FATTY ACID SYNTHASE, A NOVEL TARGET FOR THE TREATMENT OF DRUG RESISTANT BREAST CANCERS

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Dedicated to my parents for all the sacrifices they made for my better education, to my husband for his endless love, support and faithful prayers, and to my two lovely sons for their heart-melting smiles and kisses.

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Abstract

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Fatty acid synthase, a novel target for the treatment of drug resistant breast cancers

Many cancers, including breast cancer, often develop resistance to chemotherapeutic drugs over a course of treatment. Many factors, including ABC transporter-mediated drug efflux, have been shown to play a role in acquired drug resistance. Fatty acid synthase (FASN), the key enzyme of lipid synthesis pathway, was found to be over-produced in an Adiamycin resistant breast cancer cell line MCF7/AdrVp3000, compared to its parental drug sensitive MCF7 cell line. Inhibition of FASN expression increased the drug sensitivity in breast cancer cells (MCF7/AdrVp3000 and MDA-MB-468), but not in the normal breast epithelia cell line MCF10A1. Enforced overexpression of FASN in MCF7 breast cancer cells decreased its drug sensitivity. These results indicated that FASN overexpression can induce drug resistance in breast cancers.

Ectopic overexpression of FASN in MCF7 cells did not affect cell membrane permeability, transporter activity, nor did it affect cell proliferation rate. However, FASN overexpression protects cancer cells from drug-induced apoptosis by decreasing caspase-8 activation. In FASN over-expressing

MCF7 cells, I discovered the positive feedback relationship between FASN and activation of Akt as previously reported. However, activation of Akt did not mediate FASN-induced drug resistance.

Together with the findings that FASN expression associates with poor prognosis in several types of cancers, my investigations suggest that FASN overexpression is a novel mechanism of drug resistance in breast cancer chemotherapy. Inhibitors of FASN can be used as sensitizing agents in breast cancer chemotherapy.

Jian-Ting Zhang, Ph.D., Committee Chairperson

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List of Abbreviations

ABCG2	ATP-binding cassette G2
ACC	Acetyl-CoA carboxylase
BCRP	Breast cancer-resistance protein
CPT-1	Carnitine palmitoyltransferase-1
DAG	Diacylglycerol
DISC	Death-inducing signaling complex
EGCG	Epigallocatechin gallate
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunosorbent assay
ERBB2	Erythroblastic leukemia viral oncogene homolog 2
ESI	Electrospray ionization
FASN	Fatty acid synthase
FLIP	FLICE like-inhibitory protein
GFR	Growth factor receptor
HER2	Human Epidermal growth factor Receptor 2
IP	Immunoprecipitation
MALDI-TOF	matrix-assisted laser absorption/ionization-time-of-flight
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MRP1	Multidrug-resistance protein-1
MS	Mass spectrometry

NADPH	nicotinamide adenine dinucleotide phosphate
OD	Optical density
PA	Palmitic acid, palmitate
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PgP	P-glycoprotein
PI3K	Phosphoinositide-3 kinase
PTEN	phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
shRNA	Small hairpin RNA
siRNA	Small interference RNA
SDS	Sodium dodecyl sulfate
SMase	Sphingomyelinase
SRB	Sulforhodamine B
SREBP	Sterol Regulatory Element Binding Proteins

I. Introduction

Altered metabolism in human cancers has been long recognized. The first observation of increased anaerobic glycolysis in cancer was made in 1920 by Otto Warburg (Warburg, 1956). The so called Warburg effect has now become a hallmark of the transformed phenotype, and is thought to provide growth advantages to cancer cells (Bui and Thompson, 2006; Shaw, 2006). Another metabolic hallmark of cancer is an altered lipogenic pathway with increased de novo fatty acid synthesis (Kuhajda, 2000).

Fatty acids serve as important substrates of energy metabolism, essential building blocks of cellular membrane, intracellular second messengers, and anchorage for membrane proteins. There are two types of fatty acids for metabolism, exogenously-derived (dietary) fatty acids and endogenously-synthesized fatty acids. The latter is synthesized by fatty acid synthase (FASN). In a well nourished individual, cells and tissues preferentially use circulating free fatty acids from diet. FASN expression and activity are minimal except in some hormone-sensitive cells such as lactating breast and cycling endometrium. In these cells, FASN expression is tightly regulated by hormonal signals (Kuhajda, 2000).

In 1994, Kuhajda and colleagues identified oncogenic antigen-519 (OA-519) – a molecular maker found in breast cancer patients with a markedly poorer prognosis – as FASN (Kuhajda et al., 1994). Thereafter, increased FASN expression level was found in various human tumors and

their pre-neoplastic lesions, including breast, colon, prostate, lung, bladder, ovary, stomach, endometrium and many other types of cancers (Kuhajda, 2000; Kuhajda, 2006; Menendez and Lupu, 2006; Menendez and Lupu, 2007). FASN overexpression and hyperactivity is an indicator of tumor aggressiveness and poor prognosis in many type of tumors (Kuhajda, 2000; Kuhajda, 2006; Menendez and Lupu, 2007). Interrupting the lipogenesis with chemical inhibitors of FASN, such as C75 and Orlistat, or by RNA interference of FASN expression, has been shown to effectively and selectively kill cancer cells (Menendez et al., 2004e; Pizer et al., 2000; Wang et al., 2005; Zhou et al., 2003). With the recent published FASN crystal structure (Asturias et al., 2005; Maier et al., 2006; Maier et al., 2008; Pemble et al., 2007) and the development of FASN inhibitors (Alli et al., 2005; Chiang et al., 2007; Kridel et al., 2004; Kuhajda et al., 2000), targeting FASN opens a new window of opportunity for metabolically combating cancer.

A. Structure of mammalian fatty acid synthase

Human fatty acid synthase (FASN) is a 270 kDa, cytoplasmic enzyme (Smith et al., 2003; Wakil, 1989). FASN can be induced to associate transiently with lipid raft membranes and form a complex with palmitoylated Caveolin-1 in human and murine prostate cancer cells, following the activation of upstream signals within the Src, Akt and EGFR family (Di Vizio et al., 2008). There is a second gene encoding a mitochondrial FASN (Zhang et al., 2003).

FASN is a multifunctional polypeptide containing seven catalytic domains, which are β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydrogenase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE) (Smith, 1994). Conventional models of mammalian FASN proposed that FASN formed a fully extended head-to-tail homodimer. However, results from mutant complementation, chemical crosslinking and monomer interaction were imcompatible with the classical model. Therefore, a revised model was proposed, in which FASN forms an interwined head-to-head dimer. Each monomer in the dimeric form of FASN adopts a coiled conformation that allows multiple intra- and inter-monomer functional domain interaction, with the KS domains located in the central portion of the structure. This model was further supported by the results from cryo-electron microscopy and crystal structure studies (Asturias et al., 2005; Maier et al., 2006; Maier et al., 2008).

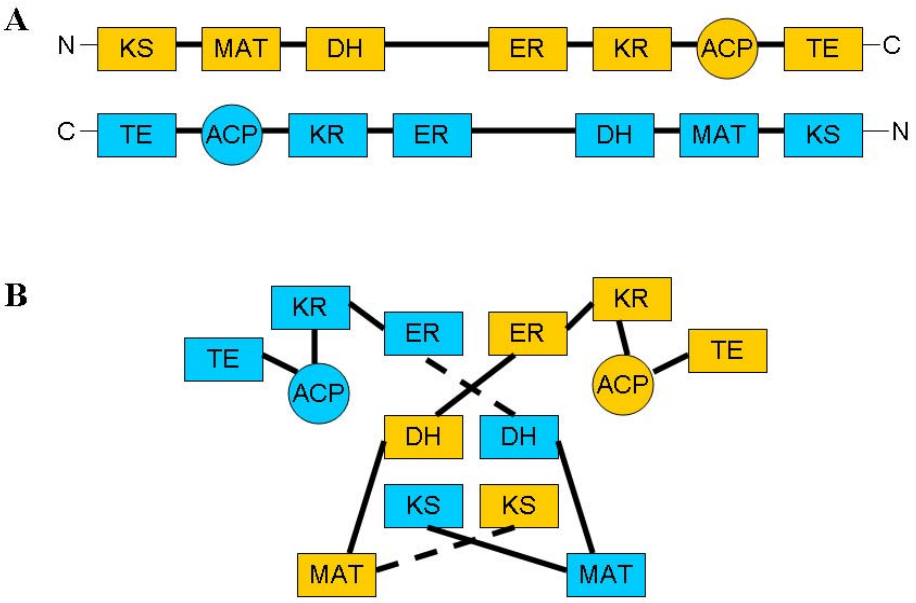

Figure 1. Two models for domain organization of FASN. (**a**) In the conventional model, two monomers in the homodimeric form of FASN are arranged in a fully extended head-to-tail orientation (**b**) A revised model for FASN, which shows an X-shaped dimeric form with each monomer adopts a coiled structure to allow multiple intra- and inter-molecular interaction. FASN catalytic domains, starting from the N-terminus, are ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), dehydrase (DH), enoyl reductase (ER), -ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE).

B. Brief overview of the fatty acid synthesis pathway

Although the synthesis of fatty acids from glucose involves about 25 enzymes, the main synthetic pathway is comprised of the following key elements: 1) acetyl-CoA carboxylase, which carboxylates acetyl-CoA to malonyl-CoA and is the rate limiting enzyme of fatty acid synthesis pathway; 2) citrate lyase, which converts citrate to acetyl-CoA; 3) nicotinamide adenine dinucleotide phosphate (NADPH), used as reducing equivalents; and 4) FASN, the enzyme that condenses acetyl-CoA and malonyl-CoA to 16-carbon palmitate. The whole fatty acid synthesis pathway consumes 14 ATP and 7 NADPH per fatty acid synthesized. (Figure 2)

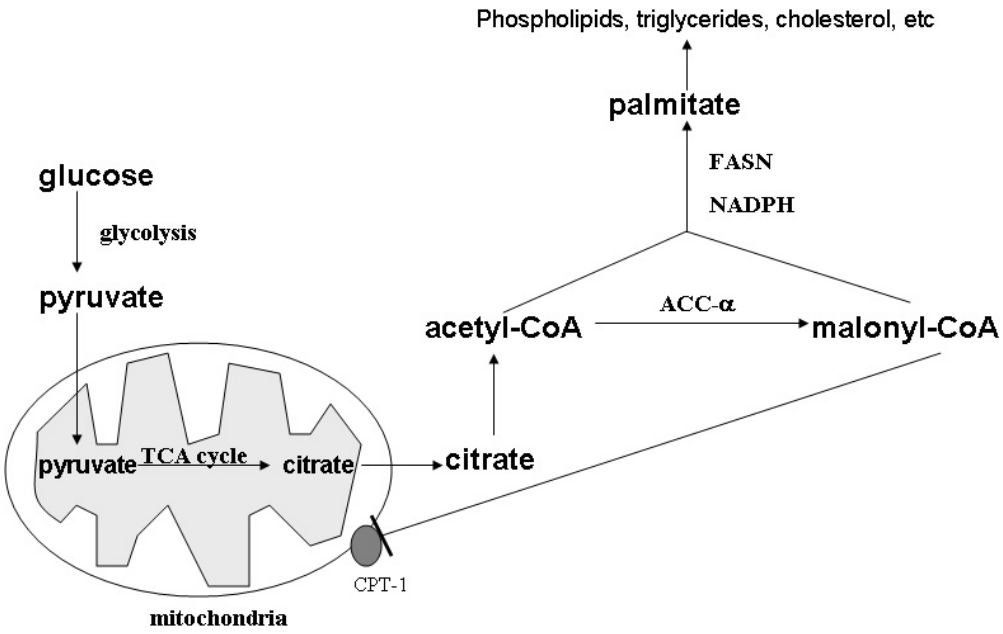

Figure 2. Fatty acid synthesis pathway. The fatty acid synthesis pathway functions in both cancers and lipogenic tissue. In both cases, excess glucose goes through glycolysis and TCA cycle, and exits the mitochondria as citrate. Citrate is transported out of mitochondria and converted to acetyl-CoA, which is then carboxylated to malonyl-CoA by Acetyle-CoA carboxylase (ACCα). FASN condenses one acetyl-CoA and seven malonyl-CoA into palmitate. Palmitate is then modified by various enzymes into various lipids such as phospholipids. Besides its role as FASN substrate, malonyl-CoA inhibits CPT-1 and thus prevents fatty acid oxidation.

Although the fatty acid synthesis pathway is identical in cancer cells and normal lipogenic tissues, the difference lies in the following three aspects:

1. The fate of palmitate, the end product of the fatty acid synthesis pathway, is different between lipogenic tissue and cancer cells. In lipogenic tissues, fatty acid synthesis occurs as a mean to store excess energy from carbohydrate, which is converted to triacylglycerol (McGarry and Brown, 1997). High level of malonyl-CoA during lipogenesis inhibits mitochondrial fatty acid oxidation, directing fatty acids to storage. During starvation, FASN expression and activity is rapidly down-regulated, malonyl-CoA levels decrease, and fatty acid oxidation is initiated to ensure survival. On the other hand, endogenously synthesized fatty acids in cancer cells are mainly converted to phopspholipid and incorporated into the cell membranes (Swinnen et al., 2003).

2. The regulation of FASN is different. In a well fed animal, diet is the main regulator of FASN, except in lactating breast and cycling endometrium, where FASN expression is under the tight hormonal control of estrogen and progesterone (Kuhajda, 2000). In contrast, FASN expression in cancer cells is transcriptionally regulated by either hormonal signals or aberrant growth factor signaling in cancer cells (Porstmann et al., 2005; Swinnen et al., 2000; Yoon et al., 2007; Zhang et al., 2005).

3. The consequence of FASN inhibition differs significantly between cancer cells and normal cells. FASN inhibition by either inhibitor or siRNA induces apoptosis selectively in human cancer cells both *in vitro* and *in vivo* (De Schrijver et al., 2003; Menendez et al., 2004a; Menendez et al., 2004c; Zhou et al., 2003), but not normal cells (Browne et al., 2006; Chajes et al., 2006; Liu et al., 2008).

C. FASN expression in cancers.

High level of FASN expression has been found in many types of cancers and their pre-neoplastic lesions, including breast, prostate, ovary, lung, colon etc. (Kuhajda, 2006; Menendez and Lupu, 2007). Elevated FASN levels have also been identified in the blood of patients with breast, prostate, colon, and ovarian cancers compared with normal subjects using ELISA (Wang et al., 2004). High FASN expression associates with poor prognosis in some type of cancers including breast, prostate, ovarian, stage I non-small cell lung cancer, malignant melanoma and soft tissue sarcoma (Kuhajda, 2000; Kuhajda, 2006). FASN expression and activity clearly confer growth advantages in human cancers and can be developed as a marker for cancer detection, prognosis and monitoring.

FASN expression in normal liver and adipose tissue is controlled by nutritional signals. Carbohydrate ingestion, thyroid hormone, insulin, and glucocorticoid coordinately upregulate FASN expression, while unsaturated fatty acids, cyclic-AMP, and glucagon downregulate FASN expression. In lactating breast and cycling endometrium, FASN expression is tightly regulated by estrogen and progestin (Kuhajda, 2000).

FASN expression in cancer is no longer responsive to the nutritional signals. In breast and prostate cancers that have functional hormone receptors, FASN expression was shown to be up-regulated upon hormone treatment (Chalbos et al., 1987; Menendez et al., 2005b; Swinnen et al.,

1997). However, in prostate cancer cells following androgen ablation, FASN expression initially decreased, only to return at higher levels following androgren ablation (Ettinger et al., 2004; Pizer et al., 2001). These results indicate that cancer cells may have novel regulatory pathways for FASN expression in cancer cells.

Cancer cells have at least two levels of regulation for FASN expression. At the transcriptional level, growth factors (GF) and growth factor receptors (GFR) have been shown to be the main contributors to FASN overexpression. Epidermal growth factor (EGF) can stimulate FASN overexpression through EGF receptor ERBB1 and ERBB2 (Kumar-Sinha et al., 2003; Swinnen et al., 2000; Zhang et al., 2005). The effect of GFs and GFRs on FASN expression involves complicated downstream signaling and cross-talk between multiple signal transduction pathways. The mitogen-activated protein kinase (MAPK) pathway has been shown to be involved in FASN expression regulation (Gao et al., 2006; Menendez et al., 2004c). The role of the PI3K/Akt pathway in FASN expression has been well studied (Chiang et al., 2007; Furuta et al., 2008; Hughes-Fulford et al., 2006; Pan et al., 2007; Porstmann et al., 2005; Uddin et al., 2008; Van de Sande et al., 2002; Van de Sande et al., 2005; Wang et al., 2005; Weng et al., 2007; Yang et al., 2002b). High levels of FASN expression were linked to activation and nuclear localization of Akt in human prostate cancer tissues (Van de Sande et al., 2005). Similarly, FASN overexpression was shown to be

associated with activated AKT in papillary thyroid cancer tissue using microarray analysis (Uddin et al., 2008). Inhibitors of PI3K pathway can decrease FASN expression, yet loss of PTEN (phosphatase and tensin homologe) and subsequently constitutive activation of Akt has been shown to correlate with increased FASN expression in prostate and ovarian cancer cells (Van de Sande et al., 2002; Wang et al., 2005). A transcriptome analysis of Her2 (ERBB2) in breast cancer cells revealed a molecular connection between FASN and Her-2 through PI3K-Akt-dependent signaling (Kumar-Sinha et al., 2003). GFs signal through MAPK and PI3K/Akt pathways, affecting FASN expression by modulation of a transcription factor, sterol regulatory element binding protein-1c (SREBP1c) (Ettinger et al., 2004; Furuta et al., 2008; Porstmann et al., 2005; Yang et al., 2002b; Yang et al., 2003). Analysis of breast cancer tissues for FASN and SREBP1c mRNA revealed a coordinate regulation of tumor-associated FASN and other lipogenic enzymes by SREBP1c (Yang et al., 2003), further supporting the role of SREBP1c in FASN expression regulation. Steroid hormones (SHs), including estrodial, progestins and androgens, also regulate FASN expression in hormone responsive cancers through aberrant activation of PI3K/Akt and MAPK pathways (Chalbos et al., 1987; Menendez et al., 2005b; Swinnen et al., 1997).

A lack of correlation between FASN gene amplification and FASN protein expression was observed in some prostate cancer cell lines and

tissues (Shah et al., 2006), suggesting the presence of post-translational regulation. In prostate cancer cells, FASN protein stability has been shown to be regulated by an ubiquitin-specific protease, USP2a (Graner et al., 2004). Knockdown of USP2a reduced FASN expression, decreased cell proliferation, and induced apoptosis. Microarray analysis from human prostate cancers has revealed a significant association between the genes in FA metabolism and high USP2a expression (Priolo et al., 2006).

D. FASN as a target for cancer chemotherapy

The differences in the FASN expression level between normal and cancer cells, together with the specific cytotoxicity of FASN inhibition in cancer cells, led to the exploration of FASN as a therapeutic target for cancer treatment.

How FASN overexpression affects the progression and potential response of cancer cells to chemotherapy is not fully understood. Results from the study of the cytotoxic effect of FASN inhibition helps to unveil the possible functions of FASN in cancer. Several mechanisms have been proposed to explain the apoptosis following FASN inhibition.

1. Toxicity from malonyl-CoA accumulation

FASN inhibition causes accumulation of its substrate malonyl-CoA. Studies showed that FASN inhibition induced apoptosis, while acetyl-CoA carboxylase (ACCα) inhibition depleted malonyl-CoA and protected cancer cells from apoptosis (Chajes et al., 2006; Pizer et al., 2000; Thupari et al., 2001). These results suggested that malonyl-CoA accumulation led to cancer cell death. The accumulation of malonyl-CoA inhibits fatty acid β-oxidation by inhibiting mitochondria outer membrane protein CPT-1 (carnitine-palmitoyltransferase 1), which in turn promotes accumulation of ceramide, followed by the induction of pro-apoptotic genes (such as BNIP3, TRAIL, and DAPK2) (Bandyopadhyay et al., 2006).

