The Role of Fatty Acid Synthase Over-Expression in Human Breast Cancer

By

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Abstract:

Fatty acid synthase (FAS) is over-expressed in many human cancers and its activity is required for cancer cell survival. To understand why FAS is over-expressed, we compared in breast cancer cells the utilization of fatty acids synthesized endogenously by FAS to those supplied exogenously in the culture medium. We found that endogenously synthesized fatty acids are esterified to the same lipid and phospholipid classes in the same proportions as those derived exogenously and that some endogenous fatty acids are excreted. Thus, FAS over-expression in cancer does not fulfill a specific requirement for endogenously synthesized fatty acids. We next investigated whether lipogenic activity mediated by FAS was, instead, involved in the maintenance of high glycolytic activity in cancer cells. By culturing breast cancer and non-cancer cells in anoxic conditions, we increased glycolysis 2-3 fold but observed no concomitant increase in lipogenesis. More research is needed to understand why FAS is over-expressed in cancer.
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~

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List of Abbreviations

ACC – Acetyl-CoA Carboxylase
ACL – ATP Citrate Lyase
ACP - Acyl Carrier Protein
ALA – Alpha-linolenic Acid
AMP – Adenosine Monophosphate
ARA – Arachidonic Acid
AT - Acetyl-CoA-ACP Transacylase
ATP – Adenosine Triphosphate
cAPK – Cyclic AMP Dependent Protein Kinase
CE – Cholesteryl Ester
CoA – Coenzyme A
DAG – Diacylglyceride
DHA – Docosahexaenoic Acid
DIM - 3, 3’-diindolylmethane
EPA – Eicosapentaenoic Acid
ER - Enoyl-ACP Reductase
ETC – Electron Transport Chain
FAD – Flavin Adenine Dinucleotide
FAME – Fatty Acid Methyl Ester
FAS – Fatty Acid Synthase
FAs – Fatty Acids
FFA – Free fatty acid
HD - β-hydroxyacyl-ACP Dehydratase
KR - β-ketoacyl-ACP Reductase
KS - β-ketoacyl ACP synthase
LA – Linolenic Acid
MCD – Malonyl-CoA Decarboxylase
MT - Malonyl-CoA-ACP Transferase
NAD – Nicotinamide Adenine Dinucleotide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate
OA – Oleic Acid
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
Pi – Inorganic Phosphate
PI – Phosphatidylinositol
PL – Phospholipid
PPS – Pentose Phosphate Shunt
PS – Phosphatidylserine
ROS – Reactive Oxygen Species
SM – Sphingomyelin
TAG – Triacylglyceride
TCA – Tricarboxylic Acid Cycle
TLC – Thin Layer Chromatography
CHAPTER 1: Introduction and Literature Review
1.1 General Introduction: Why are metabolic pathways attractive targets for cancer therapy?

Cancer treatment today relies on many of the same principles that were in use in the 1940s. In most cases, the rapid proliferative rate of cancer cells is targeted by chemo and radiation therapies (1, 2). These treatments are limited in their effectiveness by a lack of specificity to cancer, as other compartments of rapidly proliferating cells, including the skin, bone marrow and colon, are affected as well, resulting in serious side effects. Though cancer outcomes have improved greatly in the last 70 years, this is more due to improvements in diagnostics and to existing therapies than to the introduction of new targets for drug development.

1.11 Targeted Therapies – The Challenges

Targeted therapies, drugs developed to interfere with specific molecules in cancer cells, have been a dominant focus of research in cancer biology over the last 30 years but have translated into relatively few new treatments. This is perhaps because advances in genomics research have shown that cancer is a much more complicated and diverse disease than initially expected.

Over 100 thousand point mutations have been identified in cancers, over 350 of which are thought to be involved somehow in the cancer phenotype (3). Many more mutations, deletions and translocations are expected to be found as sequencing technology becomes more widespread (4). With this number of mutations, it is difficult to differentiate those that are required for cancer development, called driver mutations, from passenger mutations that do not confer any growth or survival advantage (4, 5). Driver mutations are generally connected with the hallmarks of cancer: cell growth, survival, adhesion, migration and resistance to chemotherapy (6, 7). Part of the difficulty in identifying appropriate drug targets is that passenger
mutations greatly out-number driver mutations. For example, recent genome analyses of 500 primary breast cancer samples uncovered over 27 thousand point mutations, less than 0.01% of which are thought to be potential drivers (4). In addition, most common cancers, including of the breast, prostate and colon, require multiple driver mutations for development (5, 8). This further complicates drug discovery because cancer develops slowly over many years and mutations that facilitated an essential oncogenic function at one point may no longer be required for survival at the relatively late stage when the tumor is eventually detected (Figure 1).

