/ 100 g FAs.

B. Calculated n-6/n-3 ratios and total SFA, MUFA, and PUFA in SO, LLSO, LLMOSO, and lard.

	SO	LLSO	LLMOSO	Lard
Total n-6	49.75	52.36	32.82	12.34
Total n-3	5.81	0	0.6	1.62
n-6/n-3	8.46	52.36	54.70	7.62
SFA	16.88	15.73	14.72	36.34
MUFA	24.94	26.97	48.47	40.69
PUFA	55.38	52.36	33.65	13.31

The values for total n-3 were the sum of C18:3 n-3, C20:5 n-3, and C22:6 n-3 and the values for n-6 were the sum of C18:2 n-6 and C20:4 n-6 in Table 1A. Total SFA values were the sum of C14:0, C16:0, C17:0, C18:0, C20:0, total MUFA values were the sum of C16:1 n-7, C18:1 n-7, C18:0 n-9, C20:1 n-9, and total PUFA values were the sum of C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:4 n-6. in Table 1A.

Table 2: Oligonucleotide sequences for qRT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Her-2/*neu*	GGAAACCTGGAACTCACCTACC	TGGGACCTGCCTCACTTGG
FAS	GACCGCTTCCGAGATTCC	CAGGCTCACAAACGAATGG
GAPDH	TTGGTATCGTGGAAGGACTC	TAGAGGCCAGGGATGATGTTC

PCR efficiencies between 80.3 %and 105.0%.

Table 3: FA compositions of SK-Br3 cells after oil treatments.

	Control	**SO**	**LLSO**	**Lard**	**LLMOSO**
C16:0	24.42^{a} ± 1.11	15.98^{b} ± 0.58	14.63^{b,c} ± 0.42	24.08^{a,b,e} ± 0.91	13.59^{b,c,d} ± 0.82
C16:1 n-7	2.61^{a} ± 0.14	0.54^{b} ± 0.13	0.54^{b,c} ± 0.13	2.20^{a,e} ± 0.14	0.54^{b,c,d,e} ± 0.13
C18:0	14.58^{a} ± 0.71	7.48^{b} ± 0.43	7.01^{b,c} ± 0.30	12.65^{a,e} ± 0.54	6.66^{b,c,d} ± 0.58
C18:1 n-9	29.16^{a} ± 1.45	23.97^{a,c} ± 0.27	25.62^{a,d} ± 0.13	33.66^{a,e} ± 1.42	41.67^{b,c,d} ± 1.76
C18:1 n-7	4.60^{a} ± 0.54	2.03^{b} ± 0.16	2.05^{b,c} ± 0.17	2.76^{b,c,d} ± 0.25	2.07^{b,c,d,e} ± 0.17
C18:2 n-6	5.71^{a} ± 0.05	40.11^{b} ± 1.71	43.64^{b,c} ± 2.09	11.09^{d} ± 0.32	27.92^{e} ± 1.25
C18:3 n-3	0.16^{a} ± 0.02	4.49^{b} ± 0.20	0.87^{b,c} ± 0.03	0.52^{b,c,d} ± 0.02	1.46^{a,b,c,d} ± 0.12
C20:1 n-9	0.88^{a} ± 0.03	0.21^{b} ± 0.06	0.34^{b,c} ± 0.07	0.60^{d} ± 0.06	0.34^{b,c,e} ± 0.05
C20:4 n-6	1.87^{a} ± 0.18	0.34^{b} ± 0.02	0.33^{b,e} ± 0.04	0.53^{d} ± 0.02	0.34^{b,c,e} ± 0.06
C20:5 n-3	0.22^{a} ± 0.10	0.04^{a} ± 0.04	0.07^{a} ± 0.05	0.03^{a} ± 0.01	0.01^{a} ± 0.01
C22:6 n-3	0.21^{a} ± 0.07	0.06^{a,b} ± 0.03	0.07^{a,b} ± 0.04	0.03^{a,b} ± 0.01	0.02^{b} ± 0.00
Others	15.58 ± 0.41	4.75 ± 0.42	4.83 ± 1.08	11.85 ± 3.66	5.37 ± 0.98

The FA compositions were analyzed by GC. Values are means ± SEM, n=3. Means with different letters are significantly different, $p<0.05$.

Table 4: Relative expression levels of Her-2/*neu* and FAS expression in human BC cells.

	SK-Br3	MCF10A	MCF10AT1	MCF-7	T47-D	MDA-MB-231	MDA-MB-468
ER status	(-)	(-)	(+)	(+)	(+)	(-)	(-)
Her-2/*neu*	++++	-/+	-	-/+	+	-/+	-
FAS	++++	+++/++++	+++/++++	+++/++++	++++	++	+++/++++

The table represents the relative expression of Her-2/*neu* and FAS in a panel of BC cells. The levels of Her-2/*neu* and FAS were expressed as the number of + signs (++++; overexpression, -/+; very low expression). – signs indicate no expression. The data were based on the Western blotting (Figure 5). ER status for each cell line is from a published journal (33). (-) means ER negative, (+) means ER positive cell line.

Table 5: Dietary treatment groups.

Diet groups	Cells	Mice (n)
20% LLSO	SK-Br3	10
20% SO	SK-Br3	10
20% lard	SK-Br3	10
17% SO (chow)	SK-Br3	6

Table 6. FA compositions (g/100g FAs) of diets (A), abdominal fat (B), and plasma (C) after feeding study.