2. Disturbance of membrane lipid domain

Inhibition of FASN mainly affects the synthesis of phospholipids that incorporate into a detergent-resistant membrane microdomain (lipid raft aggregates), but has a lesser effect on the synthesis of non-raft associated lipids (Swinnen et al., 2003). It is implied that FASN expression levels in colonic adenocarcinoma may contribute to the increased ratio of saturated to unsaturated C18 FAs, and thus affect the structure and function of lipid rafts (Rakheja et al., 2005). Many proteins involved in signal transduction, apoptosis, membrane transport, and cell adhesion, are associated with lipid rafts (Bollinger et al., 2005; Pike, 2003; Simons and Ikonen, 1997). Changes in lipid rafts associated with FASN overexpression in cancer cells are likely to affect the signaling proteins residing in the raft in a way to enhance cancer cell survival and resistance to drug-induced apoptosis. ERBB2 (Her2) is one of the most important oncogenes in breast cancer. It is a receptor tyrosine kinase which co-localizes with lipid rafts (Menendez et al., 2005e). It has been shown that Her2 regulated FASN expression (Kumar-Sinha et al., 2003; Menendez et al., 2005a; Menendez et al., 2005d; Menendez et al., 2006c; Yoon et al., 2007; Zhang et al., 2005), while FASN inhibition decreased Her-2 expression (Chiang et al., 2007; Menendez et al., 2004d; Menendez et al., 2006a; Menendez et al., 2005d; Menendez et al., 2005e; Menendez et al., 2006b; Menendez et al., 2004e; Vazquez-Martin et al., 2007a) and sensitized Her-2 positive cancers to Taxol and vinorelbine, 5-fluorouracil and Herceptin

(Menendez et al., 2004a; Menendez et al., 2004b; Vazquez-Martin et al., 2007a; Vazquez-Martin et al., 2007b). Clinical studies have also demonstrated the linkage between FASN, Her2 and cancer malignancy (Sebastiani et al., 2006; Silva et al., 2004; Silva et al., 2008a; Silva et al., 2008b). Most recently, Menendez and colleagues further demonstrated that enforced ectopic overexpression of FASN in breast epithelia cells can activate Her1/Her2, suggesting that increased *de novo* synthesis of fatty acids by FASN significantly affects the formation of Her1/Her2 signaling complexes at the cell membrane (Vazquez-Martin et al., 2008). Inhibition of FASN is a promising new strategy to overcome the drug resistance in Her-2 over-expressing breast cancers

3. Inhibition of cell proliferation

FASN inhibition has major influence on the synthesis of phopspholipids (Jackowski, 1994; Swinnen et al., 2003), which are important for cell proliferation in two aspects. First, phopspholipids are major components of cellular membranes. FASN inhibition, thus, affects the synthesis of cellular membranes in highly proliferating tumor cells (Pizer et al., 1996b). Second, phopspholipid biosynthesis is greatest during the G1 and S phase of the cell cycles in preparation for cell division (Jackowski, 1994). Inhibiting FASN produces rapid, potent inhibition of DNA replication, which arrests the cell cycle at the G1/S transition by affecting p21, p27, BRCA1, skp2, and NF-κB (Knowles et al., 2004; Knowles and Smith, 2007; Li et al.,

2001; Menendez et al., 2004c; Pizer et al., 1998; Zhou et al., 2003). The tumor suppressor gene p53 is the decision maker between apoptosis and growth arrest following FASN inhibition. FASN inhibition is more effective in initiating apoptosis in cells with non-functional p53, while in cells with functional p53, FASN inhibition is more likely to produce a cytostatic response (Li et al., 2001).

4. Inhibition of cell survival pathways.

Activation of the PI3K/Akt survival pathway is very important for cancer cell survival, proliferation, invasiveness and potential responses to chemo-and radiation therapy (Hu et al., 2002; Page et al., 2000; Testa and Bellacosa, 2001). It is well established that activation of the PI3K/Akt pathway can increase FASN expression in human cancers *in vivo* and *in vitro* (Chiang et al., 2007; Furuta et al., 2008; Hughes-Fulford et al., 2006; Pan et al., 2007; Porstmann et al., 2005; Uddin et al., 2008; Van de Sande et al., 2002; Van de Sande et al., 2005; Wang et al., 2005; Weng et al., 2007; Yang et al., 2002b). Inhibition of the PI3K/Akt pathway by LY 294002 abolished pAkt activity and sensitized human ovarian and breast cancer cells to FASN inhibitor induced apoptosis (Liu et al., 2006a; Wang et al., 2005), indicating that active Akt protects cancer cells against FASN inhibitor induced apoptosis. Furthermore, inhibition of FASN activity caused a decrease in the level of phosphorylated-Akt, which proceded the induction of apoptosis both in vitro and in vivo (Alli et al., 2005; Menendez et al., 2004d; Menendez et al., 2005c;

Orita et al., 2008; Wang et al., 2005; Yeh et al., 2003).

In summary, FASN overexpression confers cancer cells distinct growth advantages by modulating lipid raft domains in a way to promote cell growth, activate cell survival and proliferation signals, and/or prevent apoptosis by inhibiting the pro-apoptotic signals. Further studies of FASN inhibition in cancer cells will help to discover the pathways that modulate cell survival and/or apoptosis, and assist the development of better treatment regimens for cancers.

E. FASN inhibitors

Numerous *in viv*o and *in vitro* studies have confirmed the potential of FASN inhibitors as a novel antitumor treatment. FASN inhibitors such as cerulenin and its derivatives, C75 and C93, FDA approved anti-obesity drug Orlistat, the green tea polypenol epigallocatechine-3-gallate (EGCG) and other naturally occurring flavonoids, as well as the antibiotic triclosan, have been shown to induce cancer cell apoptosis.

The small molecule FASN inhibitor C75 was designed based on cerulenin (Kuhajda et al., 2000). C75 is more chemically stable than cerulenin, and showed significant antitumor effects on cancer cell lines of human breast (Pizer et al., 2000), prostate (Pizer et al., 2001), mesothelioma (Gabrielson et al., 2001), ovarian (Wang et al., 2005), and renal carcinoma xenografts (Horiguchi et al., 2008). Besides its inhibitory effect on FASN, C75 also increases fatty acid oxidation through direct activation of CPT-1 (Cha et al., 2005; Kuhajda et al., 2005; Landree et al., 2004; Nicot et al., 2004; Puig et al., 2008; Thupari et al., 2002; Yang et al., 2005) It also reduces food intake by blocking the production of hypothalamic neuropeptide-Y, which causes substantial weight loss in experimental animals (Thupari et al., 2004).

A newer generation of cerulenin derivatives, C93, was rationally designed and showed a FASN inhibitory effect without parallel stimulation of fatty acid oxidation in a preclinical model of lung cancer (Orita et al., 2007; Orita et al., 2008; Zhou et al., 2007). Cerulenin, C75 and C93 all target the

keto-acyl synthase domain of FASN (Christie et al., 1981; Kuhajda et al., 2000; Rendina and Cheng, 2005)

Orlistat is a newly discovered FASN inhibitor (Kridel et al., 2004). Originally a US Food and Drug Administration (FDA) approved anti-obesity drug. Orlistat works primarily on pancreatic and gastric lipase within the gastrointestinal (GI) tract (McNeely and Benfield, 1998). In 2004, Orlistat was first found to inhibit FASN in an activity-based screening for inhibitors of serine hydrolases in prostate cancer cells (Kridel et al., 2004). Studies from several groups demonstrated that Orlistat exhibits antitumor effects toward melanoma, breast and prostate cancer cells *in vitro* and *in vivo* by inhibiting FASN activity (Browne et al., 2006; Carvalho et al., 2008; Knowles et al., 2004; Knowles et al., 2008; Kridel et al., 2004; Little et al., 2007; Liu et al., 2008; Lupu and Menendez, 2006; Menendez et al., 2005d). Orlistat treatment induces endoplasmic reticulum stress in tumor cells (Little et al., 2007), inhibits endothelial cell proliferation and angiogenesis (Browne et al., 2006). Recent co-crystalization of the FASN thioesterase domain and Orlistat, together with a molecular docking study, proved that Orlistat binds and inhibits FASN thioesterase activity (Cheng et al., 2008; Pemble et al., 2007). These studies provide useful information for structure-based drug design targeting FASN.

F. Specific aims of the present work

One of the major problems in successful treatment of breast cancer is development of multidrug resistance that reduces the effectiveness of chemotherapy. Breast cancer cells can be either intrinsically drug resistant, or sometimes acquire drug resistance following chemotherapy. Research results from drug resistant model cell lines indicated that expression of plasma membrane glycoproteins, such as P-glycoprotein (Pgp or MDR1), multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP), is a frequent cause of MDR (Allen et al., 1999; Ambudkar et al., 1999; Bellamy, 1996; Cole et al., 1992; Doyle et al., 1998; Endicott and Ling, 1989; Gottesman et al., 2002; Miyake et al., 1999). Cancer cells over-expressing Pgp, MRP1, or BCRP have an ability to extrude a wide variety of unrelated cytotoxic drugs and, therefore, can survive chemotherapy.

To study the mechanisms of drug resistance, many resistant cell lines were established in vitro by selection with various anticancer agents. A series of drug resistant breast cancer cell lines (see Table 1) were generated by stepwise selection of the drug sensitive breast cancer cell line MCF7 with increasing concentrations of Adriamycin in the presence of verapmil (Chen et al., 1990; Litman et al., 2000), a MDR/MRP1 inhibitor. MCF7/AdrVp3000 cells showed the highest level of resistance, and the overexpression of BCRP in this cell line was thought to be the mechanism of drug resistance (Doyle et al., 1998; Miyake et al., 1999). Indeed, the enforced ectopic expression of BCRP

in MCF7 cells showed the same pattern of drug resistance as MCF7/AdrVp3000 cells. However, the drug resistance level was much lower in BCRP over-expressing MCF7 cells than that of MCF7/AdrVp3000 cells, even though steady-state BCRP levels in both cell lines are comparable. This result suggests that mechanisms other than BCRP overexpression also contribute to the higher drug resistance level in MCF7/AdrVp3000 cells. In fact, genomic profiling of ABC transporters (Liu et al., 2005) and a proteomic study (Liu et al., 2006b) performed by our lab discovered numerous proteins other than BCRP, which may contribute to the drug resistance phenotype in MCF7/AdrVp3000 cells.

Table 1. Summary of MCF7 and its derivative drug resistant cell lines

Cell lines	Selected with	Resistance to	
		Adriamycin	Mitoxantrone
MCF7 (parental)	N.A.	N.A.	N.A.
MCF7/AdrVp10	10ng/ml Adr/10ng/ml VP	Yes	N.D.
MCF7/AdrVp100	100ng/ml Adr/10ng/ml VP	Yes	Yes
MCF7/AdrVp3000	3000ng/ml Adr/10ng/ml VP	Yes	Yes
MCF7/BCRP (tansfected)	N.A.	Yes	Yes

N.A.=not applicable; N.D.=not determined; Adr=Adriamycin; VP=verapamil

Our preliminary data showed that FASN expression is elevated in drug resistant breast cancer cell lines and this overexpression correlates with the increased drug resistance of these cells. These observations suggest that FASN overexpression in breast cancer cells may cause resistance to chemotherapy. Indeed, a previous clinical analysis suggested that high levels of FASN expression associates with poor prognosis of breast cancer patients

(Alo et al., 1999). My study was designed to investigate the mechanism of FASN-mediated drug resistance in breast cancers. Two specific aims are addressed in the present work.

The first aim is to determine if FASN overexpression contributes to the drug resistance phenotype in breast cancer cells. Using the sulfarhodamine-B cytotoxicity assay, we showed that down-regulating FASN in drug resistant MCF7/AdrVp3000 cells decreased their resistance to Adriamycin and mitoxantrone, while ectopic overexpression of FASN in parental drug sensitive MCF7 cells increased their resistance to a panel of DNA damaging drugs, such as Adriamycin, mitoxantrone, etopside, camptothecin, and cisplatin. The FASN overexpression-induced increase in drug resistance is not limited to MCF7 cells alone, but is observed in the MDA-MB-468 breast cancer cell line as well. Down-regulation of FASN did not affect the drug resistance phenotype in the normal breast epithelia cell line MCF10A1.

The second aim is to investigate the detailed mechanism of FASN-induced apoptosis in breast cancer cells. Our findings suggest that FASN overexpression protects cancer cells from drug-induced apoptosis by inhibiting caspase-8 activation.

The outcome from this study should lead to a better understanding of how FASN contributes to the malignancy of cancer cells, and thus help to develop better antineoplastic drugs and treatment regimens that maybe more effective for drug resistant breast cancers.

II. Materials and Methods

A. Materials

All electrophoresis reagents, precast slab gel and polyvinylidene difluoride (PVDF) membrane were purchased from Bio-Rad (Hercules, CA). Adriamycin, mitoxantrone, vinblastine, paclitaxel, cisplatin, camptothecin, etopside, verapmil, dithiothreitol (DTT), Insulin, epithelial growth factor (EGF), Cholera enterotoxin and hydrocortisone were purchased from Sigma (St. Louis, MO). Orlistat (Xenical) was obtained from Roche Laboratories Inc (Nutley, NJ). SYBR Green PCR master Mix for real-time PCR was purchased from Applied Biosystems (Foster City, CA). Monoclonal antibody against fatty acid synthase (FASN) was purchased from BD Biosciences (San Jose, CA). Antibodies to phosphor-AKT, total AKT, cleaved PARP, Caspase-8 and Caspase-9 were purchased from Cell Signaling (Danvers, MA). Monoclonal antibody to FLIP (NF6) was purchased from Alexis Biochemicals (San Diego, CA). siRNA with scrambled sequence (scrambled siRNA) was purchased from Ambion (Austin, Texas). Lipofectamine 2000, Lipofectamine Plus and G418 were purchased from Invitrogen (Carlsbad, CA). FuGene 6 was purchased from Roche Applied Sciences (Indianapolis, IN). ECL Western Blotting detection reagents and SuperSignal West Dura extended duration substrate were purchased from GE Healthcare (Pittsburg, PA) and Thermo Scientific (Rockford, IL), respectively. Cell culture mediums IMEM, Opti-MEM, DMEM, DME/F12 50/50, fetal bovine serum, equine serum, and typsin-versene

mixture were purchased from BioSources International (Camarillo, CA), Media Tech (Herndon, CA), or Cambrex (Walkersvill, MD). All other chemicals were of molecular biology grade from Sigma (St. Louis, MO) or Fisher Scientific (Chicago, IL).

B. Cell cultures

Human breast cancer cell line MCF7 and its drug-resistant derivative cell lines MCF7/AdrVp10, MCF7/AdrVp100, and MCF7/AdrVp3000 (gifts from Dr. Susan E. Bates, National Cancer Institute, Bethesda, MD) were cultured at 37°C with 5% CO_2 in DMEM or IMEM medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin as previously described (Chen et al., 1990; Lee et al., 1997; Litman et al., 2000; Liu et al., 2006b). To maintain the drug resistance phenotype of MCF7/AdrVp10, MCF7/AdrVp100, and MCF7/AdrVp3000 cells, 10, 100, and 3,000 ng/ml Adriamycin were included, respectively, together with 5 μg/ml verapamil. MDA-MB-468 cell line was cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. MCF10A1 cell line (Karmanos Cancer Institute) was cultured in DME/F12 50/50 with 10% equine serum, 10 μg/ml insulin, 25 ng/ml EGF, 500 ng/ml hydrocortisone and 100 ng/ml cholera enterotoxin.

C. Stable cell transfection

To establish MCF7/AdrVp3000 stable cell clones with reduced FASN expression, a plasmid which expresses short hairpin RNAs (shRNA)

specifically targeting FASN with a sequence of AACCCTGAGATCCCAGCGCTG or a scrambled control sequence was transfected into MCF7/AdrVp3000 cells using FuGene 6 Transfection Reagents according to manufacture's suggestions. Twenty-four hours following transfection, the cells were cultured in the presence of 800 μg/ml G418 for 2 weeks. Individual stable clones selected were expanded and maintained in the presence of 200 μg/ml G418 for further analyses.

To establish MCF7 stable clones with ectopic overexpression of FASN, the cDNA encoding human FASN (a gift from Dr. Massimo Loda, Dana Farber Cancer Institute) was engineered into the pcDNA3 vector and transfected into MCF7 cells using FuGene 6 according to manufacturer's suggestions. Two days following transfection, 10% of the transfected cells were replated and selected with 800 μg/ml G418 for 2 weeks, and stable clones were propagated and maintained in the presence of 200 μg/ml G418 for further analyses

D. siRNA preparation and transfection

siRNAs targeting FASN (AACCCTGAGATCCCAGCGCTG) and the negative control siRNA with scrambled sequence (scrambled siRNA) were synthesized by Ambion (Austin, Texas) as previously described (De Schrijver et al., 2003). For siRNA transfection, 5×10^5 MDA-MB-468 cells or MCF10A1 cells were plated in a 6-well plate for 24 hours, followed by transfection with siRNAs using Lipofectamine 2000 reagent according to supplier's instructions.

Briefly, 5 μl of Lipofectamine 2000 was diluted with 100 μl of Opti-MEM medium and incubated at room temperature for 5 min. Two hundred picomoles of siRNAs were added to 100 μl of Opti-MEM medium and then mixed with the diluted Lipofectamine 2000 reagent followed by incubation at room temperature for 25 min. The siRNA-Lipofectamine 2000 reagent complex was added drop-wise into the culture containing 0.8 ml of fresh media. At different days following transfection, cell lysates were prepared for detection of FASN by Western blot analysis.

E. Cell lysate preparation

Cells were harvested and washed with phosphate-buffered saline and then lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 20mM EDTA, pH 8.0, 1 mM sodium orthovanadate, 50mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, and 1mM dithiothreitol) for 30 min on ice with constant agitation. The cell lysates were sonicated briefly followed by centrifugation (16,000g, 4°C) for 15 min to remove insoluble materials. The protein concentration of cell lysates was determined using BioRad protein assay kit.

F. Western blot analysis

Western blot analysis was performed as described previously (Pincheira et al., 2001; Yang et al., 2002a; Zhang et al., 1993). Briefly, cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a PVDF membrane. The blot was then

probed with an antibody to specific proteins (diluted according to manufacturer's suggestion), followed by reaction with horseradish peroxidase-conjugated secondary antibodies. The signal was captured by X-ray film or FluorChem HD2 immaging system (Alpha Innotech) using Amsham ECL Western Blot detection agents or Pierce Supersignal West Dura Extended Duration substrate.

G. FASN activity assay

FASN activity was determined using a protocol as previously described (Menendez et al., 2004c). Briefly, 96 μg of particle-free supernatant of cell lysate was mixed with a buffer containing 200 mM potassium phosphate, pH 6.6, 1 mM DTT, 1 mM EDTA, 0.24 mM NADPH and 30 μM acetyl-CoA in a final volume of 0.2 ml and the reaction was monitored at 340 nm for 3 min to measure background NADPH oxidation. After the addition of 50 μM of malonyl-CoA, the reaction was assayed for an additional 15 min to determine FASN-dependent oxidation of NADPH. The rates of OD_{340nm} change were corrected for the background rate of NADPH oxidation. FASN activity was expressed as nmoles NADPH oxidized/min/mg protein.

H. Cytotoxicity assay

Cytotoxicity of various anti-cancer drugs to cancer cells was determined using the sulforhodamine B (SRB) colorimetric assay as previously described (Liu et al., 2006b) with some modification. Briefly, cells were seeded in 96-well plates in triplicate and cultured at 37°C for 24 hours

before drugs were added. Cells were then cultured continuously at 37°C until the control cells became confluent (usually 72-96 hours) before SRB assay. For the study of the palmitic acid effect, Adriamycin or mitoxantrone was added to the cells 24 hours after incubation with 100 μM of palmitic acid (Chajes et al., 2006). For the study of the Orlistat effect, Adriamycin was added to the cells in the presence or absence of 30 μM Orlistat for 3 days before SRB assay (Kridel et al., 2004).

For the SRB assay (Skehan et al., 1990), the culture medium was removed and the cells were fixed and stained by addition of 0.4% (w/v) sulforhodamine B (Sigma) in 1% acetic acid solution followed by incubation at room temperature for 20 minutes. The plates were then washed three to five times with 1% acetic acid to remove the unbound SRB and then air-dried at room temperature. The bound SRB was then solubilized with 10 mM unbuffered Tris base. The $OD_{570\ nm}$ was determined using a 96-well plate reader (MRX; Dynex Technologies, Chantilly, VA). IC50 was defined as the concentration of drugs required to kill 50% of the cells relative to control condition without drugs.

I. Cell growth analysis

Cell growth rate was determined as previously described (Dong et al., 2004). Briefly, MCF7 and its derivative stable clones transfected with FASN cDNA were plated at 150 cells/well whereas MCF7/AdrVp3000 and its derivative stable clones transfected with shRNA were plated at 250 cells/well

in triplicate in 96 wells, respectively. Plates were collected every other day followed by removal of media and the fixation of cells with 100 μl/well of 1% glutaraldehyde for 30 minutes at room temperature. The plates were then washed 3 times with 200 μl PBS and air dried. Crystal violet solution (100 μl/well, 0.2% crystal violet in 20% methanol) was then added to the air-dried plates and incubated for 30 minutes at room temperature. The plates were then washed extensively with water to remove excess dye and air dried again. To dissolve the dye, 200 μl of 10% acetic acid was added to each well and the plates were incubated for 1 hour with shaking at room temperature. Finally, OD_{570nm} was determined.

J. Drug uptake assay

The drug uptake assay was performed as described previously with some modification (Lee et al., 1994). Briefly, 5×10^5 cells were resuspended in 0.5 ml PBS containing 20 μl mitoxantrone (20 μM) and incubated at 37°C for 30 minutes. Cell were then collected by centrifugation and washed twice with PBS followed by analysis using flow cytometry on a Becton Dickinson FACScalibur. The data were analyzed using Cell Quest Pro (BD Bioscience).

K. Co-immunoprecipitation

To preclear the samples, 400 μg cell lysate was diluted in 0.5 ml TNN buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.1% SDS), mixed with 1 μg normal mouse IgG,

and 40 μl Protein G-Sepharose beads, and then incubated at 4°C for 2 hours. After incubation, the mixture was centrifuged at 5000 rpm for 30 seconds, the supernatant was transferred to a new tube and incubated with primary antibodies for 3 hours at 4°C. 50 μl of Protein G-Sepharose beads was then added to the tube and incubated overnight at 4°C. The immunoprecipitates were then collected by centrifugation, washed 5 times with 1 ml TNN buffer, and finally solublized in 40 μl SDS-PAGE sample buffer for Western blot analysis.