**Figure 1:** Mutations during cancer progression

Many mutations are acquired over the course of cancer development. Some are benign passengers (circles) while others can drive cancer development (stars). By the time a cancer is detected and treatment begins, the cell has many mutations, only a few of which may still be required for its survival. Figure reproduced with permission from (4).
Most driver mutations occur at relatively low frequencies, and so vast numbers of tumor samples must be genotyped to identify potential targets. The International Cancer Genome Consortium coordinates a global effort to genotype 500 tumor samples from 50 types of cancer (9). Each set of tumor samples is estimated to cost 20 million dollars to genotype (over a billion dollars for all 50 cancer types) and will allow driver mutations that occur at frequencies as low as 3% to be identified (10). With the identification of new genetic subtypes in cancer, however, it is now thought that up to 1000 tumor samples from each cancer type will need to be genotyped to identify all the clinically relevant driver mutations (3, 11).

Because of the low frequency of individual driver mutations, targeted therapies are effective treatments for only a small subset of all cancer patients. Trastuzumab for instance, one of the best known targeted therapies currently approved, targets Her2 positive breast cancers, which accounts for 20-30% of all breast cancers (12). Of these patients, fewer than 35% will respond to treatment with Trastuzumab at all, and 70% of these initial responders will develop resistance to the drug within one year (13). Imatinib targets a protein kinase which is present in approximately 90% of chronic myelogenous leukemia patients and has been extremely effective at controlling this disease, but this cancer affects only about 500 Canadians per year (14, 15).

The high development costs and small market of targeted therapies makes them extremely expensive for patients. Treatment with Imatinib costs approximately $3600 per month in Canada while Trastuzumab costs between 30 and 40 thousand dollars per patient, per year (16, 17). The development of more targeted therapies could therefore put an immense strain on the healthcare system.

Treatments that target common features of cancer, the way radiation and chemo therapies do with proliferative rate, rather than an individual molecule, hold the possibility of helping more patients less expensively. This is part of the reason why chemo and radiation therapies have persisted in their usefulness in cancer treatment for nearly 70 years.
In light of the challenges of targeted therapies, the idea of investigating pathways rather than specific molecules for cancer treatment has become appealing. Metabolic abnormalities have been a known feature of cancer for nearly 80 years. Otto Warburg discovered a shift in ATP generation from oxidative phosphorylation to glycolysis, a phenomenon now known as the Warburg effect, in the 1930s (18, 19). Since then, several other metabolic abnormalities have been discovered in cancer, including increased uptake and metabolism of glutamine, increased activity of the pentose phosphate shunt (PPS) and increased fatty acid synthesis (20, 21). Recently, cluster analyses of driver mutations have shown that several converge on metabolic pathways. This suggests that alterations in cell metabolism are part of the development of the cancer phenotype (19, 22). These abnormalities and their interactions are summarized in Figure 2.

Metabolism re-emerged as a target for cancer therapy in the 1990s when it was discovered that oncogenic antigen-519, a protein that had been associated with poor prognosis in cancer patients, was actually fatty acid synthase (FAS) (23). Further investigation revealed that FAS over-expression is a common feature of many types of human cancers and their precursor lesions. FAS inhibition is cytotoxic to cancer cells both in vitro and in vivo while not affecting the growth and survival of non-cancer cells. Thus, FAS is an attractive target for anti-cancer therapies, though it is not yet fully understood what role FAS over-expression plays in cancer cell growth and survival.
1.14 Organization of thesis

This thesis explores the role of FAS in cancer, beginning with a literature review summarizing the function and regulation of FAS in normal cells, its expression and the effects of its inhibition in cancer and current hypotheses explaining FAS over-expression in cancer cells. The overall objective of my work was to better understand why FAS is over-expressed in cancer cells. I address this objective in Chapters 2 and 3 of this thesis, first by examining the utilization of fatty acids synthesized endogenously via FAS in breast cancer and non-cancer cells in Chapter 2, and then by exploring the connection between de novo lipogenesis and aerobic glycolysis in Chapter 3.

**Figure 2:** Summary of metabolic abnormalities in cancer

Cancer cells exhibit aerobic glycolysis, increased uptake and metabolism of glutamine, known as glutamine addiction, increased transit through the pentose phosphate shunt (PPS) and increased FAS expression and activity. These metabolic processes are all integrated via the TCA. Figure modified from (24).
1.2 Fatty Acid Synthase

1.2.1: Overview of the fatty acid synthesis pathway

FAS catalyzes the *de novo* synthesis of fatty acids, predominately palmitate, from precursors acetyl-coenzyme A (CoA) and malonyl CoA, with NADPH as a reducing agent (25). The substrates of FAS are derived primarily from end products of glucose metabolism through glycolysis, and in this way, fatty acid synthesis acts as an energy storage pathway.