A. Diet

	Lard	**SO**	**LLSO**
C14:0	0.99	0.16	0.10
C14:1 n-5	0.02	0.00	0.00
C15:0	0.05	0.02	0.02
C16:0	17.21	9.02	9.08
C16:1 n-7	1.45	0.32	0.12
C17:0	0.27	0.08	0.07
C18:0	9.02	4.15	3.78
C18:1,t6-8	0.11	0.00	0.00
C18:1,t9	0.15	0.01	0.00
C18:1,t10	0.23	0.02	0.02
C18:1,t11	0.12	0.03	0.00
C18:1 n-9	30.17	23.52	19.16
C18:1 n-7	1.88	1.31	1.20
C18:2 n-6	17.46	43.34	43.02
C18:3 n-3	0.95	0.90	0.11
C20:1 n-9	0.57	0.26	5.37
CLA	0.16	0.03	0.00
C20:4 n-6	0.19	0.02	0.00
C20:5 n-3	0.00	0.00	0.00
C22:6 n-3	0.19	0.00	0.00
Others	6.66	2.65	5.78

B. Abdominal Fat			
	Lard	**SO**	**LLSO**
C14:0	1.28	0.96	1.01
C14:1 n-5	0.07	0.05	0.05
C15:0	0.07	0.07	0.06
C16:0	18.55	16.11	16.82
C16:1 n-7	4.24	3.16	3.00
C17:0	0.19	0.14	0.14
C18:0	5.65	4.52	5.10
C18:1,t6-8	0.05	0.00	0.00
C18:1,t9	0.20	0.04	0.05
C18:1,t10	0.13	0.00	0.00
C18:1,t11	0.07	0.03	0.02
C18:1 n-9	49.35	35.12	36.85
C18:1 n-7	2.74	1.92	1.91
C18:2 n-6	11.88	31.54	30.19
C18:3 n-3	0.33	2.06	0.33
C20:1 n-9	1.35	0.89	0.95
CLA	0.29	0.15	0.14
C20:4 n-6	0.19	0.20	0.36
C20:5 n-3	0.00	0.00	0.00
C22:6 n-3	0.05	0.11	0.04
Others	5.64	5.06	5.12

C. Plasma			
	Lard	**SO**	**LLSO**
C14:0	0.29	0.16	0.18
C14:1 n-5	0.00	0.00	0.00
C15:0	0.08	0.08	0.07
C16:0	16.46	15.28	15.06
C16:1 n-7	1.52	0.94	0.85
C17:0	0.27	0.26	0.25
C18:0	10.77	10.55	9.58
C18:1,t6-8	0.07	0.04	0.04
C18:1,t9	0.12	0.08	0.06
C18:1,t10	0.13	0.06	0.06
C18:1,t11	0.09	0.09	0.06
C18:1 n-9	21.19	14.05	15.84
C18:1 n-7	2.07	1.36	1.36
C18:2 n-6	21.25	33.70	32.87
C18:3 n-3	0.23	1.22	0.24
C20:1 n-9	0.54	0.40	0.39
CLA	0.13	0.10	0.07
C20:4 n-6	16.16	12.80	16.24
C20:5 n-3	0.11	0.36	0.03
C22:6 n-3	2.93	3.53	1.94
Others	5.59	4.96	4.79

The FA compositions were analyzed by GC.

Table 7: The amounts of *trans* fat, total n-3 and n-6 in diets (A), abdominal fat (B), and plasma (C).

A. Diets

	Lard	SO	LLSO
Tans fat	0.70	0.06	0.02
Total n-3	1.35	1.07	0.13
Total n-6	20.89	51.30	50.91
n-6/n-3	15.50	48.04	396.00

B. Abdominal Fat

	Lard	SO	LLSO
Tans fat	0.45	0.07	0.07
Total n-3	0.38	2.17	0.38
Total n-6	12.07	31.75	30.55
n-6/n-3	31.75	14.64	80.82

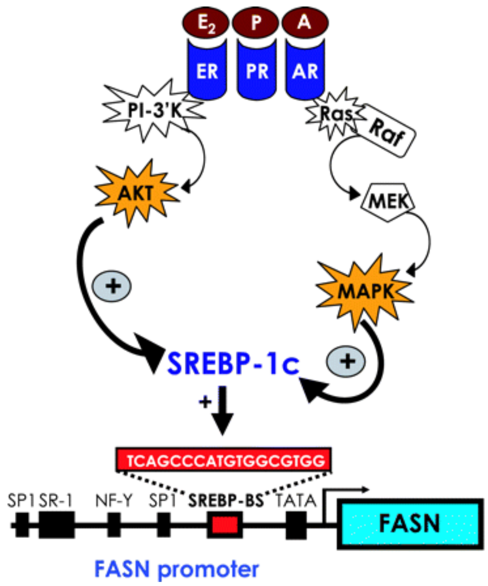
C. plasma

	Lard	SO	LLSO
Tans fat	0.41	0.27	0.22
Total n-3	3.27	5.10	2.22
Total n-6	37.41	46.50	49.12
n-6/n-3	11.43	9.11	22.17

The values for *trans* fat are the sum of C18:1,t6-8, C18:1,t9, C18:1,t10, and C18:1,t11 in Table 6. The values for total n-3 are the sum of C18:3 n-3, C20:5 n-3, and C22:6 n-3 in Table 6. The values for n-6 are the sum of C18:2 n-6 and C20:4 n-6 in Table 6.