L. Real-time quantitative reverse transcription-PCR

Total RNAs were isolated from cultured cells using a RNeasy mini kit according to the manufacturer's instruction (Qiagen, Valencia, CA) and treated with RQ1 RNase-free DNase I. Four micrograms of total RNA each were reverse transcribed using avian myeloblastosis virus Reverse Transcriptase and Oligo$(dT)_{12\text{-}18}$ primer (Invitrogen). Primers for real-time PCR were designed using Primer Express software version 2.0 (Applied Biosystem) and were synthesized by Invitrogen. The primer sequence for FASN are 5'-GCTGACCCCAGGCTGTGA-3' (forward) and 5'-TGCTCCATGTCCGTGAACTG-3' (reverse). The primer sequences for internal control glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) are 5'-AAGGACTCATGACCACAGTCCAT-3' (forward) and 5'-CCATCACGCCACAGTTTCC-3' (reverse). Real-time quantitative PCR was performed on an ABI prism@7000 Sequence Detection System (Applied

Biosystems) using SYBR Green diction according to the manufacturer's instruction. The threshold cycle (C_t) was defined as the PCR cycle number at which the reporter fluorescence crosses the threshold reflecting a statistically significant point above the calculated baseline. The C_t of FASN was determined and normalized against that of the internal control housekeeping gene GAPDH. The relative level calculated against that in MCF7 cells = $2^{\Delta\Delta Ct}$

M. DNA fragmentation assay.

FASN over-expressing MCF7 cells and vector-transfected control cells were grown in twenty four-well plates and were incubated with 1 μM Adriamycin for 48 hours. Cells were harvested to assess DNA fragmentation using the Cell Death Detection ELISA (Roche, Mannheim, Germany) according to the manufacturer's recommendations. The cytosolic fraction (20,000g for 10 minutes) from approximately 1×10^4 cells was incubated in a microtiter plate precoated with a primary antibody against histones, after which an HRP-conjugated secondary antibody against DNA was added. The relative level of DNA fragmentation was compared with that of the control that did not receive drug treatment.

N. Extraction of Ceramides

Cell pellets were re-suspended in 0.5 ml of PBS, and then added 3 ml of Methanol/chloroform (2:1) following the addition of 10 μl of 17:0 Ceramide (2.5 ng/l) as an internal standard. The samples were vortexed for 1 minute and incubated on ice for 10 minutes. Chloroform (1 ml) and water (1.3 ml)

was added to separate the phases and samples were vortexed for 1 minute prior to centrifugation at 1,750g for 10 minutes at 10˚C. The lower phase was transferred to a new glass tube. After evaporating the solvent under nitrogen at room temperature, the dried lipids were re-suspended respectively in 1 ml of MeOH for Mass spectrometry (MS) analyses.

O. LC-ESI-MS/MS

MS analyses were performed using API-4000 (Applied Biosystems/MDS SCIEX, Forster City, CA) with the Analyst data acquisition system. The instrument was equipped with a Z-spray ionization source. The nebulizer gas and desolvation gas were nitrogen and the collision gas was argon. Typical operating parameters were as follows: nebulizing gas (NEB) 15, curtain gas (CUR) 8, collision-activated dissociation (CAD) gas 35, electrospray voltage 4200, and temperature 500. Multiple reactions monitoring (MRM) mode was used for quantification. Negative monitoring ions were at m/z 550.4 (the parent ion)-294.2 (the product ion) for 17:0-CER, 536.6-280.2 for 16:0-CER, 562.6-306.2 for 18:1-CER, 564.6-308.1 for 18:0-CER, 592.6-336.2 for 20:0-CER, 646.7-390.2 for 24:1-CER and 648.6-392.4 for 24:0-Cer. The dwell time in the MRM mode was 75 ms.

Samples (10 µl each) were delivered into the electrospray ionization (ESI) source through a LC system (Agilent 1100) with an auto sampler. The mobile phase was MeOH/water/AmOH (90:10:0.1, v/v/v).

Standard curve for each ceramide were established for quantitative

analysis. Different concentrations (5-50 pg/l) of a particular form of ceramide were mixed with the same concentration (25 pg/l) of an internal standard 17:0-Cer, and then performing ESI-MS analyses. The peak intensity ratios (a ceramide form/ internal standard) versus the concentration ratios (a ceramide form/ internal standard) were plotted and fitted to a linear regression.

III. Experimental Results

A. Identification of FASN as an over-expressed protein in drug resistant MCF7/AdrVP3000 cells.

SDS-PAGE analysis was used to compare protein profiles between the parental drug sensitive cell line MCF7 and its drug resistant derivative MCF7/AdrVp3000 cells. A protein of ~260 kDa was found to be over-expressed in the drug resistant MCF7/AdrVp3000 cells (Figure 3A). This protein was excised from the gel and subjected to analysis using matrix-assisted laser absorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. This protein was identified as human fatty acid synthase (FASN) by peptide matching/protein searching of the National Center of Biotechnology Information database using the ProFound search engine. The Z-score was 1.69 with peptide coverage of 5%.

To verify that FASN is over-expressed in the drug resistant MCF7/AdrVp3000 cells, we first determined FASN expression level using Western blot. As shown in Figure 4, the FASN level in MCF7/AdrVp3000 cells is ~2 fold higher than that in the MCF7 cells.

Since FASN is an enzyme, we next determined the FASN enzymatic activity in MCF7 and MCF7/AdrVp3000 cells to make sure that FASN is functional in MCF7 and drug-selected MCF7/AdrVp3000 cells. As shown in Figure 3B, the FASN activity in MCF7/AdrVp3000 cells was increased ~5 fold compared with that in MCF7 parental cells.

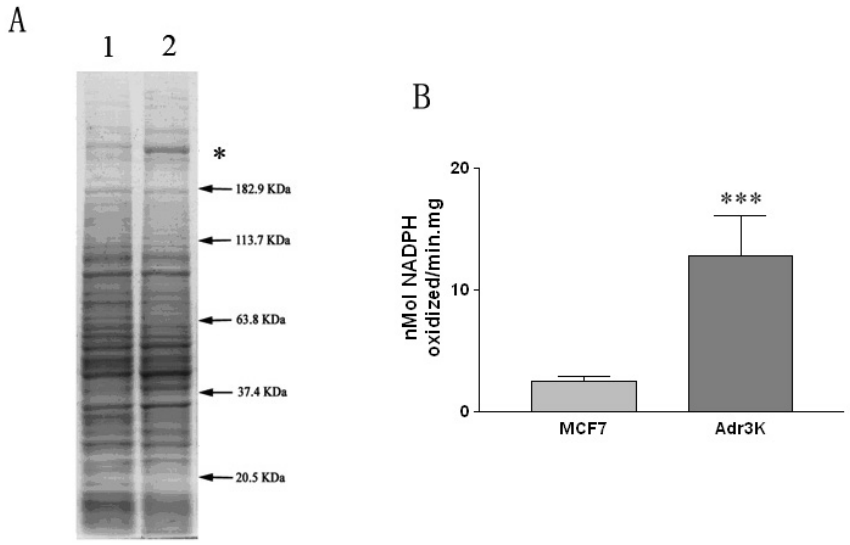

Figure 3. FASN is over-expressed in drug-resistant MCF7/AdrVp3000 cells. A. SDS-PAGE profile of cell lysates of MCF7 and its drug-resistant derivative MCF7/AdrVp3000 cells. 20 μg of protein from MCF7 and MCF7/AdrVp3000 cells were separated by a 4% to 15% gradient precast SDS-PAGE and stained with Coomassie blue. * indicates a high molecular weight protein (~260 kDa) over-expressed in MCF7/AdrVp3000 cells, which was identified as FASN by mass spectrometry (access no. G01880). B. FASN activity in MCF7 and MCF7/AdrVp3000 cells. FASN activity was determined as described in Materials and Methods (n=5, p=0.0001).

B. FASN expression level correlates with drug resistance in breast cancer cells.

To determine whether the elevated FASN expression contributes to the drug resistance of MCF7/AdrVp3000 cells, we performed a correlative analysis of FASN expression with drug resistance. During the stepwise selection to generate MCF7/AdrVp3000 cells, two MCF7 derivatives, MCF7/AdrVp10 and MCF7/AdrVp100, with low and intermediate drug resistance levels were also generated (Chen et al., 1990; Lee et al., 1997; Litman et al., 2000). As shown in Figure 4A, the FASN protein level increased as the drug resistance level of the MCF7 derivative cells increased. The relative FASN expression level in MCF7 and its drug-selected derivatives were 1.0, 1.0, 1.4, 1.9, respectively.

The mRNA level of FASN in MCF7 and its drug-selected derivatives was also assessed using real-time quantitative reverse transcription- PCR. As shown in Figure 4B, mRNA level of FASN dramatically increased in MCF7/AdrVp10 cells and was sustained in both MCF7/AdrVp100 and MCF7/AdrVp3000 cells. The discordance between FASN mRNA level and protein expression suggests that the FASN expression may be regulated at the post-transcriptional level (Priolo et al., 2006).

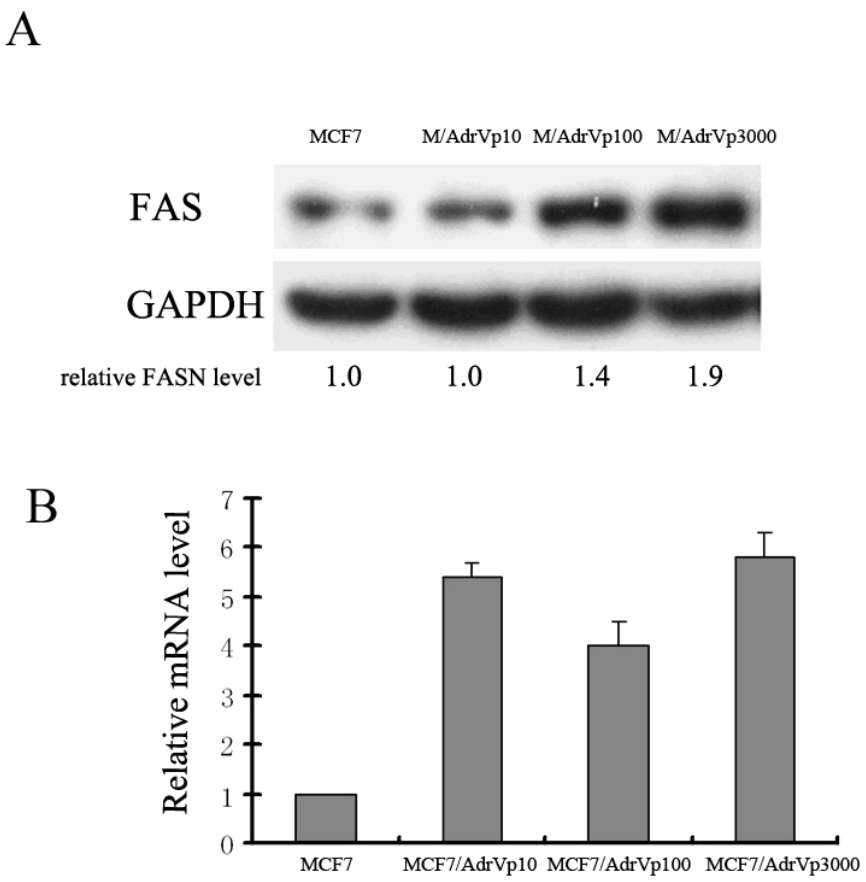

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Figure 4. FASN expression level correlates with the drug resistance level in MCF7 and its drug-selected derivative cell lines. A. FASN expression in MCF7 and its stepwise-selected drug resistance derivative cell lines. 20 μg of protein, each from MCF7, MCF7/AdrVp10, MCF7/AdrVp3000, and MCF7/AdrVp3000 cells, were separated by SDS-PAGE followed by Western blot analysis using FASN antibody. GAPDH was used as a loading control. The relative level of FASN was determined by measuring the intensity of the FASN band compared with that of GAPDH band using software Scion Image. FASN expression levels in each cell line were normalized to that of MCF7. B. Real-time quantitative reverse transcription-PCR analysis of FASN mRNA level. Real-time quantitative reverse transcription-PCR was done by measuring the mRNA level using SYBR green and calculated in the fold change ($2^{\Delta\Delta Ct}$) relative to MCF7 cells after normalization by internal control, GAPDH.

C. FASN overexpression leads to drug resistance in breast cancer cells.

To determine if FASN overexpression is the cause of drug resistance or simply a result of drug selection, FASN expression in MCF7/AdrVp3000 cells was knocked down using shRNA. A hairpin DNA segment targeting FASN was cloned into pRNA-U6.1/Neo vector (GeneScript, Piscataway, NJ) to constitutively express FASN siRNA. The FASN shRNA construct was then transfected into MCF7/AdrVp3000 cells and stable clones were selected using G418. As shown in Figure 5, the FASN protein level was decreased in two independent stable clones (Si18 and Si19) compared with the control clone (Scr) transfected with scrambled shRNA. The responses of these clones to various anticancer drugs were determined using the SRB cytotoxicity assay. The results showed that knock down of FASN expression significantly decreased the resistance level of MCF7/AdrVp3000 cells to both Adriamycin and mitoxantrone (Figure 6). Two other drugs, vinblastine and paclitaxel, were also tested. However, knocking down FASN expression in MCF7/AdrVp3000 cells did not affect the drug resistance level to these two drugs (data not shown).

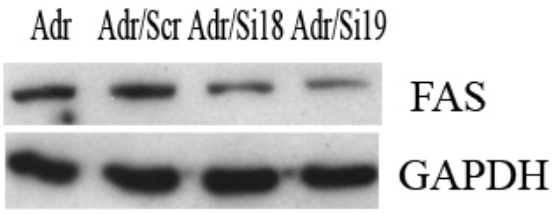

Figure 5. Down-regulation of FASN expression in MCF7/AdrVp3000 cells using stable shRNA transfection. Drug resistant MCF7/AdrVp3000 cells were stably transfected with shRNA targeting FASN for silencing and scrambled shRNA as a control. 20 μg of protein each, from MCF7/AdrVp3000, MCF7/AdrVp3000 cells transfected with scramble shRNA control, and 2 stable MCF7/AdrVp3000 clones with reduced FASN expression were separated with SDS-PAGE followed by Western blot analysis using FASN monoclonal antibody. GAPDH was used as a loading control.

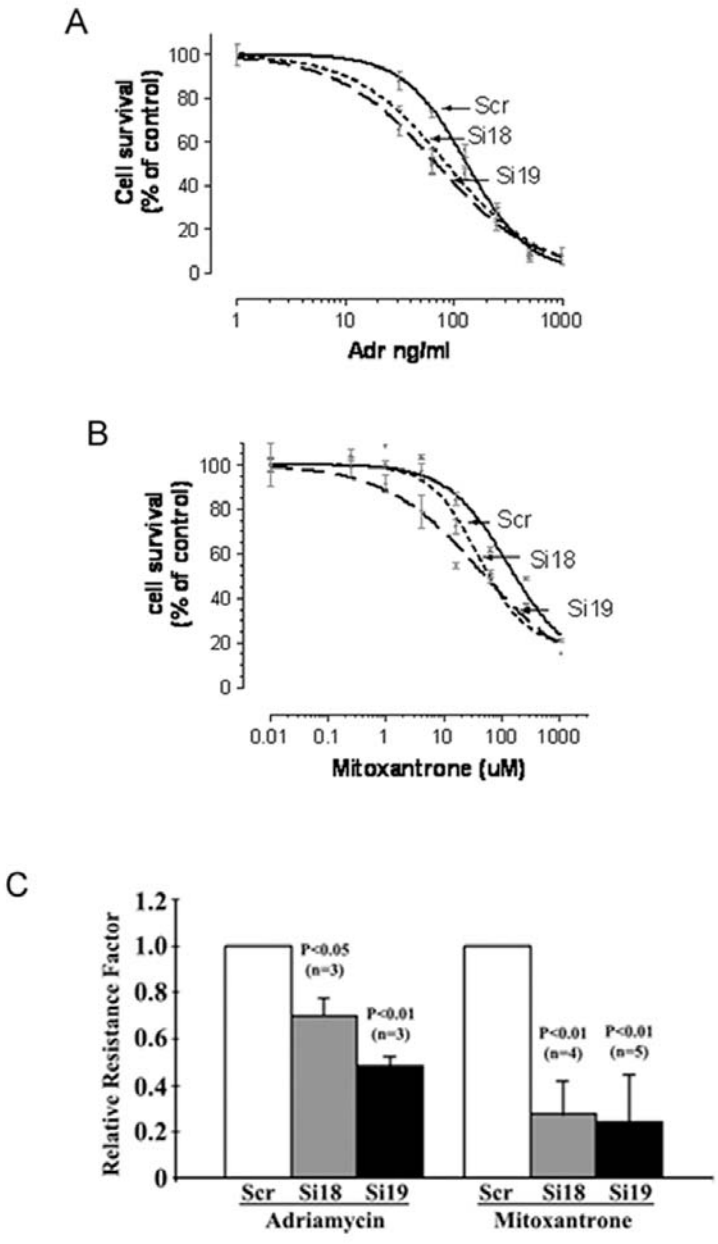

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Figure 6. Down-regulation of FASN expression in MCF7/AdrVp3000 cells by stable shRNA transfection decreased their drug resistance. MCF7/AdrVp3000 cells were stably transfected with either sh-RNA specifically targeting FASN or control scrambled shRNA. Stable clones were tested for their resistance to various anticancer drugs by SRB cytotoxicity assay. Results were analyzed by GraphPad Prism (version 3.02). A. A representative curve of those used to obtain IC50 value for Adriamycin, B. representative curve of those used to obtain IC50 value for mitoxantron, C. Summary of multiple experiments. Relative resistance factor = IC50 of FASN-shRNA transfected cells/IC50 of scrambled sh-RNA transfected cells.

To further investigate the role of FASN in drug resistance, parental drug sensitive MCF7 cells were transfected with a plasmid containing FASN cDNA for ectopic overexpression. Stable clones were selected using G418 for analysis of FASN expression and drug resistance. As shown in Figure 7, two stable clones (F and F2) showed higher levels of FASN compared with the control vector-transfected MCF7 cells. As shown in Figure 8, the stable clones with FASN overexpression showed significantly higher resistance to several anti-cancer drugs including Adriamycin, mitoxantrone, cisplatin, camptothecin and etoposide than the vector-transfected MCF7 control cells. However, FASN overexpression did not change the drug resistance levels of FASN over-expressing MCF7 cells to vinblastine and paclitaxel (data not shown).

Taken together, our data suggested that the increased FASN expression in MCF7/AdrVp3000 cells likely contributes to the drug resistance phenotype of this cell line.

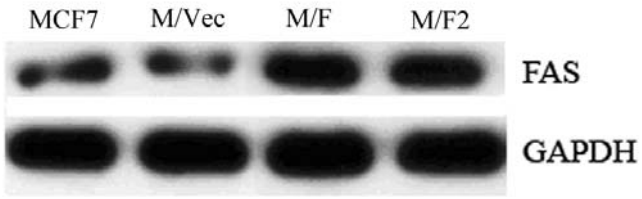
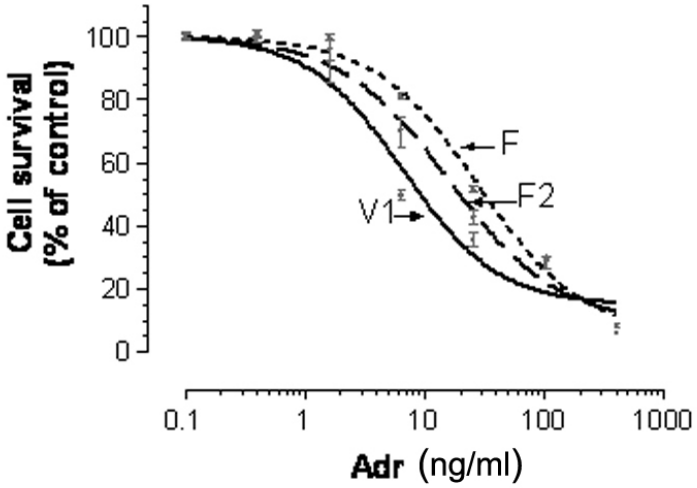


Figure 7. Ectopic overexpression of FASN in MCF7 cells. Drug sensitive MCF7 cells were stably transfected with FASN cDNA for overexpression or transfected with empty vector as control. 20 μg of protein each, from MCF7, MCF7 cells transfected with vector control (M/Vec), and 2 stable MCF7 clones over-expressing FASN (M/F and M/F2) were separated by SDS-PAGE followed by Western blot analysis with FASN antibody. GAPDH was used as a loading control.