Glycolysis converts glucose to pyruvate in a series of 6 reactions summarized in Figure 3. Pyruvate can then enter one of two pathways depending on the oxygen status of the cell. In aerobic conditions, pyruvate is transported into the mitochondria via pyruvate dehydrogenase where it enters the tricarboxylic acid cycle (TCA) (26, 27). Pyruvate can then be completely oxidized to CO$_2$ by the TCA, donating its electrons to NAD and FAD which go on to produce ATP through the electron transport chain (ETC). The complete oxidation of one molecule of glucose by glycolysis, the TCA and the ETC produces up to 34 molecules of ATP (27). In anaerobic conditions, pyruvate cannot be oxidized by the TCA and therefore builds up in the cytosol where it is converted to lactate by lactate dehydrogenase and secreted.

Under conditions in which glycolysis proceeds at an accelerated rate, the TCA intermediate citrate exits the mitochondria to the cytosol via tricarboxylate translocase where it is converted to acetyl-CoA by ATP citrate lyase (ACL). Acetyl-CoA is then converted to malonyl-CoA by acetyl-CoA carboxylase (ACC), the rate limiting step in fatty acid synthesis. FAS joins 7 molecules of malonyl-CoA and 1 molecule of acetyl-CoA in a series of condensation reactions, to produce palmitate.
**Figure 3:** Enzymes involved in glycolysis and fatty acid synthesis

The enzymes in the boxes are discussed in detail in the text. Figure reproduced with permission from (28).

### 1.22: Reactions of Fatty Acid Synthesis

FAS is a homodimer of two 250 kilodalton subunits, each consisting of 7 active domains that together catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA (29, 30). These reactions are described briefly below.

The acetyl group of acetyl-CoA is transferred to the thiol group of the β-ketoacyl-ACP synthase (KS) subunit of FAS by the enzyme acetyl-CoA-ACP transacylase (AT). The malonyl group of malonyl-CoA is then transferred to the thiol group of the acyl carrier protein (ACP) subunit by malonyl-CoA-ACP transferase (MT) bringing it in close proximity to the acetyl CoA molecule. The following five steps are repeated 7 times in the synthesis of a molecule of palmitate, each time extending the fatty acyl chain by 2 carbons.
First, KS catalyzes the condensation of the acetyl and malonyl groups to form acetoacetyl-ACP, releasing a molecule of CO₂. Acetoacetyl-ACP is then reduced by β-ketoacyl-ACP reductase (KR) using NADPH as an electron donor to form D- β-hydroxybutyryl-ACP. β-Hydroxyacyl-ACP dehydratase (HD) then catalyzes a dehydration reaction by removing H₂O from the C-2 and C-3 carbons to form a double bond, producing trans-Δ²-butenoyl-ACP. This double bond is then reduced by enoyl-ACP reductase (ER) using NADPH as the electron donor. Finally, the growing fatty acid is transferred back to the KS subunit of FAS, which held the original acetyl-CoA, MT adds another malonyl-CoA to ACP and the whole process repeats to elongate the fatty acid.

The overall synthesis of palmitate occurs in two parts. In the first, 7 molecules of acetyl-CoA are converted into malonyl-CoA by ACC using ATP. In the second, 7 molecules of malonyl-CoA are joined to one molecule of acetyl-CoA by FAS using NADPH. The reactions for these two steps as well as the balanced equation for the overall process are shown in Figure 5.

It is important to note that fatty acid synthesis is an energetically expensive process, utilizing 7 ATP and 14 NADPH per molecule of palmitate synthesized, as well as diverting acetyl-CoA from the TCA and the ETC where it could be further metabolized to produce additional ATP.
Figure 4: Equations of fatty acid synthesis

(A) The synthesis of malonyl-CoA by ACC (B) The synthesis of palmitate from acetyl-CoA and malonyl-CoA by FAS (C) The overall equation of fatty acid synthesis, the combination of reactions (A) and (B).

1.23: Fatty acid synthesis in normal tissues:

Most fatty acid synthesis occurs in specialized, lipogenic, tissues such as the liver and adipose. These tissues exhibit rates of fatty acid synthesis which are substantially higher than those found in most other tissues. The difference in FAS activity between these tissues is difficult to estimate; measurements in mice suggest that the liver FAS activity can be up to 1000 fold higher than that of the skeletal muscle, although rodents in general have higher FAS activity than humans, so the difference in humans is likely less pronounced (31, 32).