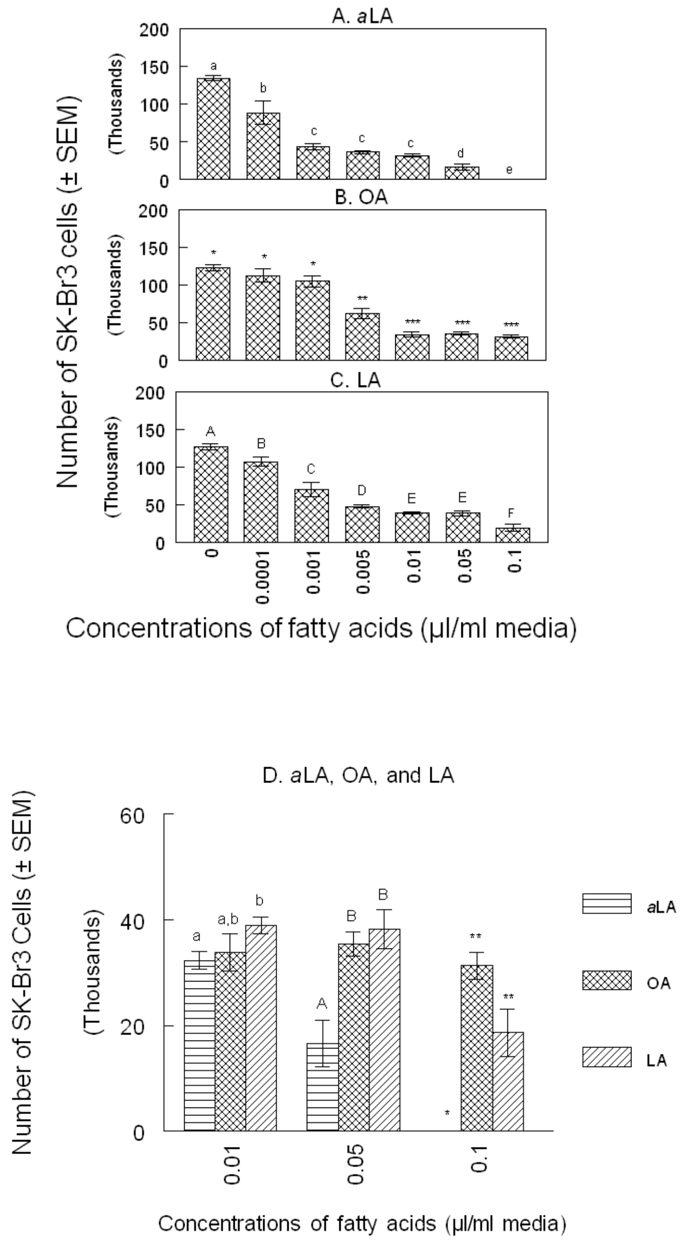
Figures

Figure 1 Hormonal regulation of FAS gene expression in cancer cells.



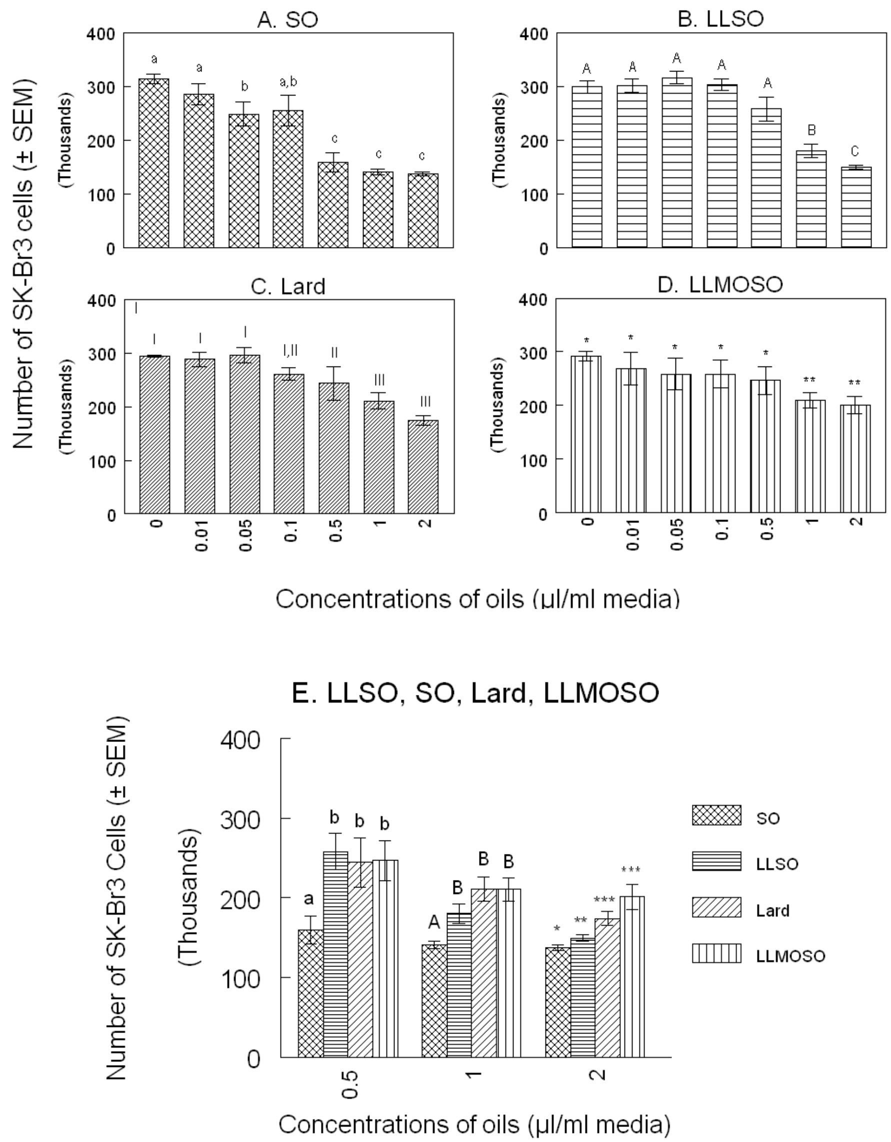
FAS gene regulation in hormone-sensitive neoplastic cells seems to occur through modulation of the transcription factor SREBP-1c, a critical intermediate in the pro- and anti-lipogenic actions of several hormones and nutrients that binds to sterol regulatory elements (SREBP-BS) in the promoter region of the target gene FAS. SREBP-1c up-regulation and nuclear maturation appears to be driven by the activation of MEK1/MEK2 → ERK1/2 MAPK, and PI-3'K → Akt signaling cascades that occurs in response to the specific binding of steroid hormones such as androgens (A), progestins (P), and E_2 to their receptors (AR, PR and ER, respectively). Published by Lupu et al., 2006 (86).