A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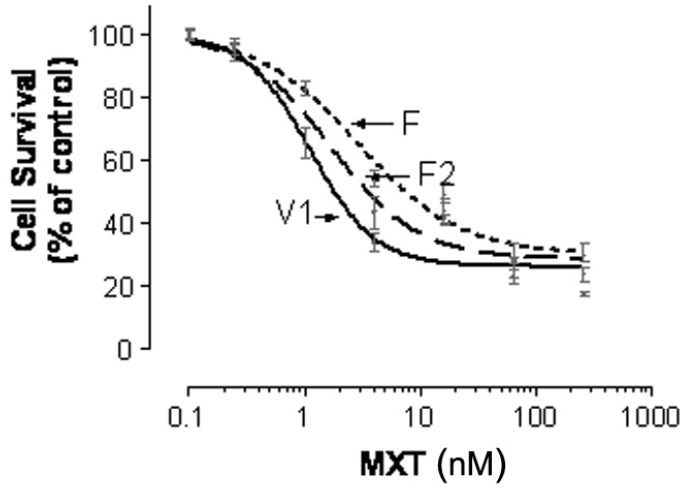
Cell Survival
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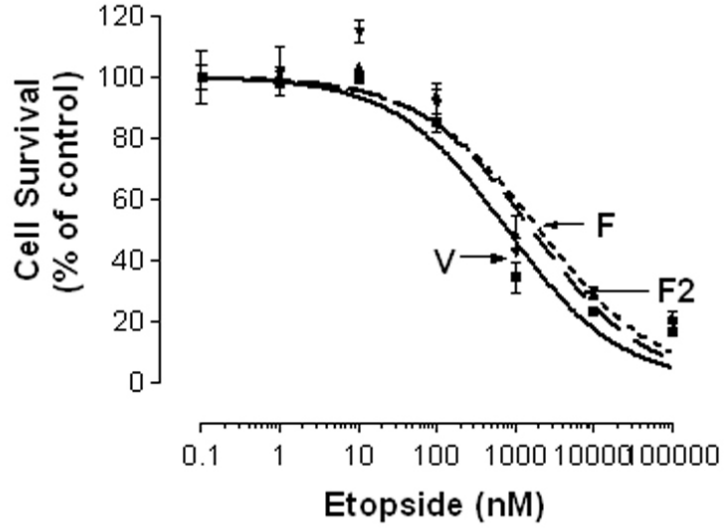
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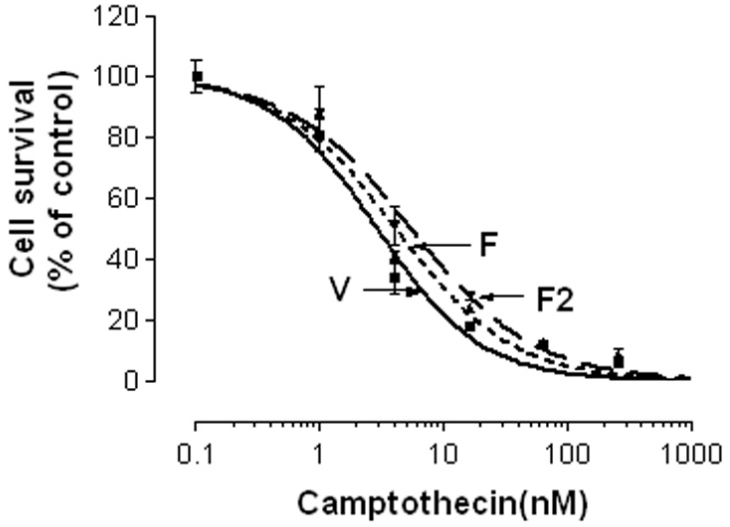
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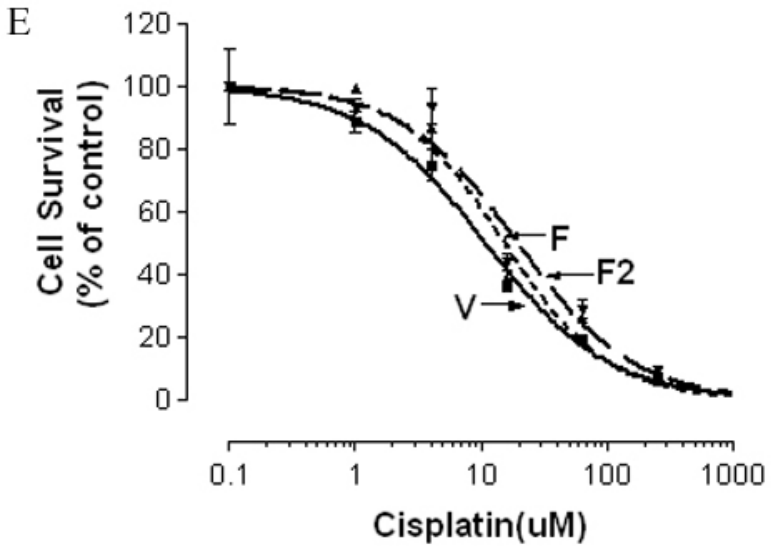

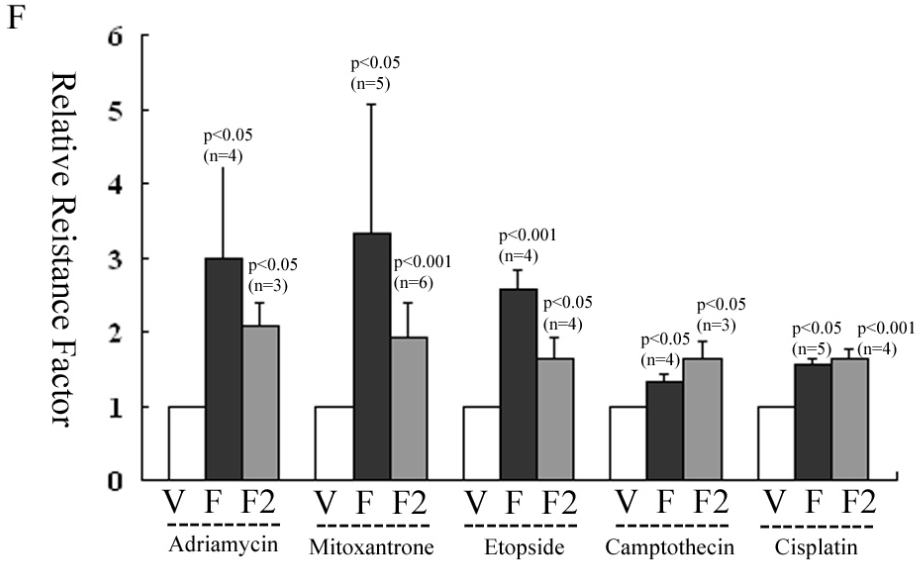

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Figure 8. Increased FASN expression in MCF7 cells increased their resistance to various anticancer drugs. MCF7 cells were stably transfected with either plasmid harboring FASN cDNA (F and F2) or empty vector (V). Stable clones were tested for their resistance to various anticancer drugs by SRB cytotoxicity assay. Results were analyzed by GraphPad Prism (version 3.02). A-E, representative curves of those used to obtain IC50 values for Adriamycin (A), mitoxantrone (B), Etoposide (C), Camptothecin (D), and Cisplatin (E). F, Summary of multiple experiments. Relative resistance factor = IC50 of FASN over-expressing cells/IC50 of vector-transfected control cells.

Orlistat is a U. S. Food and Drug Administration (FDA)-approved drug used for treating obesity. It works primarily on pancreatic and gastric lipase within the gastrointestinal (GI) tract (McNeely and Benfield, 1998). Orlistat was also found to be a rather selective FASN inhibitor targeting the thioesterase domain (Cheng et al., 2008; Kridel et al., 2004; Pemble et al., 2007). Inhibition of FASN activity by Orlistat induces endoplastic reticulum stress and tumor cell death (Little et al., 2007), inhibits tumor growth, and prevents angiogenesis (Browne et al., 2006).

As shown in Figure 6, reducing FASN expression can sensitize breast cancer cells to various anticancer drugs. We wanted to investigate whether Orlistat can also sensitize FASN over-expressing cancer cells. For this purpose, MCF7 stable clones with FASN overexpression were tested for their response to Adriamycin and mitoxantrone in the presence of 30 μM Orlistat. At this concentration, Orlistat has no toxicity to cells (data not shown). As shown in Figure 9, Orlistat was able to reverse FASN-mediated drug resistance in both MCF7 stable clones with FASN overexpression and the vector-transfected control cells, although the sensitization of vector-transfected cells by Orlistat is less than that of FASN over-expressing cells. MCF7 cells alone express low level of FASN, so it is not surprising to see the inhibitory effect of Orlistat in vector-transfected MCF7 cells. Thus, FASN inhibitors may be used as sensitizers in combination therapy of drug-resistant human breast cancers.

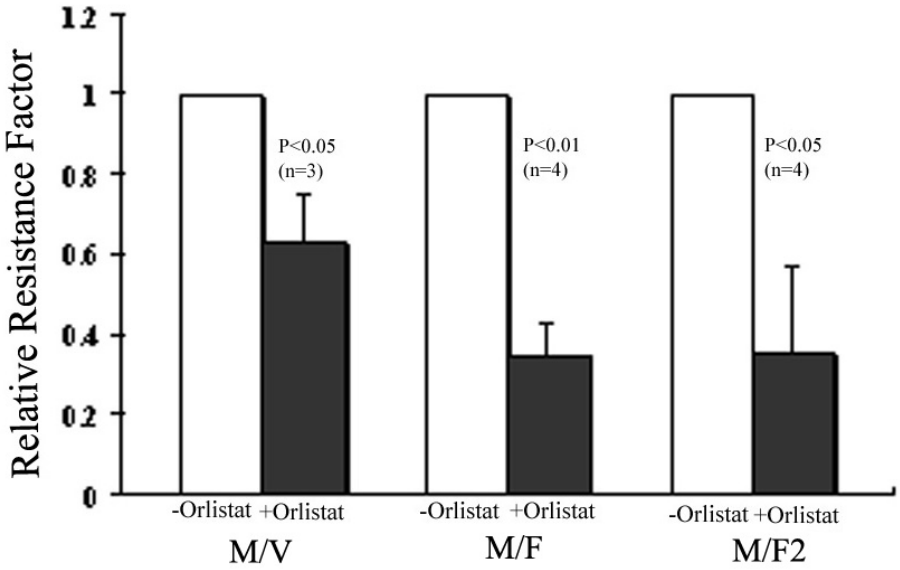


Figure 9. FASN inhibitor, Orlistat, can reverse FASN-mediated drug resistance. Two stable MCF7 clones with overexpression of FASN (M/F and M/F2) and a vector-transfected control (M/V) were treated with Adriamycin in the absence or presence of 30 μM Orlistat for 3 days followed by SRB cytotoxicity assay. Relative resistance factor=IC50 of Orlistat treated cells/IC50 of non-treated cells.

To determine if the FASN-mediated drug resistance is specific to MCF7 cells, siRNA specifically targeting FASN was transfected into another breast cancer cell line MDA-MB-468, which expresses high levels of endogenous FASN. As shown in Figure 10, decrease of FASN protein level in MDA-MB-468 cells by transient siRNA transfection persisted for at least 8 days. The effect of FASN-knock-down on drug sensitivity of MDA-MB-468 cells was next determined using the the SRB cytotoxicity assay. As shown in Figure 11, MDA-MB-468 cells transfected with FASN siRNA are significantly more sensitive to Adriamycin and mitoxantrone than the control cells transfected with scrambled siRNA. Thus, FASN overexpression-mediated drug resistance is not restricted to MCF7 cells.

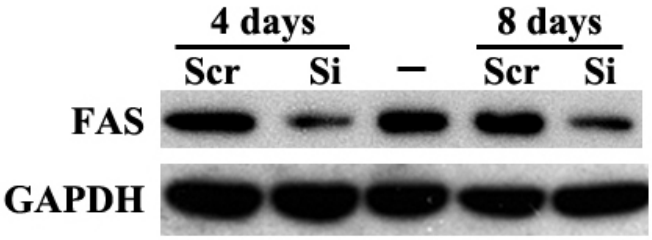


Figure 10. Western blot analysis of FASN expression in MDA-MB-468 cells. MDA-MB-468 cells were transiently transfected with either FASN siRNA (Si) or a negative control scrambled siRNA (Scr). Cells were collected at various days after transfection for Western blot analysis. 20 μg of protein from each set of transfected cells were separated with SDS-PAGE followed by Western blot analysis with FASN monoclonal antibody. GAPDH was used as a loading control.

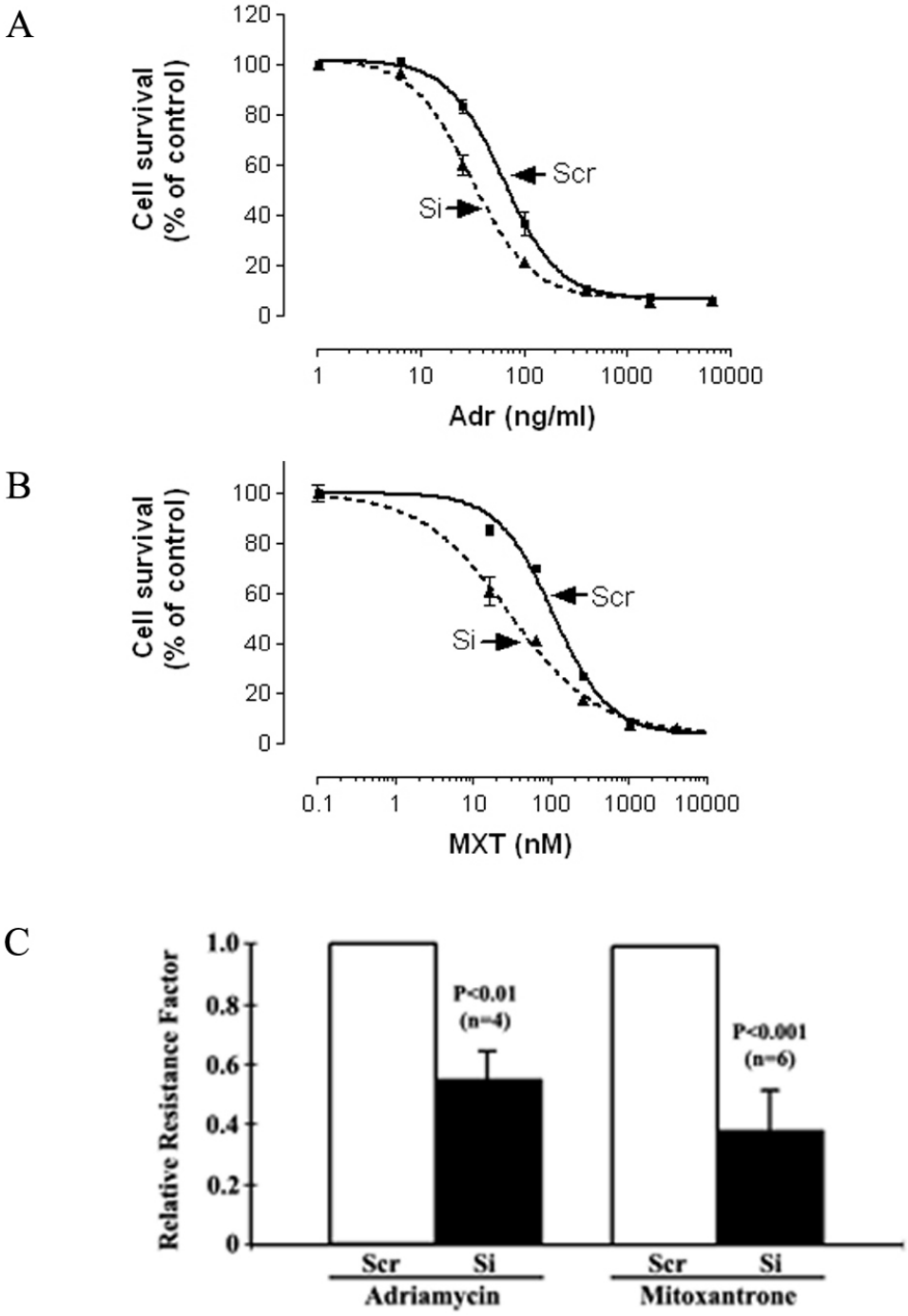


Figure 11. (Legend on next page)

Figure 11. Decreased FASN expression can sensitize MDA-MB-468 cells to anticancer drugs. MDA-MB-468 cells were transiently transfected with either FASN siRNA (Si) or control scrambled siRNA (Scr). Cells were collected at different time post transfection and tested for their resistance to Adriamycin or mitoxantrone by SRB cytotoxicity assay. Results were analyzed by GraphPad Prism (version 3.02). A. A representative curve of those used to obtain IC50 value for Adriamycin, B. A representative curve of those used to obtain IC50 value for mitoxantrone, C. Summary of multiple experiments. Relative resistance factor = IC50 of FASN-siRNA transfected cells/IC50 of scrambled si-RNA transfected cells.

To determine if decreased FASN expression in non-cancerous mammary epithelial cells would decrease their sensitivity to anticancer drugs, similar knock-down experiments were performed using the human normal breast epithelial cell line MCF10A1. As shown in Figure 12A, FASN siRNA was able to knock-down FASN expression for at least 6 days post transfection. However, the sensitivity to Adriamycin and mitoxantrone of siRNA-transfected MCF10A1 cells exhibited no significant difference compared with the control cells transfected with scrambled siRNAs (Figure 12B). This observation is interesting and indicates that FASN may be used as a target for chemo-sensitization of breast cancer cells without affecting the drug sensitivity of normal mammary epithelial cells.

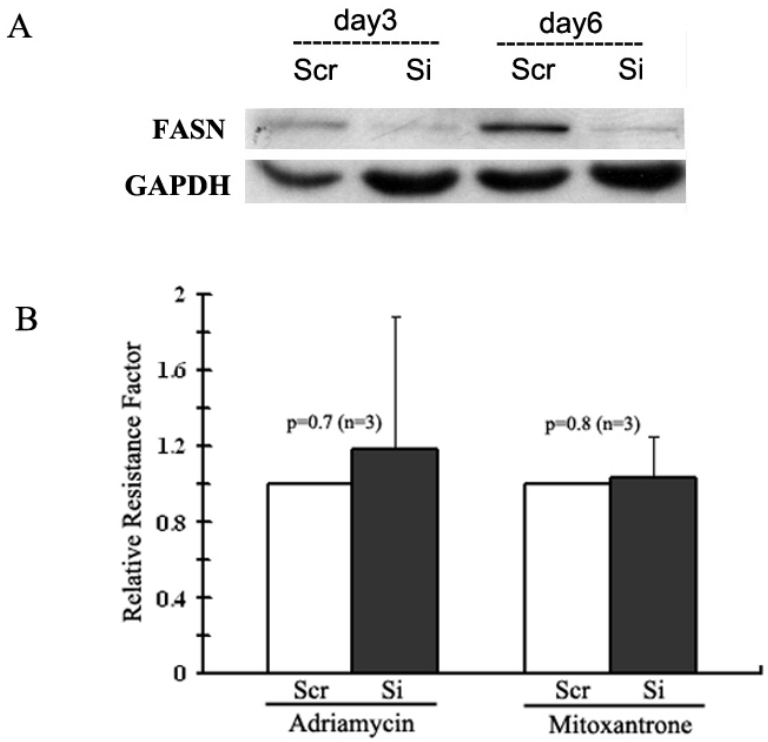


Figure 12. FASN silencing does not affect drug response of the normal breast epithelial cell line MCF10A1. A. MCF10A1 cells were transiently transfected with either FASN siRNA (Si) or a control scrambled siRNA (Scr). Cells were collected at various days after transfection for Western blot analysis of FASN expression. GAPDH was used as a loading control. B. MCF10A1 cells transfected with FASN siRNA or control scrambled siRNA were tested for their resistance to Adriamycin and mitoxantrone using SRB cytotoxicity assay. Relative resistance factor = IC50 of FASN-siRNA transfected cells/IC50 of scrambled si-RNA transfected cells.

D. FASN overexpression does not affect intracellular drug accumulation.

FASN overexpression in cancer cells affects membrane lipid composition, and thus possibly affects drug uptake through cell membrane or ABC transporter mediated drug efflux, both of which will decrease the intracellular drug accumulation.

To determine if FASN expression level affects drug uptake through cell membrane, mitoxantrone accumulation was determined and compared between MCF7 cells with FASN overexpression and vector-transfected control cells using FACS analysis. As shown in Figure 13A, there is no difference in mitoxantrone accumulation between these cells. Thus, FASN overexpression likely does not affect drug uptake of mitoxantrone through cell membrane.

To determine if FASN expression levels affect ABC transporter-mediated drug efflux, MCF7/AdrVp3000 cells, which are known to over-express several ABC transporters including ABCG2 (Liu et al., 2005), and its derivative stable clones with reduced FASN expression were tested for their ability to accumulate mitoxantrone. As shown in Figure 13B, knocking-down FASN expression in MCF7/AdrVp3000 cells did not affect mitoxantrone accumulation, indicating that FASN expression did not affect the efflux activity of ABC transporters in these cells. We also found that FASN overexpression did not affect sensitivity of cells to paclitaxel and vinblastine, substrates of multidrug-resistant ABC transporters. This finding further

confirmed our conclusion that FASN expression does not affect ABC transporter activity.