Lipogenic tissues are heavily involved in the energy balance of an organism as they allow excess carbohydrate and amino acid to be converted to lipid for storage in the adipose. The mechanisms involved in regulating FAS activity specifically will be discussed in detail in section 1.24. In the liver, where the majority of post-prandial glucose uptake occurs, glucose is first used to replenish stores of glycogen (33). When the carbohydrate available is in excess
of the glycogen storage capacity of the liver, glucose is metabolized via glycolysis and the TCA, after which it can be oxidized for energy or directed into the synthesis of non-essential amino acids and fatty acids.

Besides the liver and adipose, there is a small group of other tissues that also exhibit high levels of FAS. In the fetal lung, FAS provides the fatty acid needed to produce surfactant (34). FAS expression is also high in the lactating breast where it provides triacylglycerides and medium chain fatty acids for milk production (35). Finally, the endometrium exhibits high FAS expression in the proliferative and secretory phases of its cycle, where it seems to correlate with estrogen and progesterone signaling (36).

**Fate of endogenously synthesized fatty acids in normal cells**

The end product of FAS is predominately palmitate, though smaller quantities of myristate, laureate and stearate are produced (33, 37). Palmitate can undergo further modifications at the endoplasmic reticulum, with the addition of acetyl-CoA molecules by elongases and double bonds by desaturases, to yield a variety of different fatty acids with diverse metabolic functions (38).

Fatty acids face three broad metabolic fates: they can be stored as triacylglyceride, β-oxidized for energy or incorporated into lipids. As will be discussed further in the following section, β-oxidation and fatty acid synthesis are reciprocally regulated, so while endogenous fatty acids may eventually be used for energy, this is rarely their immediate fate after synthesis. In the case of the liver, where most fatty acid synthesis occurs, endogenous fatty acids are predominately packaged into lipoproteins and transported to the adipose for storage (39). Those that remain in the cell enter one of the five cellular neutral lipid or phospholipid classes, illustrated in Figures 5 and 6. It is important to note that in normal non-lipogenic cells, endogenously synthesized fatty acids make up a very small proportion of the cell lipids. Even normal cells with high proliferative rates obtain most of their structural fatty acids from the circulation (31, 40).
Immediately after synthesis, palmitate enters the free fatty acid (FFA) pool. From here it can be esterified to phospholipid (PL), the pool that makes up the cell and organelle membranes, triacylglyceride (TAG), the fatty acid storage pool of the cell, diacylglyceride (DAG), a cell signaling intermediate and precursor to TAG, or cholesteryl ester (CE), a storage form of cholesterol (27).

The PL pool is made up of five major classes of phospholipid, including phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidyl ethanolamine (PE). All five phospholipid classes make varying contributions to the structure of the cell membrane and all play roles in signal transduction, depending in part on the identity of their fatty acid tails (41). To differentiate these phospholipids functionally in a general way, PC is the major structural component of the plasma membrane (26). SM is enriched in the myelin sheath surrounding nerve tissue and plays a role in cell signaling through the ceramide pathway (26). PI is an anchor for membrane associated proteins and is also a signaling intermediate in variety of pathways (26). PS is enriched on the inner leaflet of the cell membrane (42). PE is thought to decrease the fluidity of cell membranes and is found particularly in neural tissue (27). The neutral lipid and phospholipid classes can be separated from total cell lipids by thin layer chromatography (TLC), as shown in Figures 6b. This will be discussed in more detail in the experimental methods, section 2.2 of the thesis.
Figure 5: The neutral lipid classes

(A) The cell lipids can be divided into five classes. FFA represents the un-esterified fatty acids in the cell and undergoes the most turnover. Two free fatty acids can be esterified to glycerol to produce DAG while three fatty acids and glycerol produce the storage molecule TAG. Free fatty acids can also be esterified to phosphatidic acid, derived from glycerol and phosphate, to produce PL the major component of cell membranes. Fatty acid esterified to cholesterol produces CE which is the storage form of cholesterol in the cell.
Figure 6: The phospholipid classes

A) PC, PS, PI and PE are all derived from glycerol via phosphatidic acid. SM is derived from PC and ceramide. Figure modified from (41). B) Illustration of the distribution of the neutral lipid classes on a TLC plate. Figure 7A reproduced with permission from (41).
1.24: Regulation of Fatty Acid Synthesis in Normal Tissues:

Short term nutritional control

Short term regulation of fatty acid synthesis occurs primarily in response to nutritional signals and involves the covalent or allosteric modification of several enzymes, summarized in Figure 7. After a meal, insulin stimulates glucose uptake by the adipose and muscle via the insulin responsive glucose transporter GLUT4 (43). The liver lacks GLUT4, although hepatic glucose uptake has been shown to increase up to 125% post-prandially and is thought to be responsible for the clearance of up to 60% of glucose delivered in a meal (44-46). The mechanism behind this is not entirely clear, though post-prandial concentrations of glucose, fatty acids, amino acids, hormones and neural mediators have all been implicated (33).