Figure 2: The effect of αLA, OA, and LA on cell viability of SK-Br3.



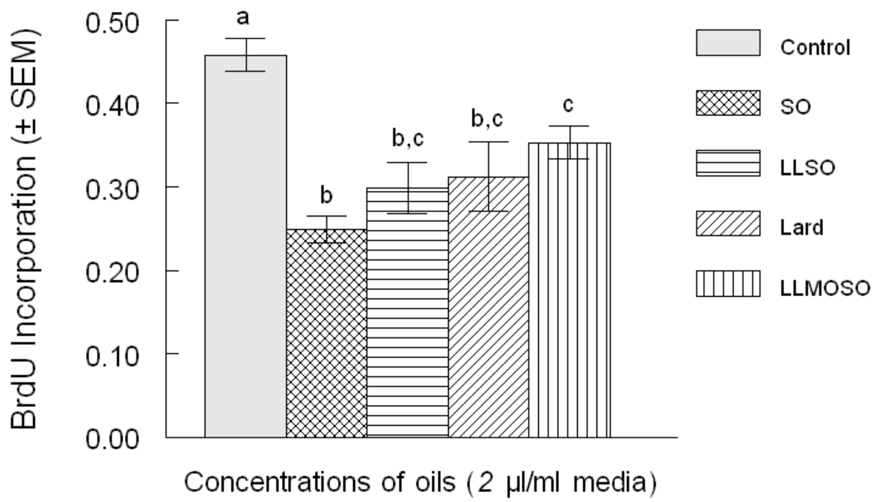
αLA (A), OA (B), and LA (C) at concentrations between 0.0001- 0.1 µl/ml media were evaluated for their effects on SK-Br3 cell viability using MTT assay. The data from higher concentrations (0.01 – 0.1 µl/ml) of individual FA treatments were selected for statistical analysis between the FAs (D). SK-Br3 cell viability was expressed as mean number of SK-Br3 cells ± SEM from three experiments. Bars with different letters are significantly different, $p<0.05$.

Figure 3: The effects of SO, LLSO, LLMOSO and lard on cell viability of SK-Br3.



SO (A), LLSO (B), lard (C), and LLMOSO (D) at concentrations between 0-2 µl/ml media were evaluated for their effects on SK-Br3 cell viability using MTT assay. The data from higher concentrations (0.5-2 µl/ml) of oil treatments were selected for statistical analysis between oil treatments (E). SK-Br3 cell viability was expressed as mean numbers of SK-Br3 cells ± SEM from three experiments. Bars with different letters are significantly different, $p<0.05$.

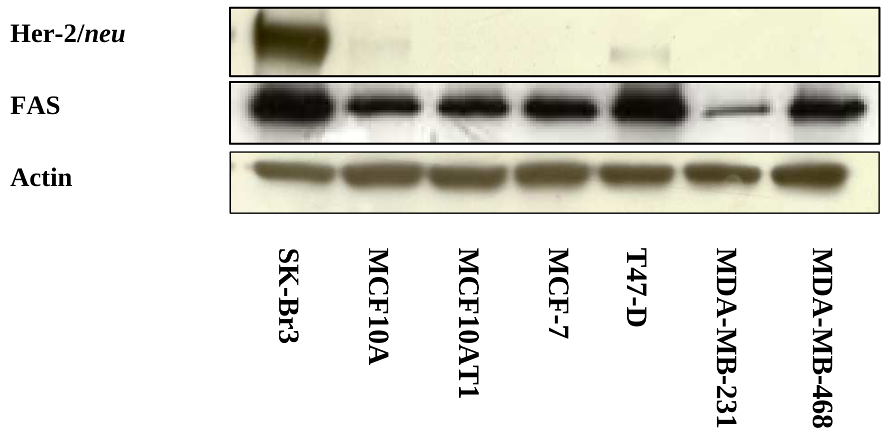
Figure 4: The effects of SO, LLSO, lard, or LLMOSO on cell proliferation of SK-Br3.



The SK-Br3 (1×10^4 cells/100µl/well) cells treated with 2 µl/ml of SO, LLSO, lard, or LLMOSO for 96 h before BrdU incorporation. No oil treatment was included as a control. SK-Br3 cell proliferation was expressed as mean values of BrdU incorporation in SK-Br3 cells ± SEM from three independent experiments. Bars with different letters are significantly different, $p<0.05$.