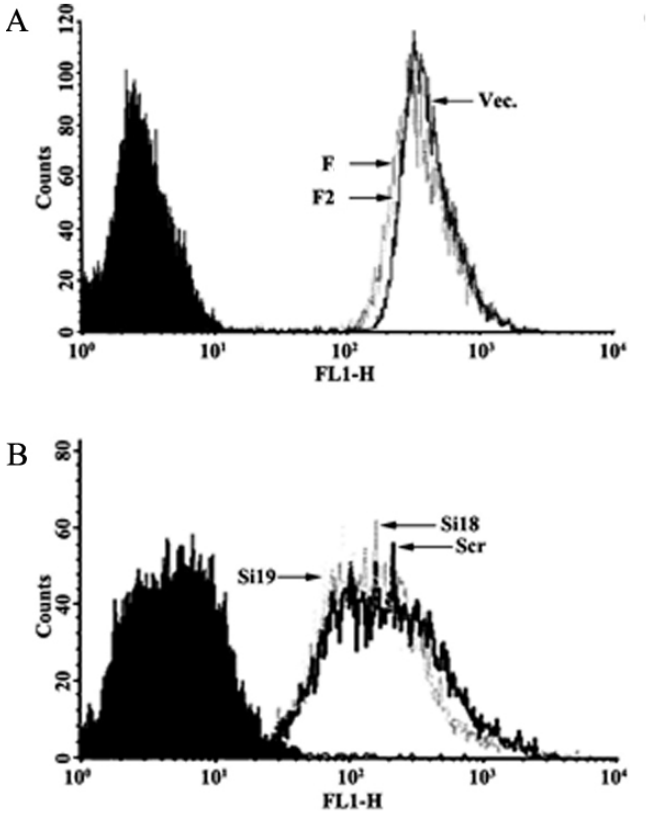


Figure 13. FASN expression levels do not affect intracellular drug accumulation. Vector transfected MCF7 (Vec) and two FASN over-expressing stable clones (F and F2) (A) and MCF7/AdrVp3000 cell line transfected with scrambled siRNA (Scr) and two derivative clones with down-regulated FASN expression (si18 and Si19) (B) were tested for their ability to accumulate anticancer drug mitoxantrone using FACS analysis.

E. FASN overexpression does not affect cell proliferation.

Another possible mechanism of FASN-mediated drug resistance is that FASN expression may affect cell proliferation. To test this possibility, the cell growth rate was compared among FASN over-expressing MCF7-derived stable cell clones, as well as in FASN knock-down MCF7/AdrVp3000 clones and the vector-transfected control cell line. As shown in Figure 14, overexpression or decreasing FASN expression does not affect the growth rate of these cells. Therefore, the drug responses mediated by FASN overexpression is unlikely due to its effect on cell proliferation.

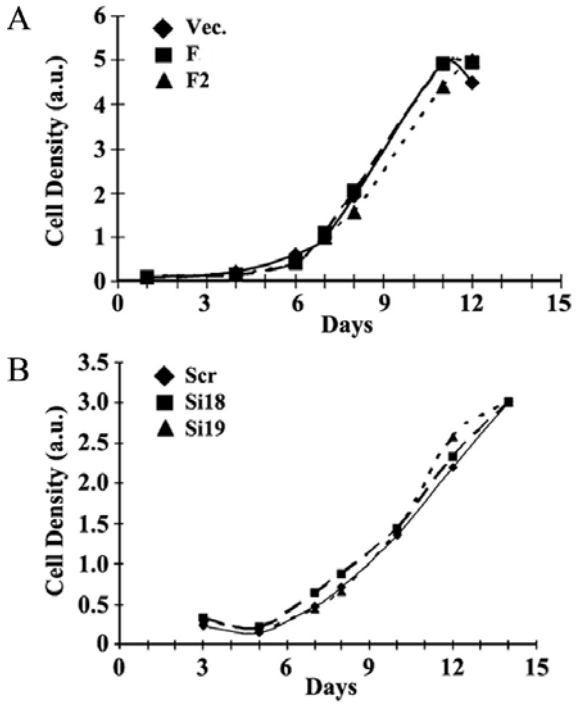


Figure 14. FASN expression levels have no effect on cell proliferation. Vector transfected MCF7 (Vec) and its two FASN over-expressing stable clones (F and F2) (A) and MCF7/AdrVp3000 cell line transfected with scrambled siRNA (Scr) and its two derivative stable clones with down-regulated FASN expression (si18 and si19) (B) were determined for their cell growth rate over a period of two weeks. Cell density was determined by crystal violet staining.

F. FASN overexpression protects cancer cells from drug-induced apoptosis.

Many anticancer drugs, such as Adriamycin, cause apoptosis in cancer cells. To determine if the overexpression of FASN protects cancer cells from drug-induced apoptosis, Poly ADP-Ribose Polymerase (PARP) cleavage was analyzed. PARP is a 116 kDa protein whose cleavage serves as a marker of cells undergoing apoptosis (Oliver et al., 1998). PARP cleavage by caspases during apoptosis generates two fragments, the PARP amino-terminal DNA binding domain (24 kDa) and the carboxy-terminal catalytic domain (89 kDa) (Lazebnik et al., 1994; Nicholson et al., 1995). As shown in Figure 15A, the 89-kDa PARP cleavage product was produced in both FASN over-expressing MCF7 cells and control cells following treatment with 1 μM Adriamycin for 48 hours. However, in the two stable MCF7 clones which over-express FASN, the drug-induced PARP cleavage was much lower. Adriamycin induced apoptosis was also quantified by the level of DNA fragmentation using Cell Death Detection ELISA (Roche, Indianapolis, IN). As shown in Figure 15B, FASN over-expressing MCF7 cells showed a significantly lower level of DNA fragmentation compared with control cells. Together, these results indicate that MCF7 cells with FASN overexpression are more resistant to drug-induced apoptosis.

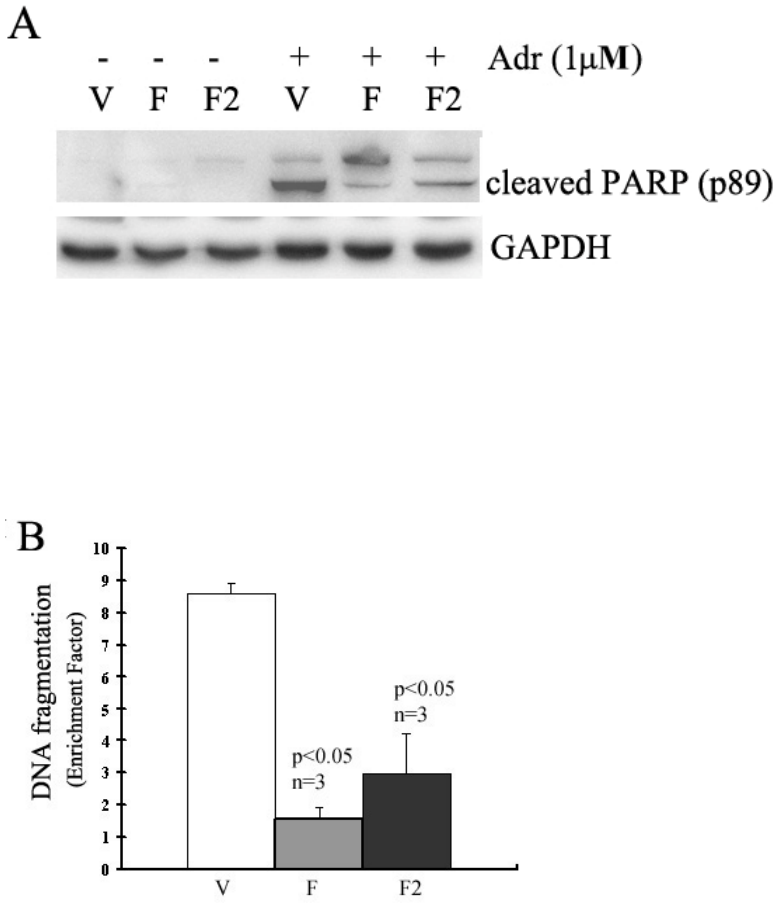
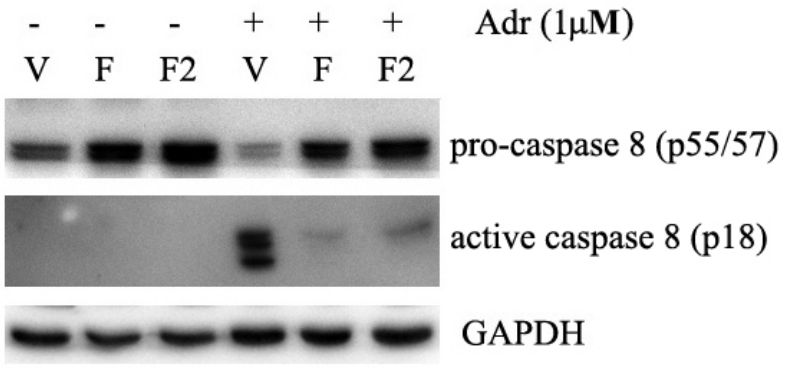


Figure 15. (Legend on next page)

Figure 15. FASN overexpression protects MCF7 cells from drug induced apoptosis. Two stable MCF7 clones with FASN overexpression (F and F2) and a control clone transfected with empty vector (V) were treated with 1μM of Adriamycin for 48 hours, A. PARP cleavage was analyzed using a Western blot. GADPH was used as a loading control. B. Apoptosis at 48 hours after Adriamycin treatment was estimated by DNA fragmentation ELISA. Enrichment factor= Absorbance of cells treated with Adriamycin/absorbance of corresponding cells without Adriamycin treatment.

G. FASN overexpression inhibits caspase-8 activation, and thus apoptosis.

After anti-cancer drug treatment, apoptosis occurs in cancer cells through two distinctive pathways, the mitochondrial pathway followed by activation of Caspase-9, or cell death receptor pathway followed by activation of Caspase-8. To determine which pathway is affected by FASN oeve-rexpression, cells were treated with 1 μM of Adriamycin for 48 hours. Cleavage of Caspase-8 and Caspase-9 to their corresponding active forms was determined using Western blot analysis. As shown in Figure 16, Caspase-9 failed to be activated in both FASN over-expressing clones and vector-transfected control cells after Adriamycin treatment. However, Caspase-8 activation was much higher in vector-transfected control cells, compared with FASN over-expressing clones. These results suggest that FASN overexpression likely blocks the drug-induced activation of caspase-8, and subsequently apoptosis.



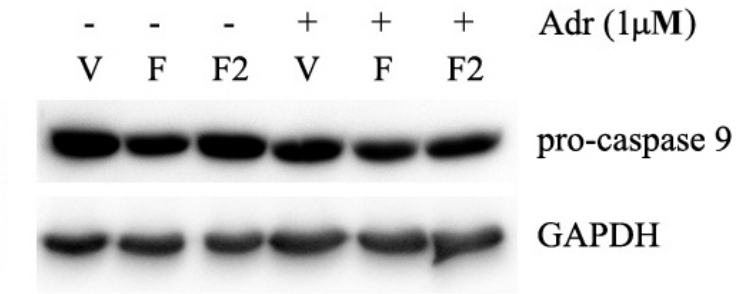


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Figure 16. FASN overexpression in MCF7 cells blocks the activation of caspase-8, but not caspase-9. Two stable MCF7 clones with FASN overexpression (F and F2) and a control clone transfected with vector (V) were plated at 3×10^6 cells/plate on 10cm plates for 24 hours and then treated with 1 μM of Adriamycin for 48 hours followed by analysis of caspase-8 and caspase-9 cleavage using a Western blot. Actin was used as a loading control.

Caspase-8 exists in cells as a pro-caspase. After drug treatment, pro-caspase-8 is recruited to death-inducing signal complex (DISC) and activated to initiate a cascade of proteolytic events resulting in apoptosis. The lack of caspase-8 activation in FASN over-expressing clones suggested a possible binding of caspase-8 by an inhibitor even under drug treatment conditions.

One of the possible ways to inhibit caspase-8 activation is that FASN binds to caspase-8 as an inhibitor. Therefore, in FASN over-expressing clones, more caspase-8 is sequestered by binding to FASN, and less caspase-8 is available to be activated. To rule out this possibility, however, co-immunoprecipitation was used to determine if FASN and caspase-8 can be found in association. Cell lysates from FAS over-expressing MCF7 cells and vector-transfected control cells were first subjected to immunoprecipitation with mouse anti-caspase 8 monoclonal antibody, followed by Western blot analysis using mouse anti-FASN monoclonal antibody. As shown in Figure 17, FASN and caspase-8 did not co-immunoprecipitate, indicating that they do not bind to each other. Furthermore, examination of FASN sequence did not show any caspase-8 binding motif in the FASN protein, confirming that FASN likely does not bind to caspase-8.

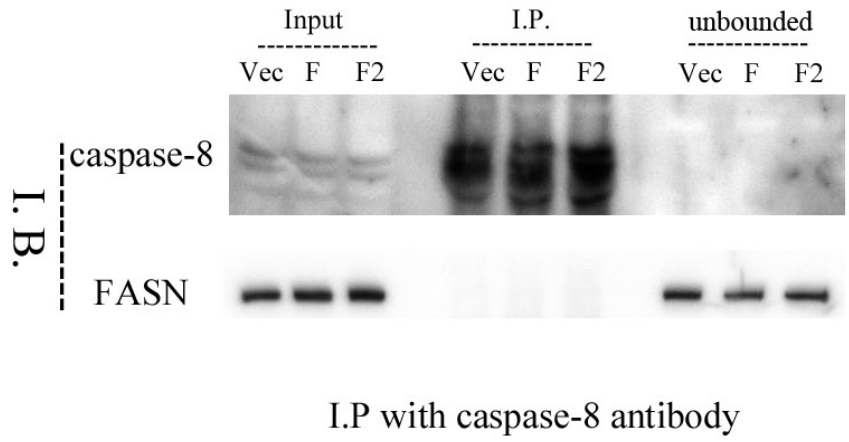

Figure 17. FASN does not bind caspase-8 in MCF7 cells. 200 μg cell lysates from two MCF7 stable clones over-expressing FASN (F and F2) and vector-transfected control (Vec) were immunoprecipitated with anti-caspase-8 monoclonal antibody followed by Western blot (I.B) analysis with either caspase-8 monoclonal antibody or FASN monoclonal antibody.

c-FLIP is a well known caspase-8 inhibitor, it degrades after drug treatment to release caspase-8 for activation of apoptosis. There are two forms of c-FLIP, FLIPs and $FLIP_L$. FLIPs has been shown to inhibit apoptosis, yet $FLIP_L$ has both pro- and anti-apoptotic activities depending on its expression level (Kataoka, 2005). To determine if FASN overexpression blocks c-FLIP degradation after drug treatment, c-FLIP expression level in FASN over-expressing MCF7 and vector-transfected control cells was compared by Western blot analysis. As shown in Figure 18, both c-$FLIP_L$ and c-FLIPs protein levels were similar in FASN over-expressing clones compared with vector-transfected cells. After Adrimaycin treatment, c-$FLIP_L$ was totally degraded, and a smaller size (~43 kDa) product was generated. However the level of this smaller product showed no differences between FASN over-expressing clones and vector-transfected controls at any time points. Similarly, c-FLIPs was also degraded or down-regulated after drug treatment, although to a lesser extent compared with c-$FLIP_L$. Again no difference was found in the level of c-FLIPs in FASN over-expressing clones compared with vector-transfected controls at any time points after Adriamycin treatment. These results indicate that FASN overexpression does not inhibit caspase-8 activation through modulating c-FLIP, but may affect upstream signals that activate caspase-8.

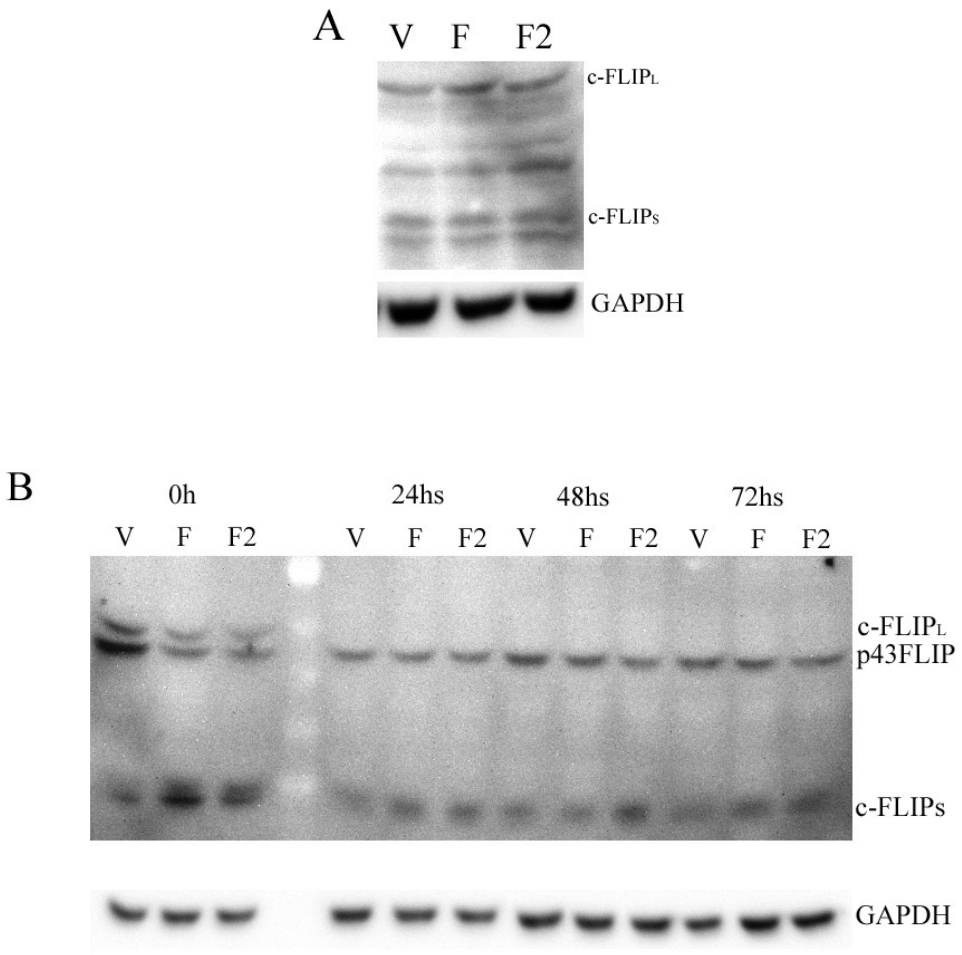

Figure 18. FASN overexpression does not affect expression or degradation of c-FILP$_L$ and C-FLIPs. A. Western blot analysis of c-FLIP expression in MCF7 cells with FASN overexpression (F and F2) and vector-transfected control cells (V), B. Two stable MCF7 clones with FASN overexpression (F and F2) and a control clone transfected with vector (V) were treated with 1 μM of Adriamycin for up to 72 hours followed by analysis of c-FLIP expression using a Western blot. GAPDH was used as a loading control.

H. FASN overexpression decreases Adriamycin-induced ceramide generation.

Because FASN overexpression likely increases the production of palmitate, it is possible that increasing cellular plamitate level would increase the level of drug resistance. To test this hypothesis, MCF7 cells were treated with or without palmitate and tested for their responses to Adriamycin and mitoxantrone using the SRB cytotoxicity assay. As shown in Figure 19, the palmitate-treated cells were significantly more resistant to both Adriamycin and mitoxantrone compared to the vehicle-treated control cells. However, the degree of increase in resistance is less than that induced by FASN overexpression (0.3-0.4 fold increase compared to 2-3 fold increase). This limited increase in resistance by palmitate may be due to the difficulties in delivering palmitate into cells.

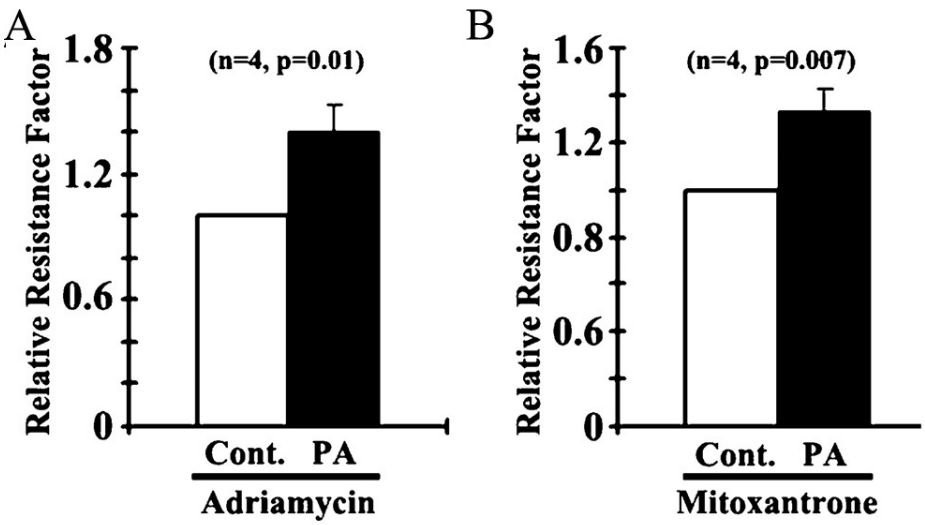

Figure 19. Supplementation of palmitate to MCF7 cells increases their resistance to anticancer drug treatment. MCF7 cells were first treated with 100 μM of palmitic acid (PA) for 24 hours followed by treatment with Adriamycin (A) or mitoxantrone (B) and subjected to a SRB cytotoxicity assay. Relative resistance factor = IC50 of palmitate treated cells/IC50 of non-treated cells.