Insulin up-regulates the activity of many enzymes involved in glucose metabolism including glycogen synthase, phosphofructokinase-1, pyruvate dehydrogenase, ACL and ACC (27, 47). The increase in glycolysis and traffic of pyruvate through the TCA results in increased production of ATP which decreases the AMP: ATP ratio in the cell. This inactivates cAMP dependent protein kinase (cAPK). As a result, enzymes of β-oxidation are de-phosphorylated and inactivated, while enzymes of fatty acid synthesis, ACL and ACC, are de-phosphorylated and activated (26, 27).

As concentrations of ATP and acetyl-CoA increase in the mitochondria, citrate is transported to the cytosol by tricarboxylate translocase. Here, citrate can be converted to acetyl-CoA by ACL. The affinity of ACL for citrate is further increased by allosteric binding by fructose 6-phosphate, an intermediate of glycolysis (48). Citrate in turn allosterically modifies ACC, increasing its affinity for acetyl-CoA. Citrate and malonyl-CoA allosterically bind and inactivate CPT-1, a rate limiting step in β-oxidation (49).

Under fasting conditions, the opposite occurs. Glucagon causes an intracellular increase in cAMP levels, activating cAPK. cAPK phosphorylates and activates the enzymes of β-oxidation and inactivates ACL and ACC.
Long term nutritional control

In addition to providing short term control of fatty acid synthesis through allosteric and covalent enzyme modifications, insulin and glucagon also alter the expression of the FAS genes (50). In vitro studies of human adipocytes and liver cells have shown that FAS mRNA transcription increases in response to insulin stimulation and decreases in response to treatment with glucagon. In vivo, humans fed with a eucaloric, high carbohydrate, low fat diet exhibit high levels of FAS in the liver and adipose compared those fed a regular diet (51). This is particularly pronounced after fasting and re-feeding (52). This increase is attributed to insulin, as FAS levels do not increase in diabetic rats unless they are treated with insulin (53, 54). Further, hyper-insulinemia has been shown to increase FAS expression in mice livers (54). There is evidence that diets high in saturated fat can also induce FAS expression, though the mechanism of this is unclear (55).

In non-lipogenic tissues, FAS gene expression is modulated by hormonal signaling. This is particularly apparent in the cycling endometrium, where FAS expression is associated with the expression of estrogen and progesterone receptors at various stages in the cycle (36, 56). In the lactating breast, FAS expression is associated with increases in prolactin and insulin that occur during pregnancy (57).

Transcription factors such as sterol regulatory element binding protein 1-c (SREBP1-c), upstream stimulatory factors and specificity protein 1 (Sp1) mediate the regulation of FAS transcription by hormonal signals (28, 53, 58-61).
Figure 7: Summary of changes in the activity of enzymes of FAS and glycolysis

(A) Post-prandial changes (B) Post-absorptive changes. Circles represent changes in enzymatic activity in response to hormone levels while dashed arrows represent changes in enzymatic activity in response feedback activation or inhibition. Figures reproduced with permission from (28). Post-prandial and post-absorptive enzyme activity added for this thesis.
**FAS regulation by exogenous fatty acids**

Fatty acid synthesis in the liver and adipose tissues is potently down-regulated by small amounts of exogenously supplied fatty acid. Induction of FAS activity and mRNA transcription after a carbohydrate-heavy meal is abrogated by addition of corn-oil to the diet in a rat model (62). In another study, liver enzymatic activity after a carbohydrate meal was shifted from fatty acid synthesis towards oxidation by oleate perfusion (39). In the brain, another tissue that exhibits high FAS activity, treatment with oleic acid decreased the incorporation of C-14 acetate into glioma cells by 80% (63).

Polyunsaturated fatty acids (PUFAs), particularly alpha-linolenic (ALA), docosahexaenoic (DHA), eicosapentaenoic (EPA) and arachidonic (ARA), have also been shown to down-regulated FAS expression in the liver and adipose tissue (64-66). Exogenous fatty acids are thought to down-regulate FAS transcription by interfering with the activation of SREBP1-c by insulin (65).