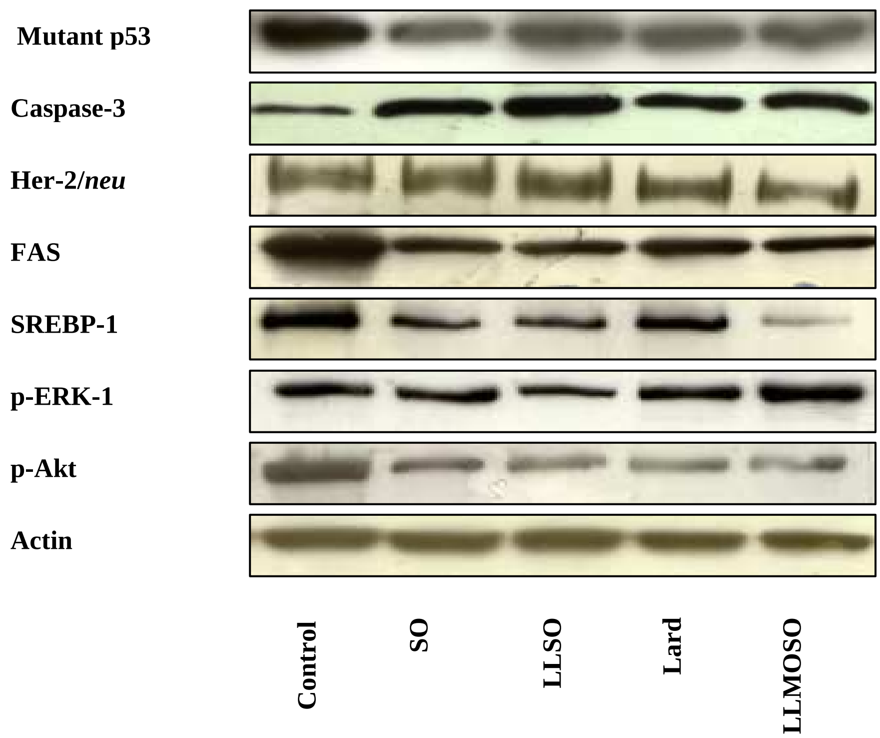
Figure 5: Her-2/*neu* and FAS expression in human BC cells.