Palmitate is the precursor of many other lipids in cells, the above observation that palmitate supplementation increase MCF7 cells' resistance to anticancer drug treatment implies that FASN overexpression likely affects the production of cellular lipids that involve in cell survival, proliferation and/or apoptosis.

One way by which Adriamycin induces apoptosis in cancer cells is to increase ceramide generation (Senchenkov et al., 2001). Inhibition of FASN expression in MCF7 cells has been shown to increase ceramide level (Bandyopadhyay et al., 2006). Based on these observations, I hypothesized that FASN overexpression may decrease Adriamycin-induced ceramide production. To test this hypothesis, amount of ceramide was measured using mass spectrometry in two FASN over-expressing MCF7 clones and vector-transfected control cells at different time point after 1 μM of Adriamycin treatment. As shown in Figure 20, ceramide level increased in both FASN over-expressing MCF7 clones and vector-transfected control at a time-dependent manner. At 48 hours after Adriamycin treatment, the level of ceramide in the FASN over-expressing clones was significantly lower compared with that of vector-transfected cells. This result indicates that FASN overexpression in MCF7 cells inhibits the Adriamycin-induced ceramide generation, and thus apoptosis.

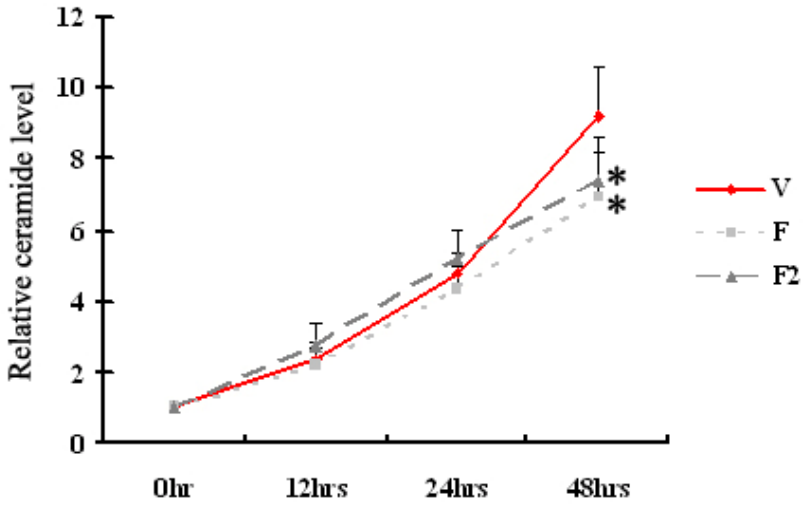

Figure 20. FASN overexpression inhibits Adriamycin-induced ceramide generation. FASN over-expressing MCF7 cells and the vector-transfected control cells were treated with 1 μM of Adriamycin for different time and the level of ceramide in cells was measured using mass spectrometry. Relative ceramide level = amount of ceramide at different time point after Adriamycin treatment/ amount of ceramide at 0 hours.

I. FASN overexpression increases the level of AKT activation in MCF7 cell lines.

There are several reports about the positive feedback relationship between FASN and activation of Akt (Orita et al., 2008; Uddin et al., 2008; Wang et al., 2005). To determine if the FASN overexpression in our stable MCF7 clones may increase the activation of AKT compared with vector-transfected control cells, Western blot analysis was performed to compare the phosphorylated-AKT level in two MCF7 stable clones with FASN overexpression with vector-transfected control MCF7 cells. Cells were cultured under serum free condition for 24 hours and then serum was added for 30min before collecting cells for cell lysate preparation. As shown in Figure 21, the phosphorylated-AKT level was higher in two FASN over-expressing clones compared with vector-transfected control. This result suggested that there is a positive feedback between FASN overexpression and AKT activation in MCF7 cells.

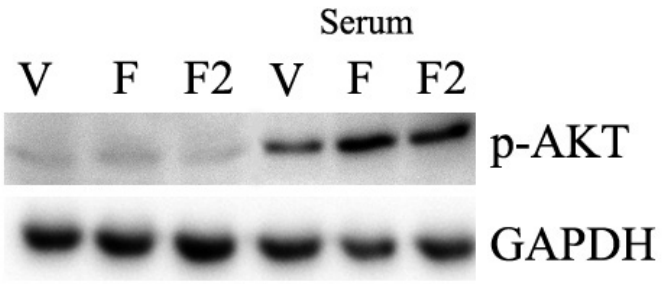

Figure 21. FASN overexpression increases the activation of AKT in MCF7 cells after serum stimulation. Two FASN over-expressing MCF7 clones (F and F2) and vector-transfected control (V) were cultured with serum free DMEM for 24 hours before addition of serum for 30 min. Cells were then collected for analysis of phosphorylated AKT level using Western blot. GAPDH was used as loading control.

J. Activation of AKT does not contribute to increased drug resistance in FASN over-expressing MCF7 cells.

AKT activation promotes cell survival and enhances tumor cell growth and invasiveness. Moreover, active AKT has been shown to confer resistance to chemotherapy and radiation in cell lines derived from a variety of tumor type (Datta et al., 1999; Page et al., 2000; Testa and Bellacosa, 2001). It has been shown that in MCF7 cells, Adriamycin increased PI3K dependent AKT phosphorylation as early as 1hr after treatment with a peak at 24 hours. This increase in AKT activation may contribute to the resistance of MCF7 cells to Adriamycin (Li et al., 2005). PI3K/AKT activation occurs at the plasma membrane and FASN overexpression has been shown to alter membrane lipid composition specifically within the membrane raft domains, where many signaling molecules reside. Based on the above knowledge, we hypothesized that FASN overexpression in MCF7 cells may increase the level and/or prolong the duration of AKT activation after Adriamycin treatment. To test this hypothesis, phophorylated-AKT levels were determined using Western blot in two stable MCF7 clones with FASN overexpression compared with vector-transfected cells at different times following Adriamycin treatment. As shown in Figure 22, phosphorylated-AKT levels gradually increased within 24 hours and started to decrease after 48 hours of 1μM Adriamycin treatment. However, the level of phosphorylated-AKT in FASN over-expressing clones was not higher than that of vector-transfected control cells.

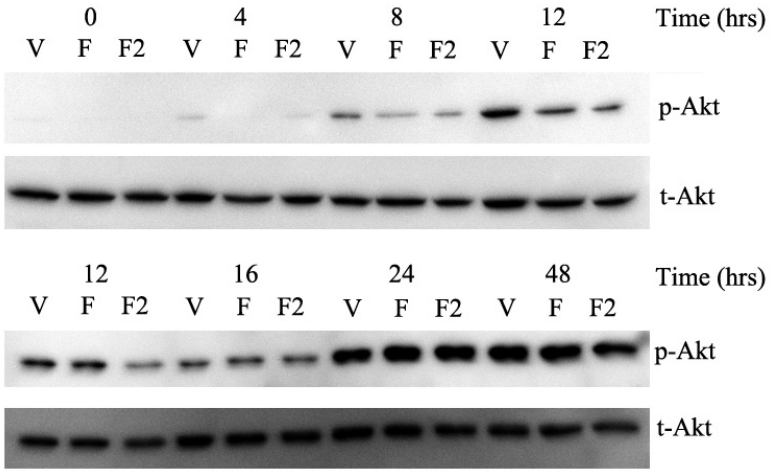

Figure 22. FASN overexpression does not affect Adriamycin-induced phosphorylation of Akt in MCF7 cells. Two stable MCF7 clones with FASN overexpression (F and F2) and a control clone transfected with empty vector (V) were treated with 1μM of Adriamycin for up to 48 hours followed by analysis of phopsphorylated-Akt (p-AKT) and total-Akt (t-AKT) using Western blot analysis.

To further confirm the effect of AKT activation on FASN induced drug resistance, MCF7 cells were transiently transfected with siRNA specifically targeting FASN or scrambled siRNA as control. As shown in Figure 23, FASN expression levels were successfully decreased in MCF7 cells. The effect of FASN knock-down on Adriamycin-induced phosphorylated-AKT levels were determined by Western blot analysis. As shown in Figure 23, the phosphorylated-AKT levels were not lower in FASN siRNA transfected MCF7 cells compared with scrambled siRNA trasfected control cells, regardless of the concentration of Adriamycin used or duration of treatment.

Together, these results indicated that activation of AKT in FASN over-expressing MCF7 cells does not contribute to their higher resistance to various anticancer drugs.

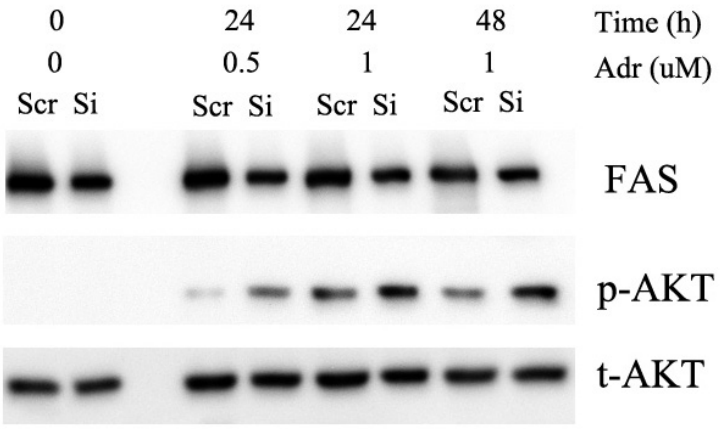

Figure 23. Decreased FASN expression does not affect Adriamycin-induced AKT activation in MCF7 cells. MCF7 cells were transfected with either siRNA specifically targeting FASN (Si) or scrambled siRNA (Scr) as control. 48 hours after transfection, cells were treated with different concentrations of Adriamycin for the indicated time. The level of FASN, cleaved PARP, phosphorylated-AKT and total AKT was determined using Western blot analysis.

IV. Discussion

Increased lipogenesis in tumor tissues was found as early as the 1950s (Medes et al., 1953), and by the mid-1980, Baker and colleagues found that endogenous fatty acid synthesis provides a major source of fatty acids for the growth of tumor cells (Ookhtens et al., 1984). However, the fatty acid synthesis pathway did not become a focus of interest until 1994, when Kuhjada and colleagues identified the oncogenic antigen-519 (OA-519), a molecule found in tumor cells from breast cancer patients with a markedly poorer prognosis, as fatty acid synthase (FASN) (Kuhajda et al., 1994). It gradually became clear that FASN expression is high in a biologically aggressive subset of human carcinomas, including breast cancers (Kuhajda, 2000; Kuhajda, 2006; Menendez and Lupu, 2007). FASN overexpression and hyperactivity were also found to correlate with poor prognosis (Kuhajda, 2000; Kuhajda, 2006; Menendez and Lupu, 2007). In this study, we demonstrated FASN overexpression as a new mechanism of drug resistance in a series of Adriamycin-selected MCF7 breast cancer cell lines. We propose that one of the mechanisms of FASN-induced drug resistance is that FASN overexpression protects cancer cells from drug-induced apoptosis by blocking the activation of caspase-8. FASN-mediated drug resistance is not specific to MCF7 cells. More interestingly, inhibition of FASN expression in normal breast epithelial cells does not affect their drug responsiveness, suggesting that FASN can be used as a target for developing chemosensitizing agents in

combinational therapy without affecting normal cells. The above observations strongly suggest that overexpression of FASN in breast cancer cells likely plays an important role in drug resistance and thus poor prognosis of breast cancer patients. Inhibition of FASN, by inhibitors or siRNA specifically targeting FASN, preferentially induces tumor cell apoptosis and retards tumor growth in xenograft models (Bandyopadhyay et al., 2006; Baron et al., 2004; Browne et al., 2006; Gao et al., 2006; Kridel et al., 2004; Kuhajda et al., 2000; Li et al., 2001; Little et al., 2007; Menendez et al., 2004c; Menendez et al., 2004d; Menendez et al., 2005d; Menendez et al., 2005e; Menendez et al., 2004e; Pan et al., 2007; Pizer et al., 1998; Pizer et al., 1996a; Thupari et al., 2001; Vazquez-Martin et al., 2007a; Wang et al., 2005; Yeh et al., 2003; Zhou et al., 2007; Zhou et al., 2003). Thus, FASN is now considered a potential target for anti-cancer therapy.

In normal cells, FASN expression is under tight control by nutritional signals in liver and adipose tissues and hormonal signals in lactating breast and cycling endometrium (Kuhajda, 2000). However, in cancer cells, FASN overexpression is thought to be the result of inappropriate activation of growth factors receptors such as epidermal growth factor receptor, keratinocyte growth factor receptor, and HER-2 receptor tyrosine kinase (Kumar-Sinha et al., 2003; Menendez et al., 2004d; Oskouian, 2000; Swinnen et al., 2000; Yoon et al., 2007; Zhang et al., 2005). These growth factors activate downstream signaling pathways, particularly the PI3K/Akt pathway, to

stimulate transcriptional upregulation of FASN through activation of SREBP-1c (Ettinger et al., 2004; Furuta et al., 2008; Porstmann et al., 2005; Swinnen et al., 2000; Yang et al., 2002b; Yang et al., 2003). Also, in prostate cancer cells, FASN protein was shown to be stabilized by USP2a, an ubiquitin-specific protease (Priolo et al., 2006). However, the mechanism for further up-regulation of FASN in drug-resistant cancer cells, compared with their parental sensitive ones, remains to be determined. In this study, I show that both mRNA and protein levels of FASN increase in drug-selected MCF7/Adr cell lines compared with drug sensitive MCF7 parental cells. However, there is an obvious discordance in the mRNA and protein expression level of FASN in the drug-selected MCF7 cells lines. The mRNA level increased very early in the drug selection process in MCF7/AdrVp10 cells, while the protein level is similar to that of MCF7 cells. Protein levels started to increase later in MCF7/AdrVp100 cells, with the mRNA level of FAS remaining steady. It is reasonable to conclude that in drug resistant MCF7 cell lines, FASN expression is controlled not only at the transcriptional level, but also at the post-transcriptional level, including the regulation of translation and/or protein stability. Moreover, increased FASN expression in the early stepwise-selected breast cancer cell lines suggests that FASN may be an early response gene for drug insult.

Overexpression of FASN in MCF7 cells increased their drug resistance to a broad range of anti-cancer drugs such as Adriamycin,

mitoxantrone, etoposide, camptothecin and cisplatin, but did not affect the drug resistance levels to vinblastine and paclitaxel. Adriamycin and mitoxantrone are anthracyclines which act as DNA intercalators, and inhibitors of topoisomerase II. Etoposide is a classic topoisomerase II inhibitor, Camptothecin is a topoisomerase I inhibitor, while cisplatin is a DNA alkylating-mimetic agent which can produces intra-stand DNA crosslink. All of the above drugs can produce DNA strand breaks that lead to cancer cell apoptosis through the activation of caspase-8. In this study, we showed that FASN overexpression blocks the activation of caspase-8 in FASN over-expressing MCF7 cells, which helps to explain the increased resistance to these drugs seen in the FASN over-expressing cells. Vinblastine inhibits the assembly of microtubules and paclitaxel interferes with normal microtubule breakdown, thus blocking cells in mitosis. FASN overexpression did not increase resistance to these two drugs. Further investigation of the apoptotic mechanisms of these drugs will help to better define how FASN contributes to the increased drug resistance phenotype in cancer cells.

Inhibition of FASN expression using siRNA or inhibition of FASN activity by inhibitors C75 or cerulinin have been reported to cause apoptosis in a variety of cancer cell lines (Bandyopadhyay et al., 2006; Baron et al., 2004; Browne et al., 2006; De Schrijver et al., 2003; Gao et al., 2006; Horiguchi et al., 2008; Knowles et al., 2004; Kridel et al., 2004; Li et al., 2001; Little et al., 2007; Menendez et al., 2004c; Menendez et al., 2004d; Menendez et al.,

2004e; Pizer et al., 1998; Thupari et al., 2001; Wang et al., 2005; Zhou et al., 2003). Interestingly, we did not observe apoptosis of drug-resistant breast cancer cells following reduction of FASN expression using shRNA. The reason for the difference between these studies is currently unknown. One possibility is that FASN expression in drug resistant cells was so high that the reduction of FASN expression in the drug-resistant cells was insufficient to induce apoptosis as observed in the previous studies using non-drug-selected cancer cell lines. Another reason is that during the selection of stable clones with FASN down-regulation, clones which could undergo apoptosis can happen were eliminated during the selection procedure.

The mechanism of FASN-mediated drug resistance is not yet understood. There are several possible ways that FASN may affect cancer cell responsiveness to drugs.

First, the newly synthesized fatty acids by FASN in cancer cells are saturated and mono-unsaturated. In cancer cells, these fatty acids are mainly incorporated into cellular membrane. Therefore, the increased lipid synthesis in cancer cells may not only increase the quantity of lipids in cell membranes required for cell growth but also change the lipid composition of the membrane. In fact, Rakheja and colleagues showed that there is an increased ratio of saturated to unsaturated C18 fatty acids in colonic adenocarcinoma with high levels of FASN expression (Rakheja et al., 2005; Swinnen et al.,

2003). These changes in membrane composition may result in a decrease in the membrane permeability to anticancer drugs so that the intracellular drug concentration is below the effective therapeutic levels, which in turn causes drug resistance. The changes in membrane composition may also enhance the activity of membrane drug efflux pumps, such as ABCG2, which actively pumps out anticancer drugs and hence effectively reduces intracellular drug concentration. However, we have ruled out these possibilities. It is also unlikely that ABCG2 plays any role in FASN-mediated drug resistance because knocking down FASN expression in MDA-MB-468 cells or over-expressing FASN in MCF7 cells changed the drug response level of these cells, whereas they don't express any detectable level of ABCG2.

Second, FASN has been suggested to promote cancer cell proliferation (Browne et al., 2006; Kuhajda, 2006). It is possible that FASN overexpression affects cell growth rate or cell cycle distribution, which in turn decreases the cellular responses to anticancer drugs. However, we showed that changing the FASN expression level did not change the cell proliferation rate under normal growth conditions. Hence, the change in cell proliferation or cell cycle distribution due to FASN overexpression is not likely the reason for FASN-mediated drug resistance. Menendez and colleagues recently showed that enforced overexpression of FASN in HBL100 and NIH-3T3 cells significantly increased cell proliferation under low-serum (0.1% FBS) conditions, suggesting that increased FASN expression may increase the

proliferative potential of the cells (Vazquez-Martin et al., 2008). It is intriguing to test whether the FASN over-expressing MCF7 cells changes their growth speed under low serum conditions.

Third, most anticancer drugs exert their effect by inducing apoptosis in cancer cells. There are two distinctive apoptotic pathways, the mitochondria pathway with subsequent activation of caspase-9, or cell death receptor pathway, and alternatively activation of caspase-8. Our results indicated that FASN overexpression blocks the activation of capase-8. Caspase-8 exists as a pro-caspase and binds to its inhibitor, c-FLIP, under normal condition. After drug treatment, c-FLIP is degraded and caspase-8 is then cleaved to an active form, initiating a cascade of proteolytic events resulting in apoptosis (Kataoka, 2005). However, FASN overexpression did not change the expression level or degradation of c-FLIP after drug treatment. These results suggested that FASN overexpression does not affect the caspase-8 inhibitor, c-FLIP, but affects either the upstream signals that activate caspase-8 or the formation of death-inducing signaling complex (DISC). Recent studies suggest that lipid rafts serve as the plasma membrane platforms for death-receptor initiated signaling (Muppidi et al., 2004). FASN expression has been shown to affect the composition of lipid rafts (Swinnen et al., 2003). It is very likely that FASN overexpression in MCF7 cells affects the formation of DISC and subsequently downstream signaling (Song et al., 2007).

Supplementation of palmitate, the precursor for the synthesis of many lipid molecules, to MCF7 cells significantly increased their drug resistance. The level of drug resistance in palmitate-treated MCF7 cells, however, is not as substantial as seen in FASN over-expressing MCF7 cell line, which may be due to the difficulty of delivering long chain fatty acids into cells. Palmitate is synthesized by FASN using malonyl-CoA and acetyl-CoA in the presence of NADPH. FASN overexpression and hyperactivity will thus increase the production of palmitate. Previous results from other groups have shown that supplementation of palmitate can rescue FASN knockdown-induced apoptosis, suggesting a critical role of palmitate or its metabolic derivatives in the survival of breast cancer cells (Chajes et al., 2006). Clinically, dietary saturated palmitic acid was associated with an increased risk of breast cancer (Chajes et al., 1999; Saadatian-Elahi et al., 2002). The *de novo* synthesized fatty acids in cancer cells are mostly incorporated into cellular membranes, especially lipid raft aggregates (Swinnen et al., 2003), where many cellular signaling events initiate. In breast cancers, the fatty acid composition of cell membrane is significantly altered compared with the surrounding healthy tissues (Chajes et al., 1995). Such an alteration could have profound effects on many cellular processes including initiation of cellular signaling events such as growth factor signaling, biosynthesis of signaling lipids, and maintenance of mitochondrial membrane potential, etc. Another interesting aspect of cellular palmitate is that palmitate is an allosteric inhibitor of fatty

acid synthesis in lactating mammary gland. Yet in breast cancer cells, it is obvious that this feedback inhibitory effect of palmitate is deficient.