### 1.25 FAS in Cancer

FAS over-expression is a common feature of many types of human cancers, including the breast, prostate, colon, ovary, thyroid, endometrium, bladder, melanoma, meninges, head and neck, esophagus, pancreas, lung and stomach (23, 67-79). In these cancers, FAS expression is considerably higher in the tumor than in the adjacent tissue and its expression is often a predictor of tumor aggressiveness. FAS over-expression has been detected in pre-malignant as well as infiltrating cancerous lesions, and thus it is likely that FAS over-expression is not an adaptation of the cancer cell to the poor perfusion environment that can exist in an established tumor (80). Further, it has been suggested that FAS itself can act as an oncogene, in that inducing FAS expression has been shown to initiate the transformation of non-tumorigenic breast cells (81). FAS protein can be detected in the serum of cancer patients and has been investigated as a potential diagnostic test for breast, prostatic and colorectal cancers.
High levels of FAS in the serum is associated with advanced cancer stage and poor prognosis in many types of human cancers, including a 4-fold increased risk of death in breast cancer patients (67). The expression of FAS in the three common cancers in North America is summarized in the Figure 9.

**Table 1:** Summary of FAS expression in three common cancers

<table>
<thead>
<tr>
<th></th>
<th>Pre-malignant</th>
<th>Infiltrating Cancer</th>
<th>Normal Tissue</th>
<th>Prognostic Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast (67, 85, 86)</td>
<td>+ + in non-proliferative benign breast disease (BBD)</td>
<td>+ + + expression increases with increasing cancer stage</td>
<td>Detectable in ~30% of normal cells compared to ~50% of BBD cells and &gt;80% of malignant cells</td>
<td>&gt; Four-fold increased risk of death if expressed in stage I</td>
</tr>
<tr>
<td>Prostate (76) (87) (23)</td>
<td>+ + in intraepithelial neoplasia</td>
<td>+ + + in adenocarcinoma</td>
<td>Detectable in 60% of benign cells compared to 95% of malignant; 81% accurate as diagnostic indicator</td>
<td>&gt; Four fold increased risk of disease progression, metastasis</td>
</tr>
<tr>
<td>Colon (84) (88) (89, 90) (70)</td>
<td>+ in 80% of aberrant crypt foci</td>
<td>+ + + in ~60% of colon cancers</td>
<td>Low or absent expression</td>
<td>Serum levels predictive of tumor stage, tumor protein expression associated with increased risk of death in high BMI patients, decreased risk of death in low BMI patient</td>
</tr>
</tbody>
</table>
1.26 Regulation of FAS in Cancer

**De-regulation from nutritional control**

As discussed in section 1.24, FAS expression and activity is tightly regulated by nutritional stimuli, and most normal tissues express FAS at very low, often undetectable levels. The fact that FAS is expressed at constitutively high levels in cancer compared to the adjacent non-cancerous tissues is, therefore, evidence that FAS has become de-regulated from normal nutritional controls as otherwise, circulating insulin and fatty acids would down-regulate its activity and expression.

Despite this, the same transcription factors that regulate FAS in normal cells seem to regulate its activity in cancer, in particular Sp1 and SREBP-1c. Breast and prostate cancer cells transfected with Sp1 siRNA exhibit decreased proliferation and FAS expression (28, 91). SREBP1-c has been implicated in the regulation of FAS in cancer because the expression of FAS and SREBP1-c mRNA increases concomitantly during H-ras transformation and decreases concomitantly upon MAPK or PI3K inhibition in a breast cancer model (92). It is possible, however, that this activity is mediated by Sp1, since in normal cells, inhibition of Sp1 reduces insulin-induced binding of SREBP1-c to the FAS promoter (28, 92, 93).

Aberrant growth factor and hormonal signaling via the epidermal growth factor, androgen, estrogen and progesterone receptors have all been implicated as upstream activators of FAS over-expression in cancer. There is as yet, however, no encompassing theory to explain FAS over-expression in the wide variety of cancer types in which it occurs (93). It is possible that this is an example of convergent evolution, in which different cancers arrive at the same phenotype via different pathways because of a similar evolutionary pressure. Improved understanding of how FAS over-expression confers a survival advantage in cancer could help to explain how and why so many varieties of cancer over-express FAS.
**Regulation by exogenous fatty acids**

FAS in cancer cells is largely un-responsive to control by exogenous fatty acids. This is most apparent in cultured cancer cell lines that over-express FAS, of which there are many, as they are exposed to an abundance of fatty acids from fetal bovine serum (FBS) in the culture medium and yet continue to exhibit high expression and activity of FAS (94). It is possible that in an established tumor, the cancer cells may be poorly perfused, and thus would not be exposed to high levels of circulating exogenous fatty acids. However, since FAS over-expression has been detected at early and even pre-malignant stages in cancer development, before blood supply is limited, it is unlikely that this is a major factor in FAS regulation in cancer.