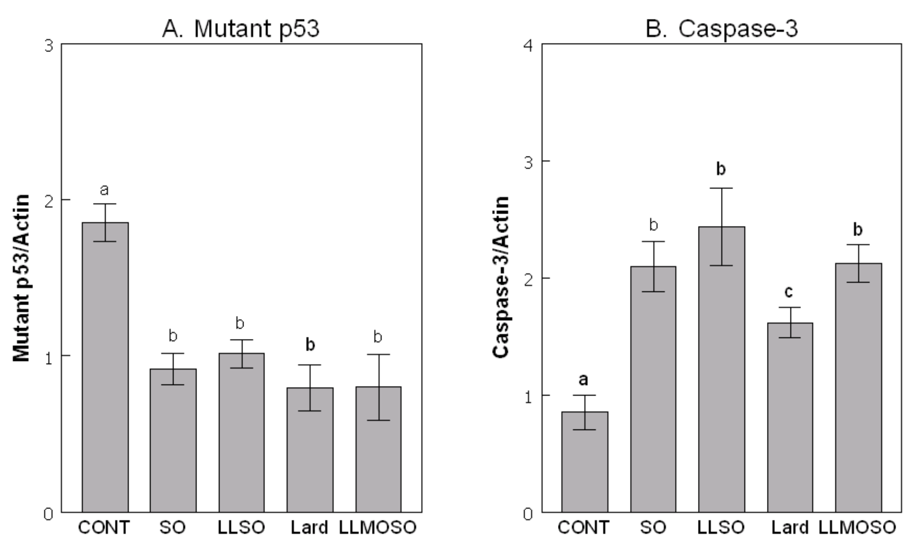
A. Western Blot

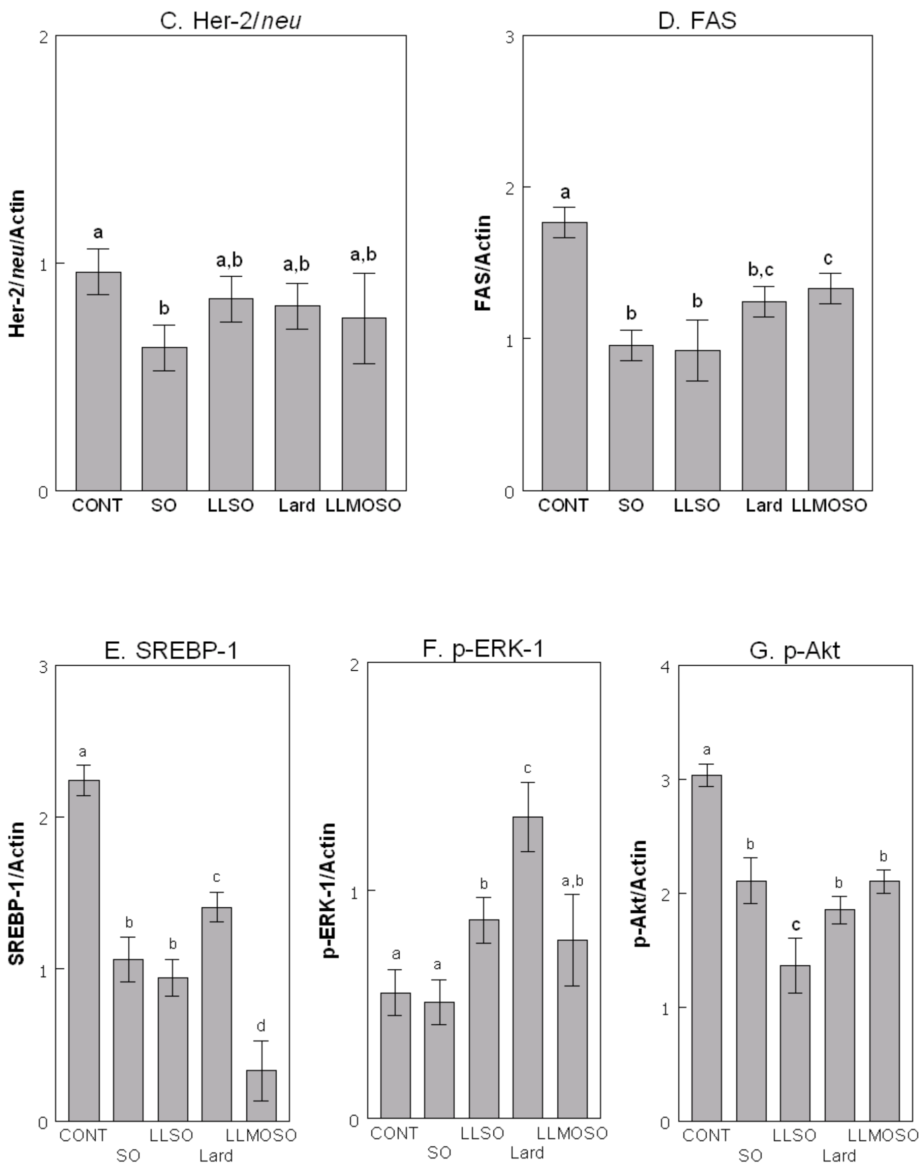


Western blotting was employed for relative analysis of Her-2/*neu* and FAS protein levels. 10 µg of total protein were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The proteins were probed with a rabbit anti-Her-2/*neu* polyclonal antibody or a rabbit anti-FAS polyclonal antibody. Beta-actin was used as an internal control. Similar results were obtained in three independent experiments. Figure shows a representative immunostaining analysis.

Figure 6: Protein expression levels in SK-Br3 cells after oil treatments.

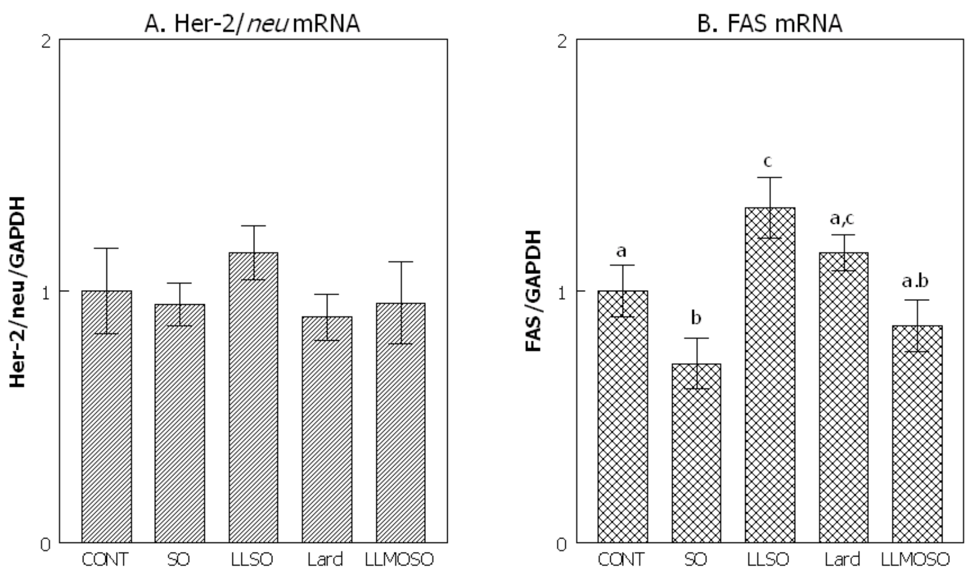






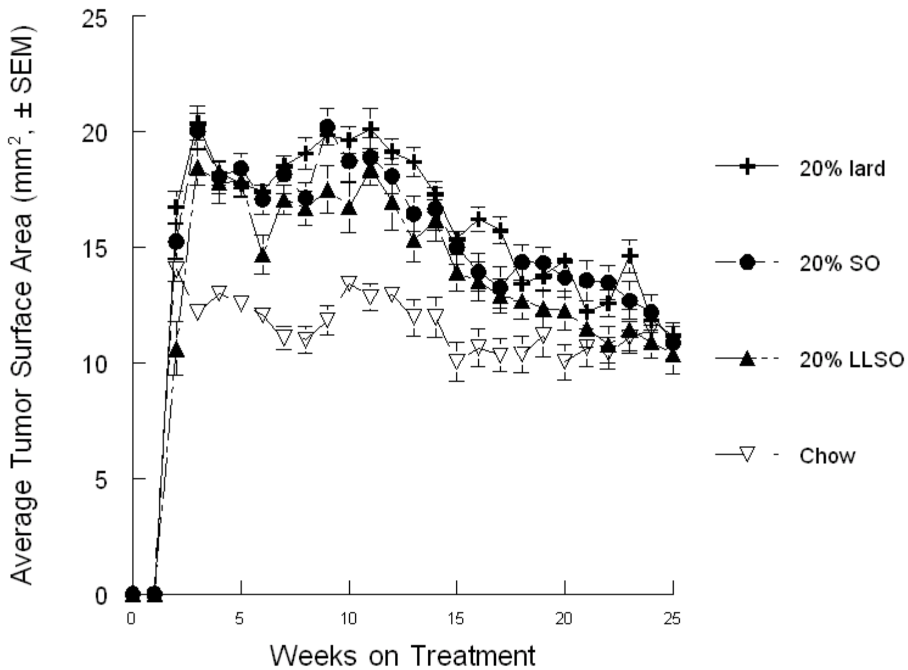
Western blotting analysis of mutant p53 (A), caspase-3 (B), Her-2/*neu* (C), FAS (D), SREBP-1 (E), p-ERK-1 (F), and p-Akt (G). Representative immunoblot of target protein was shown. Relative expression (mean ± SEM) of target protein was shown in graphs (A-G). The relative expression level was calculated as the ratio of each protein to beta-actin. CONT: control, no oil treatment. Bars with different letters are significantly different, $p<0.05$.