Over-production of palmitate in FASN over-expressing MCF7 cells may affect the biosynthesis of signaling lipids that involve in cell survival, proliferation, and/or apoptosis. Knocking down FASN expression in MCF7 breast cancer cells using siRNA significantly increased the synthesis of ceramide and up-regulated the expression of several pro-apoptotic genes, such as BNIP3, TRAIL, and DAPK2 (Bandyopadhyay et al., 2006). It is thus tempting to propose that FASN overexpression in drug-resistant cells causes decreased expression of these proapoptotic genes and ceramide levels, which in turn causes drug resistance. It has been shown that Adriamycin induces apoptosis in cancer cells by generating ceramide from sphingomyelin breakdown or *de novo* synthesis (Bollinger et al., 2005; Reynolds et al., 2004). Results from current study showed that the increased FASN expression level in drug-resistant cells decreased the level of Adriamycin-induced ceremide, thus may protect cancer cells from drug-induced apoptosis.

On one hand, FASN overexpression blocks the apoptotic signals generated after anti-cancer drug treatment. On the other hand, it may also activate cellular survival pathways, such as PI3K/Akt pathway, to promote cell survival after drug insult. In this study, I showed that elevated FASN expression in MCF7 cells increased the serum-stimulated phosphorylated-Akt

levels as predicted based on previous research results (Wang et al., 2005). However, increased active Akt levels did not have any protection against Adriamycin induced apoptosis since level of active Akt in FASN over-expressing clones showed no higher level compared with vector-transfected control cells after Adriamycin treatment.

As I and others have shown, decreasing FASN expression or inhibiting its activity, sensitizes the drug-resistant cancer cells to traditional anticancer drugs (Menendez et al., 2004a; Menendez et al., 2005c; Menendez et al., 2005e; Menendez et al., 2006b). These observations suggest that FASN inhibitor may be used in combination therapy. The fact that inhibition of FASN expression in normal breast epithelial cells does not affect their drug responsiveness further supports the above idea. One concern is that inhibiting FASN may affect bone marrow or other critical normal cells, thus limiting the use of FASN inhibitors. However, a study using FASN inhibitor cerulenin or C75 on diet-induced obese mice showed that FASN inhibitors substantially decreased fatty liver and adipose mass without hepatocellular injury or fat necrosis (Loftus et al., 2000). Another study using a novel FASN inhibitor Orlistat in a prostate cancer xenograft model with PC-3 cells showed that Orlistat prevented the growth of PC-3 tumors, while mice exhibited no outward signs of toxicity, experienced no loss of weight, nor were there any effects of Orlistat on hematocrit or white blood cell levels (Kridel et al., 2004). These data imply that targeting FASN may not have significant side

effect on normal cells. Orlistat is a US Food and Drug Administration–approved drug for treating obese patients (McNeely and Benfield, 1998). It has also found to be an inhibitor of FASN (Kridel et al., 2004). Recent co-crystalization study of Orlistat and the FASN thioesterase domain further confirmed the inhibitory effect of Orlistat on FASN (Pemble et al., 2007). Orlistat induces endoplasmic reticulum stress and tumor cell death, inhibits tumor growth and prevents angiogenesis (Browne et al., 2006; Little et al., 2007). In this study, we showed that Orlistat can sensitize FASN over-expressing MCF7 cells to various anticancer drugs. Even though Orlistat has low oral bioavailability, and it is rapidly degraded after i.v. injection (Kridel et al., 2004), it provides a starting backbone for the design of new compounds as sensitizers in combination therapy for cancers.

Based on the results from this study, I proposed a model of FASN-induced drug resistance (Figure 24). In cancer cells, anticancer drugs such as Adriamycin induce ceramide production either through *de novo* synthesis or breakdown of sphigomyelin (Bollinger et al., 2005; Reynolds et al., 2004; Senchenkov et al., 2001). Increased ceramide leads to activation of caspase-8 and then apoptosis. On the other hand, Adriamycin can also induce the production of diacylglycerol (DAG) by breakdown of phospholipids via phospholipases (Bettaieb et al., 1999). DAG is a lipid second messenger that promotes cell survival. DAG also inhibits sphingomyelinase (SMase), thus inhibit ceramide generation through sphigomyelin breakdown (Hannun

and Obeid, 1995). Ceramide and DAG counterbalance in cells to determine cell survival or apoptosis. In drug sensitive cells, Adriamycin-induced ceramide production overpowers DAG production, so cells go to apoptosis. However, in FASN over-expressing cancer cells, palmitate production increased. And since palmitate is incorporated into membrane phospholipids, phospholipids level goes up, and Adriamycin-induced DAG generation will increase. This Increase in DAG generation inhibits ceramide production by inhibiting SMase, resulting in the inhibition of the downstream caspase-8 activation and apoptosis. In this case, survival signals overpower the apoptotic signals and cells with FASN overexpression can survive the chemotherapy. Clearly, further experiments are needed to test this hypothesis.

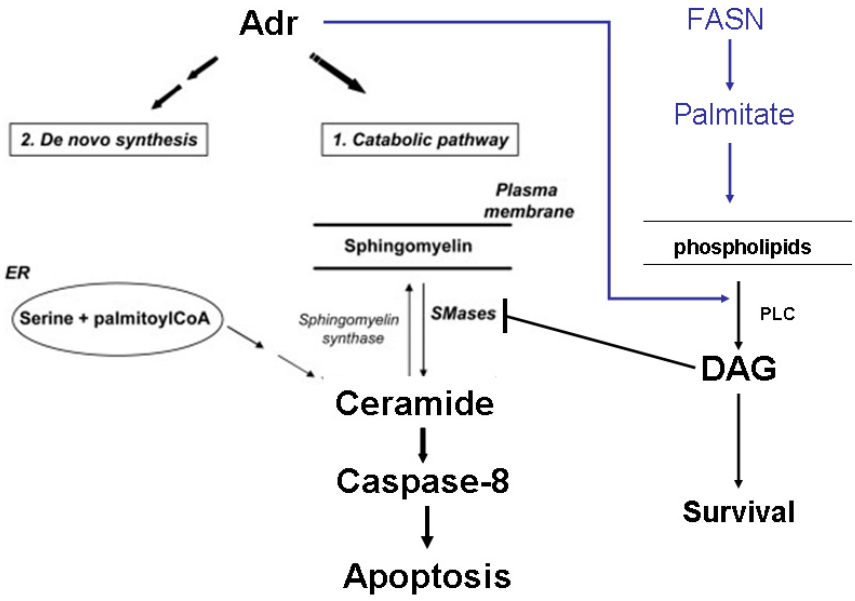

Figure 24. Mechanism of FASN-mediated drug resistance. Adriamycin induces generation of both ceramide and diacylglycerol (DAG), which counterbalance each other to decide the fate of the cells after drug treatment. In FASN over-expressing cells, generation of DAG and downstream survival signals overpower the generation of ceramide induced apoptotic signals, thus cells with FASN overexpression become resistant to chemotherapy.

V. Summary and Conclusion

The experimental results of this dissertation can be summarized as follows:

1. The level of drug resistance in the drug-selected MCF7 cell lines correlates with the level of FASN expression.

2. Down-regulation of FASN expression in the drug resistant MCF7/AdrVp3000 cells reverses Adriamycin and mitoxantrone resistance phenotype.

3. Ectopic overexpression of FASN in the parental drug sensitive MCF7 cells increases resistance to Adriamycin, mitoxantrone, etoposide, camptothecin, and cisplatin.

4. FASN overexpression does not alter the sensitivity to vinblastine and paclitaxel in MCF7 and MCF7/AdrVp3000 cell lines with altered FASN expression.

5. Down-regulation of FASN expression in the breast cancer cell line MDA-MB-468 decreases the drug resistance level to Adriamycin and mitoxantrone.

6. Down-regulation of FASN expression levels in the normal breast epithelial cell line MCF10A1 does not alter its drug resistance level.

7. FASN overexpression does not affect drug uptake or membrane transporter activity in MCF7 and drug-selected MCF7/AdrVp3000 cells.

8. FASN overexpression does not affect cancer cell proliferation

under normal growth conditions.

9. FASN overexpression protects cancer cells from drug-induced apoptosis by blocking the drug-induced caspase-8 activation.

10. FASN overexpression likely causes increased palmitate production.

11. FASN overexpression decreases Adriamycin-induced ceramide production.

VI. Future Plans

My present work showed that FASN overexpression contributes to the increased drug resistance phenotype in breast cancer cells. One advantage of increased FASN expression in cancer cells is to protect cells from anticancer drug-induced apoptosis through blocking caspase-8 activation. Future directions that may extend the current work are:

1. FASN synthesizes palmitate, which is the precursor of many lipid molecules. FASN overexpression may affect the de novo synthesis or generation of some important lipid messengers that involves in regulation of cellular survival and apoptosis upon drug insult. As shown in this study, FASN overexpression inhibits the Adriamycin-induced ceramide production. It will be intresting to determine if FASN overexpression affects the level of other signaling lipids such as diacylglycerol (DAG), that promote cell survival (Bettaieb et al., 1999).

2. In cancer cells, FASN mainly involves in the production of phospholipids partitioning into detergent resistant membrane microdomain (lipid raft aggregate). Lipid raft domains house many molecules involved in signal transduction, apoptosis, membrane transport, etc. Since FASN overexpression protects cancer cells from drug-induced apoptosis, it will be interesting to determine if there are any changes in membrane signaling molecules that either promote cell survival (such as growth factor receptors) or apoptosis (such as the assembly of DISC).

VII References

Allen, J. D., Brinkhuis, R. F., Wijnholds, J., and Schinkel, A. H. (1999). The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res *59*, 4237-4241.

Alli, P. M., Pinn, M. L., Jaffee, E. M., McFadden, J. M., and Kuhajda, F. P. (2005). Fatty acid synthase inhibitors are chemopreventive for mammary cancer in neu-N transgenic mice. Oncogene *24*, 39-46.

Alo, P. L., Visca, P., Trombetta, G., Mangoni, A., Lenti, L., Monaco, S., Botti, C., Serpieri, D. E., and Di Tondo, U. (1999). Fatty acid synthase (FAS) predictive strength in poorly differentiated early breast carcinomas. Tumori *85*, 35-40.

Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol *39*, 361-398.

Asturias, F. J., Chadick, J. Z., Cheung, I. K., Stark, H., Witkowski, A., Joshi, A. K., and Smith, S. (2005). Structure and molecular organization of mammalian fatty acid synthase. Nat Struct Mol Biol *12*, 225-232.

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

Baron, A., Migita, T., Tang, D., and Loda, M. (2004). Fatty acid synthase: a metabolic oncogene in prostate cancer? J Cell Biochem *91*, 47-53.

Bellamy, W. T. (1996). P-glycoproteins and multidrug resistance. Annu Rev Pharmacol Toxicol *36*, 161-183.

Bettaieb, A., Plo, I., Mansat-De Mas, V., Quillet-Mary, A., Levade, T., Laurent, G., and Jaffrezou, J. P. (1999). Daunorubicin- and mitoxantrone-triggered phosphatidylcholine hydrolysis: implication in drug-induced ceramide generation and apoptosis. Mol Pharmacol *55*, 118-125.

Bollinger, C. R., Teichgraber, V., and Gulbins, E. (2005). Ceramide-enriched membrane domains. Biochim Biophys Acta *1746*, 284-294.

Browne, C. D., Hindmarsh, E. J., and Smith, J. W. (2006). Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor. Faseb J *20*, 2027-2035.

Bui, T., and Thompson, C. B. (2006). Cancer's sweet tooth. Cancer Cell *9*, 419-420.

Carvalho, M. A., Zecchin, K. G., Seguin, F., Bastos, D. C., Agostini, M., Rangel, A. L., Veiga, S. S., Raposo, H. F., Oliveira, H. C., Loda, M.*, et al.* (2008). Fatty acid synthase inhibition with Orlistat promotes apoptosis and reduces cell growth and lymph node metastasis in a mouse melanoma model. Int J Cancer *123*, 2557-2565.

Cha, S. H., Hu, Z., Chohnan, S., and Lane, M. D. (2005). Inhibition of hypothalamic fatty acid synthase triggers rapid activation of fatty acid oxidation in skeletal muscle. Proc Natl Acad Sci U S A *102*, 14557-14562.

Chajes, V., Cambot, M., Moreau, K., Lenoir, G. M., and Joulin, V. (2006). Acetyl-CoA carboxylase alpha is essential to breast cancer cell survival. Cancer Res *66*, 5287-5294.

Chajes, V., Hulten, K., Van Kappel, A. L., Winkvist, A., Kaaks, R., Hallmans, G., Lenner, P., and 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.

Chajes, V., Lanson, M., Fetissof, F., Lhuillery, C., and Bougnoux, P. (1995). Membrane fatty acids of breast carcinoma: contribution of host fatty acids and tumor properties. Int J Cancer *63*, 169-175.

Chalbos, D., Chambon, M., Ailhaud, G., and Rochefort, H. (1987). Fatty acid synthetase and its mRNA are induced by progestins in breast cancer cells. J Biol Chem *262*, 9923-9926.

Chen, Y. N., Mickley, L. A., Schwartz, A. M., Acton, E. M., Hwang, J. L., and Fojo, A. T. (1990). Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. J Biol Chem *265*, 10073-10080.

Cheng, F., Wang, Q., Chen, M., Quiocho, F. A., and Ma, J. (2008). Molecular docking study of the interactions between the thioesterase domain of human fatty acid synthase and its ligands. Proteins *70*, 1228-1234.

Chiang, C. T., Way, T. D., Tsai, S. J., and Lin, J. K. (2007). Diosgenin, a naturally occurring steroid, suppresses fatty acid synthase expression in HER2-over-expressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation. FEBS Lett *581*, 5735-5742.

Christie, W. W., Hunter, M. L., and Clegg, R. A. (1981). The effects of cerulenin on lipid metabolism in vitro in cellular preparations from the rat. Biochim Biophys Acta *666*, 284-290.

Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G. (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science *258*, 1650-1654.

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes Dev *13*, 2905-2927.

De Schrijver, E., Brusselmans, K., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2003). RNA interference-mediated silencing of the fatty acid synthase gene attenuates growth and induces morphological changes and apoptosis of LNCaP prostate cancer cells. Cancer Res *63*, 3799-3804.

Di Vizio, D., Adam, R. M., Kim, J., Kim, R., Sotgia, F., Williams, T., Demichelis, F., Solomon, K. R., Loda, M., Rubin, M. A.*, et al.* (2008). Caveolin-1 interacts with a lipid raft-associated population of fatty acid synthase. Cell Cycle *7*, 2257-2267.

Dong, Z., Liu, L. H., Han, B., Pincheira, R., and Zhang, J. T. (2004). Role of eIF3 p170 in controlling synthesis of ribonucleotide reductase M2 and cell growth. Oncogene *23*, 3790-3801.

Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. (1998). A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A *95*, 15665-15670.

Endicott, J. A., and Ling, V. (1989). The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem *58*, 137-171.

Ettinger, S. L., Sobel, R., Whitmore, T. G., Akbari, M., Bradley, D. R., Gleave, M. E., and Nelson, C. C. (2004). Dysregulation of sterol response element-binding proteins and downstream effectors in prostate cancer during progression to androgen independence. Cancer Res *64*, 2212-2221.

Furuta, E., Pai, S. K., Zhan, R., Bandyopadhyay, S., Watabe, M., Mo, Y. Y., Hirota, S., Hosobe, S., Tsukada, T., Miura, K.*, et al.* (2008). Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1. Cancer Res *68*, 1003-1011.

Gabrielson, E. W., Pinn, M. L., Testa, J. R., and Kuhajda, F. P. (2001). Increased fatty acid synthase is a therapeutic target in mesothelioma. Clin Cancer Res *7*, 153-157.

Gao, Y., Lin, L. P., Zhu, C. H., Chen, Y., Hou, Y. T., and Ding, J. (2006). Growth arrest induced by C75, A fatty acid synthase inhibitor, was partially modulated by p38 MAPK but not by p53 in human hepatocellular carcinoma. Cancer Biol Ther *5*, 978-985.

Gottesman, M. M., Fojo, T., and Bates, S. E. (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer *2*, 48-58.

Graner, E., Tang, D., Rossi, S., Baron, A., Migita, T., Weinstein, L. J., Lechpammer, M., Huesken, D., Zimmermann, J., Signoretti, S., and Loda, M. (2004). The isopeptidase USP2a regulates the stability of fatty acid synthase in prostate cancer. Cancer Cell *5*, 253-261.

Horiguchi, A., Asano, T., Asano, T., Ito, K., Sumitomo, M., and Hayakawa, M. (2008). Pharmacological inhibitor of fatty acid synthase suppresses growth and invasiveness of renal cancer cells. J Urol *180*, 729-736.

Hu, L., Hofmann, J., Lu, Y., Mills, G. B., and Jaffe, R. B. (2002). Inhibition of phosphatidylinositol 3'-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. Cancer Res *62*, 1087-1092.

Hughes-Fulford, M., Li, C. F., Boonyaratanakornkit, J., and Sayyah, S. (2006). Arachidonic acid activates phosphatidylinositol 3-kinase signaling and induces gene expression in prostate cancer. Cancer Res *66*, 1427-1433.

Hannun, Y. A., and Obeid, L. M. (1995). Ceramide: an intracellular signal for apoptosis. Trends Biochem Sci *20*, 73-77.

Jackowski, S. (1994). Coordination of membrane phospholipid synthesis with the cell cycle. J Biol Chem *269*, 3858-3867.

Kataoka, T. (2005). The caspase-8 modulator c-FLIP. Crit Rev Immunol *25*, 31-58.

Knowles, L. M., Axelrod, F., Browne, C. D., and Smith, J. W. (2004). A fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating Skp2. J Biol Chem *279*, 30540-30545.

Knowles, L. M., and Smith, J. W. (2007). Genome-wide changes accompanying knockdown of fatty acid synthase in breast cancer. BMC Genomics *8*, 168.

Knowles, L. M., Yang, C., Osterman, A., and Smith, J. W. (2008). Inhibition of fatty acid synthase induces caspase 8-mediated tumor cell apoptosis by Up-regulating DDIT4. J Biol Chem *283*,31378-31384.

Kridel, S. J., Axelrod, F., Rozenkrantz, N., and Smith, J. W. (2004). Orlistat is a novel inhibitor of fatty acid synthase with antitumor activity. Cancer Res *64*, 2070-2075.

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

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

Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., and Pasternack, G. R. (1994). Fatty acid synthesis: a potential selective target for antineoplastic therapy. Proc Natl Acad Sci U S A *91*, 6379-6383.

Kuhajda, F. P., Landree, L. E., and Ronnett, G. V. (2005). The connections between C75 and obesity drug-target pathways. Trends Pharmacol Sci *26*, 541-544.

Kuhajda, F. P., Pizer, E. S., Li, J. N., Mani, N. S., Frehywot, G. L., and Townsend, C. A. (2000). Synthesis and antitumor activity of an inhibitor of fatty acid synthase. Proc Natl Acad Sci U S A *97*, 3450-3454.

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

Landree, L. E., Hanlon, A. L., Strong, D. W., Rumbaugh, G., Miller, I. M., Thupari, J. N., Connolly, E. C., Huganir, R. L., Richardson, C., Witters, L. A.*, et al.* (2004). C75, a fatty acid synthase inhibitor, modulates AMP-activated protein kinase to alter neuronal energy metabolism. J Biol Chem *279*, 3817-3827.

Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994). Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature *371*, 346-347.

Lee, J. S., Paull, K., Alvarez, M., Hose, C., Monks, A., Grever, M., Fojo, A. T., and Bates, S. E. (1994). Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol Pharmacol *46*, 627-638.

Lee, J. S., Scala, S., Matsumoto, Y., Dickstein, B., Robey, R., Zhan, Z., Altenberg, G., and Bates, S. E. (1997). Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP overexpression. J Cell Biochem *65*, 513-526.

Li, J. N., Gorospe, M., Chrest, F. J., Kumaravel, T. S., Evans, M. K., Han, W. F., and 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.

Li, X., Lu, Y., Liang, K., Liu, B., and Fan, Z. (2005). Differential responses to doxorubicin-induced phosphorylation and activation of Akt in human breast cancer cells. Breast Cancer Res *7*, R589-597.

Litman, T., Brangi, M., Hudson, E., Fetsch, P., Abati, A., Ross, D. D., Miyake, K., Resau, J. H., and Bates, S. E. (2000). The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci *113 (Pt 11)*, 2011-2021.

Little, J. L., Wheeler, F. B., Fels, D. R., Koumenis, C., and Kridel, S. J. (2007). Inhibition of fatty acid synthase induces endoplasmic reticulum stress in tumor cells. Cancer Res *67*, 1262-1269.

Liu, H., Liu, Y., and Zhang, J. T. (2008). A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction. Mol Cancer Ther *7*, 263-270.

Liu, X., Shi, Y., Giranda, V. L., and Luo, Y. (2006a). Inhibition of the phosphatidylinositol 3-kinase/Akt pathway sensitizes MDA-MB468 human breast cancer cells to cerulenin-induced apoptosis. Mol Cancer Ther *5*, 494-501.

Liu, Y., Liu, H., Han, B., and Zhang, J. T. (2006b). Identification of 14-3-3sigma as a contributor to drug resistance in human breast cancer cells using functional proteomic analysis. Cancer Res *66*, 3248-3255.