Supra-physiologic concentrations of both arachidonic and linolenic acid (ARA and LA) failed to inhibit fatty acid synthesis in the SK-Br3 breast cancer cell line. Alpha-linolenic and gamma-linolenic acid (ALA and GLA), however, did have inhibitory effects (95). It is not clear why these fatty acids modulate FAS in cancer when other fatty acids have no effect.

1.27 Inhibition of FAS

Almost 20 years ago, it was found that FAS inhibition by cerulenin, an anti-fungal agent, decreased proliferation in a human breast cancer cell line (37). Shortly after, it was found that FAS inhibition also causes cell cycle arrest in the S phase of the cell cycle and induces programmed cell death by apoptosis (37, 96, 97). Since then, experiments with many cancers including breast, prostate, colon, ovarian, lung and melanoma have shown similar growth inhibitory and cytotoxic effects of FAS inhibition (98). Experiments in which FAS activity was targeted specifically with RNAi have confirmed that the cytotoxic activity of these agents are indeed due to FAS inhibition and not other off-target effects (99). These observations are striking because FAS inhibition does not appear to affect the growth and survival of non-cancer
cells or to cause symptoms of toxicity \textit{in vivo} \cite{37,99-102}. Research on the effects of the synthetic FAS inhibitor C75 on various rapidly proliferating tissues \textit{in vivo}, including the bone marrow, gastrointestinal tract and skin, has also shown no adverse effects \cite{101}. Together, these results suggest that FAS inhibitor-induced cytotoxicity is specific to cancer and not a function of the increased proliferative rate of cancer cells.

In view of these findings, FAS inhibition has emerged as a promising target for the prevention and treatment of cancer. Feeding of the FAS inhibitor triclosan decreases tumorigenesis in rats treated with a mammary carcinogen, suggesting that it can act as a chemopreventative agent \cite{103}. FAS inhibition also decreases tumor growth and initiates apoptosis in rodent models of breast and prostate cancer \cite{37,100}. More recently, FAS inhibition has been shown to decrease metastasis by over 50\% in a mouse model of melanoma \cite{104}. FAS inhibition may also have applications as an adjuvant therapy as it has been shown to sensitize breast cancer cells to treatment with chemotherapeutic drugs \cite{105,106}.

\textbf{Pharmacological inhibitors of FAS}

Because of these promising anti-cancer effects, dozens of synthetic and natural inhibitors of FAS have been patented. These synthetic FAS inhibitors work by binding to various active sites of FAS \cite{98}. Most, including cerulenin, C75 and C93, interact with the β-ketoacyl synthase domain. Orlistat blocks the thioesterase domain of FAS while triclosan binds the enoyl reductase domain \cite{98,107}.

The earliest inhibitors, cerulenin and triclosan, are anti-fungal agents that were re-purposed for use in cancer-biology \cite{98,103}. Cerulenin was found to be chemically unstable and undesirable for use \textit{in vivo}, so C75 was developed as a more stable alternative \cite{101}. Orlistat was re-purposed from its original use as an anti-obesity drug \cite{100}.

Though these ‘first generation’ FAS inhibitors were shown to potently decrease FAS activity, reduce proliferation and induce apoptosis in cancer cells, they are not ideal for use in cancer patients. Orlistat was found to have low solubility, low oral bioavailability and a narrow
therapeutic index (98, 102, 108). C75 and cerulenin cause appetite suppression and weight loss in mouse models, possibly via off-target stimulation of CPT-1 which initiates β-oxidation and autophagia (109).

To address these issues, a new FAS inhibitor, C93, was designed not to activate CPT-1. C93 has been shown to prevent tumor growth without causing weight loss and appetite suppression in vivo and may therefore be the most promising synthetic FAS inhibitor developed so far (110).

In addition to these synthetic agents, there are several naturally occurring dietary substances, mostly phytochemicals with anti-proliferative or cytotoxic effects in cancer that are thought to be mediated, at least in part, by FAS inhibition. These include 3,3'-diindolylmethane (DIM), luteolin, quercetin, epigallocatechin-3-gallate, resveratrol, cacalol and genistein (95, 111-113). In contrast to the synthetic FAS inhibitors, many of these dietary components do not bind FAS but rather down-regulate its activity via modulation of the upstream AMPK and PI3K/Akt pathways and SREBP1-c (98, 113, 114).

Though these results are promising, the effectiveness of FAS inhibitors in human cancer patients has yet to be evaluated in clinical trials (98).