Figure 7: Her-2/*neu* and FAS mRNA expression levels in SK-Br3 cells treated with oils.



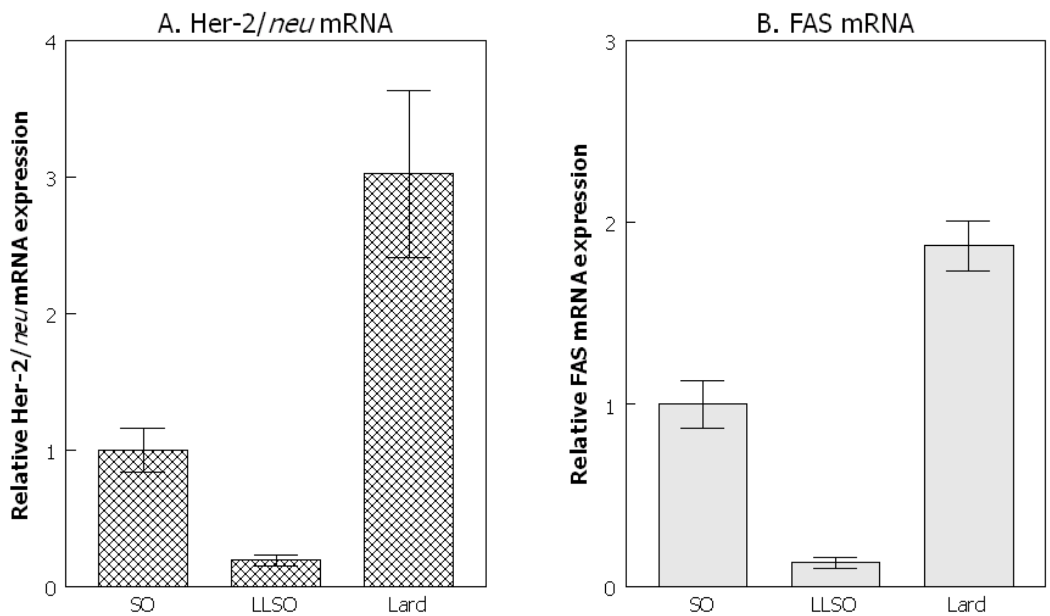
A: Her-2/*neu* mRNA expression levels. B: FAS mRNA expression levels after oil treatments. SK-Br3 cells were incubated with 2 µl/ml of SO, LLSO, lard, or LLMOSO for 48 h. Results were expressed as the mean ± SEM (n=3). Bars with different letters are significantly different, $p<0.05$. CONT: control, no oil treatment.

Figure 8: Tumor growth (surface area) of SK-Br3 fed with diets containing chow, SO, LLSO, and lard.