Liu, Y., Peng, H., and Zhang, J. T. (2005). Expression Profiling of ABC Transporters in a Drug-Resistant Breast Cancer Cell Line Using AmpArray. Mol Pharmacol *68*, 430-438.

Loftus, T. M., Jaworsky, D. E., Frehywot, G. L., Townsend, C. A., Ronnett, G. V., Lane, M. D., and Kuhajda, F. P. (2000). Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors. Science *288*, 2379-2381.

Lupu, R., and Menendez, J. A. (2006). Pharmacological inhibitors of Fatty Acid Synthase (FASN)--catalyzed endogenous fatty acid biogenesis: a new family of anti-cancer agents? Curr Pharm Biotechnol *7*, 483-493.

Maier, T., Jenni, S., and Ban, N. (2006). Architecture of mammalian fatty acid synthase at 4.5 A resolution. Science *311*, 1258-1262.

Maier, T., Leibundgut, M., and Ban, N. (2008). The crystal structure of a mammalian fatty acid synthase. Science *321*, 1315-1322.

McGarry, J. D., and Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem *244*, 1-14.

McNeely, W., and Benfield, P. (1998). Orlistat. Drugs *56*, 241-249; discussion 250.

Medes, G., Thomas, A., and Weinhouse, S. (1953). Metabolism of neoplastic tissue. IV. A study of lipid synthesis in neoplastic tissue slices in vitro. Cancer Res *13*, 27-29.

Menendez, J. A., Colomer, R., and Lupu, R. (2004a). Inhibition of tumor-associated fatty acid synthase activity enhances vinorelbine (Navelbine)-induced cytotoxicity and apoptotic cell death in human breast cancer cells. Oncol Rep *12*, 411-422.

Menendez, J. A., and Lupu, R. (2006). Oncogenic properties of the endogenous fatty acid metabolism: molecular pathology of fatty acid synthase in cancer cells. Curr Opin Clin Nutr Metab Care *9*, 346-357.

Menendez, J. A., and Lupu, R. (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer *7*, 763-777.

Menendez, J. A., Lupu, R., and Colomer, R. (2004b). Inhibition of tumor-associated fatty acid synthase hyperactivity induces synergistic chemosensitization of HER -2/ neu -over-expressing human breast cancer cells to docetaxel (taxotere). Breast Cancer Res Treat *84*, 183-195.

Menendez, J. A., Lupu, R., and Colomer, R. (2005a). Targeting fatty acid synthase: potential for therapeutic intervention in her-2/neu-over-expressing breast cancer. Drug News Perspect *18*, 375-385.

Menendez, J. A., Mehmi, I., Atlas, E., Colomer, R., and Lupu, R. (2004c). 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.

Menendez, J. A., Mehmi, I., Verma, V. A., Teng, P. K., and Lupu, R. (2004d). Pharmacological inhibition of fatty acid synthase (FAS): a novel therapeutic approach for breast cancer chemoprevention through its ability to suppress Her-2/neu (erbB-2) oncogene-induced malignant transformation. Mol Carcinog *41*, 164-178.

Menendez, J. A., Oza, B. P., Colomer, R., and Lupu, R. (2005b). The estrogenic activity of synthetic progestins used in oral contraceptives enhances fatty acid synthase-dependent breast cancer cell proliferation and survival. Int J Oncol *26*, 1507-1515.

Menendez, J. A., Papadimitropoulou, A., Vellon, L., and Lupu, R. (2006a). 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.

Menendez, J. A., Vellon, L., Colomer, R., and Lupu, R. (2005c). Pharmacological and small interference RNA-mediated inhibition of breast cancer-associated fatty acid synthase (oncogenic antigen-519) synergistically enhances Taxol (paclitaxel)-induced cytotoxicity. Int J Cancer *115*, 19-35.

Menendez, J. A., Vellon, L., and Lupu, R. (2005d). Antitumoral actions of the anti-obesity drug orlistat (XenicalTM) in breast cancer cells: blockade of cell cycle progression, promotion of apoptotic cell death and PEA3-mediated transcriptional repression of Her2/neu (erbB-2) oncogene. Ann Oncol *16*, 1253-1267.

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

Menendez, J. A., Vellon, L., and Lupu, R. (2006b). The antiobesity drug Orlistat induces cytotoxic effects, suppresses Her-2/neu (erbB-2) oncogene overexpression, and synergistically interacts with trastuzumab (Herceptin) in chemoresistant ovarian cancer cells. Int J Gynecol Cancer *16*, 219-221.

Menendez, J. A., Vellon, L., and Lupu, R. (2006c). DNA topoisomerase IIalpha (TOP2A) inhibitors up-regulate fatty acid synthase gene expression in SK-Br3 breast cancer cells: in vitro evidence for a 'functional amplicon' involving FAS, Her-2/neu and TOP2A genes. Int J Mol Med *18*, 1081-1087.

Menendez, J. A., Vellon, L., Mehmi, I., Oza, B. P., Ropero, S., Colomer, R., and Lupu, R. (2004e). Inhibition of fatty acid synthase (FAS) suppresses HER2/neu (erbB-2) oncogene overexpression in cancer cells. Proc Natl Acad Sci U S A *101*, 10715-10720.

Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T., and Bates, S. E. (1999). Molecular cloning of cDNAs which are highly over-expressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res *59*, 8-13.

Muppidi, J. R., Tschopp, J., and Siegel, R. M. (2004). Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity *21*, 461-465.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37-43.

Nicot, C., Napal, L., Relat, J., Gonzalez, S., Llebaria, A., Woldegiorgis, G., Marrero, P. F., and Haro, D. (2004). C75 activates malonyl-CoA sensitive and insensitive components of the CPT system. Biochem Biophys Res Commun *325*, 660-664.

Oliver, F. J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M. C., de Murcia, G., and Murcia, J. M. (1998). Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. J Biol Chem *273*, 33533-33539.

Ookhtens, M., Kannan, R., Lyon, I., and Baker, N. (1984). Liver and adipose tissue contributions to newly formed fatty acids in an ascites tumor. Am J Physiol *247*, R146-153.

Orita, H., Coulter, J., Lemmon, C., Tully, E., Vadlamudi, A., Medghalchi, S. M., Kuhajda, F. P., and Gabrielson, E. (2007). Selective inhibition of fatty acid synthase for lung cancer treatment. Clin Cancer Res *13*, 7139-7145.

Orita, H., Coulter, J., Tully, E., Kuhajda, F. P., and Gabrielson, E. (2008). Inhibiting fatty acid synthase for chemoprevention of chemically induced lung tumors. Clin Cancer Res *14*, 2458-2464.

Oskouian, B. (2000). Overexpression of fatty acid synthase in SKBR3 breast cancer cell line is mediated via a transcriptional mechanism. Cancer Lett *149*, 43-51.

Page, C., Lin, H. J., Jin, Y., Castle, V. P., Nunez, G., Huang, M., and Lin, J. (2000). Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. Anticancer Res *20*, 407-416.

Pan, M. H., Lin, C. C., Lin, J. K., and Chen, W. J. (2007). Tea polyphenol (-)-epigallocatechin 3-gallate suppresses heregulin-beta1-induced fatty acid synthase expression in human breast cancer cells by inhibiting phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase cascade signaling. J Agric Food Chem *55*, 5030-5037.

Pemble, C. W. t., Johnson, L. C., Kridel, S. J., and Lowther, W. T. (2007). Crystal structure of the thioesterase domain of human fatty acid synthase inhibited by Orlistat. Nat Struct Mol Biol *14*, 704-709.

Pike, L. J. (2003). Lipid rafts: bringing order to chaos. J Lipid Res *44*, 655-667.

Pincheira, R., Chen, Q., and Zhang, J. T. (2001). Identification of a 170-kDa protein over-expressed in lung cancers. Br J Cancer *84*, 1520-1527.

Pizer, E. S., Chrest, F. J., DiGiuseppe, J. A., and 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.

Pizer, E. S., Pflug, B. R., Bova, G. S., Han, W. F., Udan, M. S., and Nelson, J. B. (2001). Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. Prostate *47*, 102-110.

Pizer, E. S., Thupari, J., Han, W. F., Pinn, M. L., Chrest, F. J., Frehywot, G. L., Townsend, C. A., and Kuhajda, F. P. (2000). Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts. Cancer Res *60*, 213-218.

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

Pizer, E. S., Wood, F. D., Pasternack, G. R., and Kuhajda, F. P. (1996b). Fatty acid synthase (FAS): a target for cytotoxic antimetabolites in HL60 promyelocytic leukemia cells. Cancer Res *56*, 745-751.

Porstmann, T., Griffiths, B., Chung, Y. L., Delpuech, O., Griffiths, J. R., Downward, J., and Schulze, A. (2005). PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. Oncogene *24*, 6465-6481.

Priolo, C., Tang, D., Brahamandan, M., Benassi, B., Sicinska, E., Ogino, S., Farsetti, A., Porrello, A., Finn, S., Zimmermann, J.*, et al.* (2006). The isopeptidase USP2a protects human prostate cancer from apoptosis. Cancer Res *66*, 8625-8632.

Puig, T., Vazquez-Martin, A., Relat, J., Petriz, J., Menendez, J. A., Porta, R., Casals, G., Marrero, P. F., Haro, D., Brunet, J., and Colomer, R. (2008). Fatty acid metabolism in breast cancer cells: differential inhibitory effects of epigallocatechin gallate (EGCG) and C75. Breast Cancer Res Treat *109*, 471-479.

Rakheja, D., Kapur, P., Hoang, M. P., Roy, L. C., and Bennett, M. J. (2005). Increased ratio of saturated to unsaturated C18 fatty acids in colonic adenocarcinoma: implications for cryotherapy and lipid raft function. Med Hypotheses *65*, 1120-1123.

Rendina, A. R., and Cheng, D. (2005). Characterization of the inactivation of rat fatty acid synthase by C75: inhibition of partial reactions and protection by substrates. Biochem J *388*, 895-903.

Reynolds, C. P., Maurer, B. J., and Kolesnick, R. N. (2004). Ceramide synthesis and metabolism as a target for cancer therapy. Cancer Lett *206*, 169-180.

Saadatian-Elahi, M., Toniolo, P., Ferrari, P., Goudable, J., Akhmedkhanov, A., Zeleniuch-Jacquotte, A., and Riboli, E. (2002). Serum fatty acids and risk of breast cancer in a nested case-control study of the New York University Women's Health Study. Cancer Epidemiol Biomarkers Prev *11*, 1353-1360.

Sebastiani, V., Botti, C., Di Tondo, U., Visca, P., Pizzuti, L., Santeusanio, G., and Alo, P. L. (2006). Tissue microarray analysis of FAS, Bcl-2, Bcl-x, ER, PgR, Hsp60, p53 and Her2-neu in breast carcinoma. Anticancer Res *26*, 2983-2987.

Senchenkov, A., Litvak, D. A., and Cabot, M. C. (2001). Targeting ceramide metabolism--a strategy for overcoming drug resistance. J Natl Cancer Inst *93*, 347-357.

Shah, U. S., Dhir, R., Gollin, S. M., Chandran, U. R., Lewis, D., Acquafondata, M., and Pflug, B. R. (2006). Fatty acid synthase gene overexpression and copy number gain in prostate adenocarcinoma. Hum Pathol *37*, 401-409.

Shaw, R. J. (2006). Glucose metabolism and cancer. Curr Opin Cell Biol *18*, 598-608.

Silva, S. D., Agostini, M., Nishimoto, I. N., Coletta, R. D., Alves, F. A., Lopes, M. A., Kowalski, L. P., and Graner, E. (2004). Expression of fatty acid synthase, ErbB2 and Ki-67 in head and neck squamous cell carcinoma. A clinicopathological study. Oral Oncol *40*, 688-696.

Silva, S. D., Cunha, I. W., Rangel, A. L., Jorge, J., Zecchin, K. G., Agostini, M., Kowalski, L. P., Coletta, R. D., and Graner, E. (2008a). Differential expression of fatty acid synthase (FAS) and ErbB2 in nonmalignant and malignant oral keratinocytes. Virchows Arch *453*, 57-67.

Silva, S. D., Perez, D. E., Alves, F. A., Nishimoto, I. N., Pinto, C. A., Kowalski, L. P., and Graner, E. (2008b). ErbB2 and fatty acid synthase (FAS) expression in 102 squamous cell carcinomas of the tongue: correlation with clinical outcomes. Oral Oncol *44*, 484-490.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst *82*, 1107-1112.

Smith, S. (1994). The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. Faseb J *8*, 1248-1259.

Smith, S., Witkowski, A., and Joshi, A. K. (2003). Structural and functional organization of the animal fatty acid synthase. Prog Lipid Res *42*, 289-317.

Song, J. H., Tse, M. C., Bellail, A., Phuphanich, S., Khuri, F., Kneteman, N. M., and Hao, C. (2007). Lipid rafts and nonrafts mediate tumor necrosis factor related apoptosis-inducing ligand induced apoptotic and nonapoptotic signals in non small cell lung carcinoma cells. Cancer Res *67*, 6946-6955.

Swinnen, J. V., Esquenet, M., Goossens, K., Heyns, W., and Verhoeven, G. (1997). Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. Cancer Res *57*, 1086-1090.

Swinnen, J. V., Heemers, H., Deboel, L., Foufelle, F., Heyns, W., and 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.

Swinnen, J. V., Van Veldhoven, P. P., Timmermans, L., De Schrijver, E., Brusselmans, K., Vanderhoydonc, F., Van de Sande, T., Heemers, H., Heyns, W., and Verhoeven, G. (2003). Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains. Biochem Biophys Res Commun *302*, 898-903.

Testa, J. R., and Bellacosa, A. (2001). AKT plays a central role in tumorigenesis. Proc Natl Acad Sci U S A *98*, 10983-10985.

Thupari, J. N., Kim, E. K., Moran, T. H., Ronnett, G. V., and Kuhajda, F. P. (2004). Chronic C75 treatment of diet-induced obese mice increases fat oxidation and reduces food intake to reduce adipose mass. Am J Physiol Endocrinol Metab *287*, E97-E104.

Thupari, J. N., Landree, L. E., Ronnett, G. V., and Kuhajda, F. P. (2002). C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity. Proc Natl Acad Sci U S A *99*, 9498-9502.

Thupari, J. N., Pinn, M. L., and Kuhajda, F. P. (2001). Fatty acid synthase inhibition in human breast cancer cells leads to malonyl-CoA-induced inhibition of fatty acid oxidation and cytotoxicity. Biochem Biophys Res Commun *285*, 217-223.

Uddin, S., Siraj, A. K., Al-Rasheed, M., Ahmed, M., Bu, R., Myers, J. N., Alnuaim, A., Al-Sobhi, S., Al-Dayel, F., Bavi, P*., et al.* (2008). Fatty Acid Synthase and AKT Pathway Signaling in a Subset of Papillary Thyroid Cancers. J Clin Endocrinol Metab.

Van de Sande, T., De Schrijver, E., Heyns, W., Verhoeven, G., and Swinnen, J. V. (2002). Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. Cancer Res *62*, 642-646.

Van de Sande, T., Roskams, T., Lerut, E., Joniau, S., Van Poppel, H., Verhoeven, G., and Swinnen, J. V. (2005). High-level expression of fatty acid synthase in human prostate cancer tissues is linked to activation and nuclear localization of Akt/PKB. J Pathol *206*, 214-219.

Vazquez-Martin, A., Colomer, R., Brunet, J., Lupu, R., and Menendez, J. A. (2008). Overexpression of fatty acid synthase gene activates HER1/HER2 tyrosine kinase receptors in human breast epithelial cells. Cell Prolif *41*, 59-85.

Vazquez-Martin, A., Colomer, R., Brunet, J., and Menendez, J. A. (2007a). Pharmacological blockade of fatty acid synthase (FASN) reverses acquired autoresistance to trastuzumab (Herceptin by transcriptionally inhibiting 'HER2 super-expression' occurring in high-dose trastuzumab-conditioned SKBR3/Tzb100 breast cancer cells. Int J Oncol *31*, 769-776.

Vazquez-Martin, A., Ropero, S., Brunet, J., Colomer, R., and Menendez, J. A. (2007b). Inhibition of Fatty Acid Synthase (FASN) synergistically enhances the efficacy of 5-fluorouracil in breast carcinoma cells. Oncol Rep *18*, 973-980.

Wakil, S. J. (1989). Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry *28*, 4523-4530.

Wang, H. Q., Altomare, D. A., Skele, K. L., Poulikakos, P. I., Kuhajda, F. P., Di Cristofano, A., and Testa, J. R. (2005). Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. Oncogene *24*, 3574-3582

Wang, Y. Y., Kuhajda, F. P., Li, J., Finch, T. T., Cheng, P., Koh, C., Li, T., Sokoll, L. J., and Chan, D. W. (2004). Fatty acid synthase as a tumor marker: its extracellular expression in human breast cancer. J Exp Ther Oncol *4*, 101-110.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.

Weng, M. S., Ho, C. T., Ho, Y. S., and 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.

Yang, N., Kays, J. S., Skillman, T. R., Burris, L., Seng, T. W., and Hammond, C. (2005). C75 [4-methylene-2-octyl-5-oxo-tetrahydro-furan-3-carboxylic acid] activates carnitine palmitoyltransferase-1 in isolated mitochondria and intact cells without displacement of bound malonyl CoA. J Pharmacol Exp Ther *312*, 127-133.

Yang, Y., Chen, Q., and Zhang, J. T. (2002a). Structural and functional consequences of mutating cysteine residues in the amino terminus of human multidrug resistance-associated protein 1. J Biol Chem *277*, 44268-44277.

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

Yang, Y. A., Morin, P. J., Han, W. F., Chen, T., Bornman, D. M., Gabrielson, E. W., and Pizer, E. S. (2003). Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. Exp Cell Res *282*, 132-137.

Yeh, C. W., Chen, W. J., Chiang, C. T., Lin-Shiau, S. Y., and Lin, J. K. (2003). Suppression of fatty acid synthase in MCF-7 breast cancer cells by tea and tea polyphenols: a possible mechanism for their hypolipidemic effects. Pharmacogenomics J *3*, 267-276.

Yoon, S., Lee, M. Y., Park, S. W., Moon, J. S., Koh, Y. K., Ahn, Y. H., Park, B. W., and Kim, K. S. (2007). Up-regulation of acetyl-CoA carboxylase alpha and fatty acid synthase by human epidermal growth factor receptor 2 at the translational level in breast cancer cells. J Biol Chem *282*, 26122-26131.

Zhang, D., Tai, L. K., Wong, L. L., Chiu, L. L., Sethi, S. K., and Koay, E. S. (2005). Proteomic study reveals that proteins involved in metabolic and detoxification pathways are highly expressed in HER-2/neu-positive breast cancer. Mol Cell Proteomics *4*, 1686-1696.

Zhang, J. T., Duthie, M., and Ling, V. (1993). Membrane topology of the N-terminal half of the hamster P-glycoprotein molecule. J Biol Chem *268*, 15101-15110.

Zhang, L., Joshi, A. K., and Smith, S. (2003). Cloning, expression, characterization, and interaction of two components of a human mitochondrial fatty acid synthase. Malonyltransferase and acyl carrier protein. J Biol Chem *278*, 40067-40074.

Zhou, W., Han, W. F., Landree, L. E., Thupari, J. N., Pinn, M. L., Bililign, T., Kim, E. K., Vadlamudi, A., Medghalchi, S. M., El Meskini, R.*, et al.* (2007). Fatty acid synthase inhibition activates AMP-activated protein kinase in SKOV3 human ovarian cancer cells. Cancer Res *67*, 2964-2971.

Zhou, W., Simpson, P. J., McFadden, J. M., Townsend, C. A., Medghalchi, S. M., Vadlamudi, A., Pinn, M. L., Ronnett, G. V., and Kuhajda, F. P. (2003). Fatty acid synthase inhibition triggers apoptosis during S phase in human cancer cells. Cancer Res *63*, 7330-7337.

Curriculum Vitae

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PUBLICATIONS

- Kang JS, Yang YC, **Liu HL**, Li YH, Du YC, Li RX. Cloning and distribution of rRP58, a novel neuronal gene. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 33(5):563-568, 2001.
- Cai YY, **Liu H**, Kang JS, Li RX. Cloning and Expression of a Zinc Finger Protein in Rat Brain. *Journal of Shanghai Jiaotong University.* 35(7):1071-1075, 2001.
- Liu, Y, **Liu, H,** Han, B, Zhang, JT. Identification of 14-3-3-σ as a contributor to drug resistance in human breast cancer cells using functional proteomics analysis. *Cancer Research*.66(6):3248-3255,2006.
- **Liu, H**, Liu, Y, Zhang, JT. A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction. *Molecular Cancer Therapy*. 7(2):263-70, 2008.

PRESENTATIONS

Oral Presentations:

- Fatty acid synthase, a novel target in the treatment of drug resistant breast cancers. Walther Oncology Center, May 2008

Poster Presentations:

- Fatty acid synthase, a novel target in the treatment of drug resistant breast cancers, Department of Defense Breast Cancer Research Program Era of Hope Meeting 2008.
- Fatty acid synthase, a novel target in the treatment of drug resistant breast cancers, IU Simon Cancer Center Cancer Research Day 2007.
- Fatty acid synthase, a novel target in the treatment of drug resistant breast cancers, AACR Annual Meeting 2007.
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