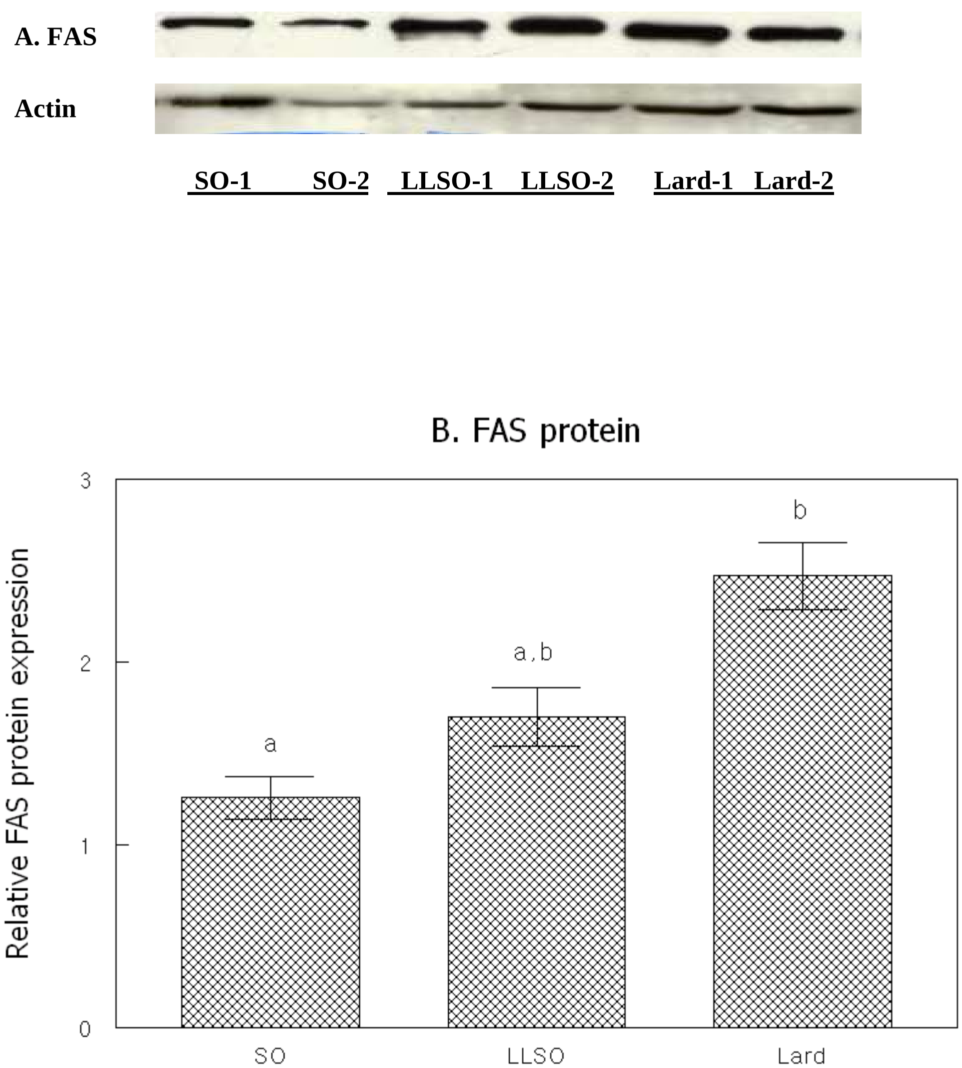
All mice received SK-Br3 cells (1×10^5/ 40µl/site, 4 sites/ animal) subcutaneously into the mice. Tumor growth and body weight were measured weekly, and tumor cross-sectional area was determined using the formula [length (mm)/2 × width (mm)/2 × π].

Figure 9: Her-2/*neu* and FAS mRNA expression levels in mice tumors fed with diets containing chow, SO, LLSO, or lard.



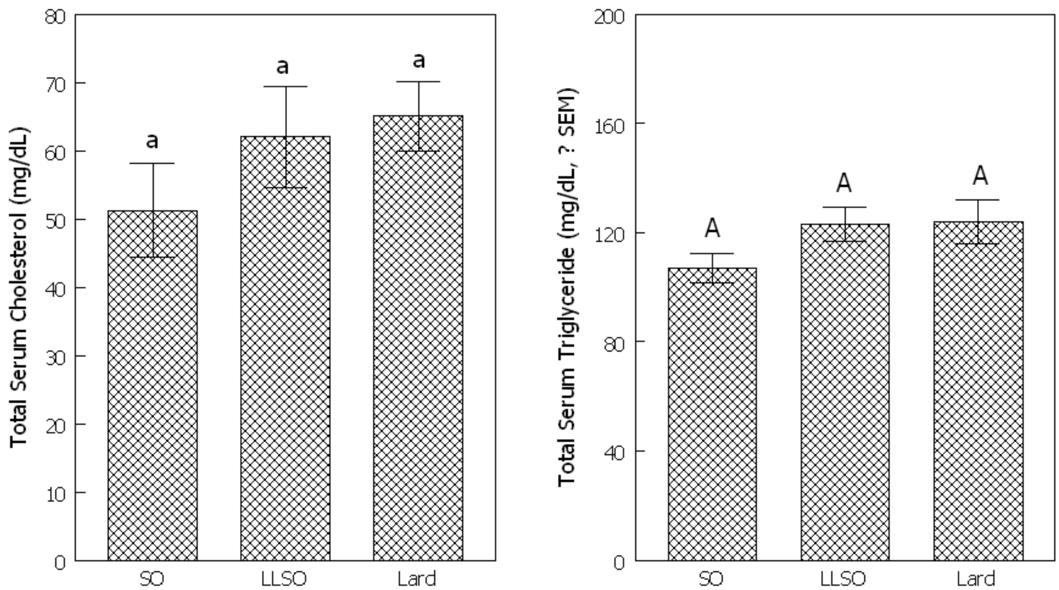
The tumors from individual mouse in the same diet treatment were combined in a tube before RNA extraction. Total RNA was prepared from the pooled tumors (100 mg) and 10 ng was used to synthesize the cDNA. Each sample was run as triplicates. GAPDH was used to calculate the relative expression.

Figure 10: FAS expression in mice liver fed by diets containing chow, SO, LLSO, or lard.



Total protein was extracted from mice liver to compare FAS protein expression levels. A.FAS protein expression level was measured by western blot using anti-FAS antibody. The figure was a representative of n=6 for chow diet, n=10 for SO, LLSO, or lard containing diets. Beta-actin was used as a loading control. The extent of FAS protein was quantified using a densitometer with Image-Pro Plus software. B. Relative expression of FAS protein. The relative level was calculated as the ratio FAS to beta-actin. Results were expressed as the mean ± SEM. Bars with different letters are significantly different, *p*<0.05.

Figure 11: Levels of total cholesterol and triglyceride in mice blood.



The plasma from each mouse was used to analyze the level of cholesterol (A) and triglyceride (B). Results are expressed as the mean ± SEM (n=6 for chow diet, n=10 for SO, LLSO, or lard containing diets). Bars with different letters are significantly different, $p<0.05$.