Rescue experiments with exogenous fatty acids

There have been four studies that have directly examined whether provision of exogenous fatty acids can prevent, or rescue, cancer cells from the anti-proliferative and cytotoxic effects of FAS inhibition. Kuhajda et al. were the first to attempt these experiments and showed that ZR-75-1 breast cancer cells could be partially rescued from cerulenin by treatment with palmitate (37). The concentrations of palmitate used to achieve rescue, however, were 10 fold higher than found in vivo. The authors noted that at these high concentrations, palmitate became cytotoxic to the cells, independent of cerulenin. In a similar experiment in HL-60 leukemia cells, they found that supraphysiologic levels of oleate could achieve complete rescue from cerulenin induced toxicity (115).
In similar experiments, Menendez et al found that treatment of MCF7 and SKBr3 breast cancer cells with oleate increased the IC₅₀ values, defined as the concentration of cerulenin required to kill 50% of the cells (94). It should be noted, however, that the IC₅₀ increase was significant only after 24 hours incubation and was not significant at 48 hours. Time course experiments have since shown that cerulenin cytotoxicity is maximal at 48 hours, so it could be argued that the negative 48 hour results are more representative of the effects of oleate on FAS inhibition (102). In addition, this rescue was achieved at extremely high concentrations of oleate, 10 fold higher than those used in the experiments by Kuhajda et al and 30 fold higher than found in vivo.

The most potent rescue effects in these studies were observed with oleate. Oleate is not a major end product of FAS either in cancer or in non-cancer cells, so it remains unclear why oleate would rescue the cells but palmitate, which makes up over 80% of endogenously synthesized fatty acids, would not (33, 37). Further, rescue was only seen at supraphysiologic concentrations of the fatty acids. It is not known whether tumor-associated FAS is capable of producing these quantities of fatty acids, and therefore whether providing such high quantities of exogenous fatty acid is truly replacing the function of FAS.

Some experiments have not shown rescue effects by exogenous fatty acid. Saati et al. found that concentrations of oleate up to 30 µM (10-fold higher than found in vivo) failed to rescue MCF-7 breast cancer cells from inhibition by cerulenin or DIM (116). Other unpublished observations from our lab have also not replicated the rescue effect of oleate.
One of the most common explanations for FAS over-expression in cancers is that fatty acids are required for membrane biosynthesis to accommodate the increased proliferative rates of cancer cells (40, 117, 118). While endogenous fatty acid biosynthesis certainly contributes to membrane production, FAS over-expression in cancer is likely more than an adaptation to an increased proliferative rate. First, FAS inhibition does not affect the viability of rapidly proliferating non-cancer cells, such as those of the small intestine and skin, which suggests that it is not needed to support proliferation in cancer (31, 97). Second, cancer cells are rescued from inhibitor cytotoxicity only by supraphysiologic (3-30 fold higher) levels of exogenous fatty acids, levels that are far higher than those necessary to support membrane biosynthesis (118). Even under these conditions, complete cell rescue (no decrease in viability) is rarely achieved, and the higher concentrations of exogenous fatty acid that could theoretically, based on dose responsiveness, completely rescue the cancer cells are independently cytotoxic (37, 94, 119). Finally, and perhaps most importantly, if FAS over-expression in cancer fulfills a requirement for fatty acids for membrane biosynthesis, its expression should be down-regulated when sufficient concentrations of fatty acids are available exogenously. Fatty acid synthesis is energetically expensive and would likely pose a survival disadvantage in cancer if it was producing fatty acid in excess of cell requirements, especially given the lack of perfusion in some established tumors that restricts cancer cell access to nutrients (120). It seems likely, therefore, that FAS plays other roles in cancer in addition to providing fatty acids for membrane production.

One possibility is that FAS affects cancer cell survival by changing the plasma membrane fatty acid composition. It has been shown that FAS activity may increase the size of lipid rafts in the plasma membranes of prostate cancer cells. Saturated fatty acids, such as those produced by FAS, partition more readily into rafts than mono and polyunsaturated fatty acids derived from dietary sources. Swinnen et al. found that RNAi mediated FAS inhibition
decreased the proportion of phospholipids involved in lipid raft formation (121). They went on to show that inhibiting FAS increased the transverse motility or ‘flip flop’ rate of the cell membranes, the rate at which proteins and phospholipids move between the outer and inner membrane leaflets. Some chemotherapeutics, such as doxorubicin, are thought to enter the cell via flip flop and this group showed that FAS inhibition increases the translocation of doxorubicin into the cell (122). Swinnen et al also showed the increased saturation of the plasma membranes protected prostate cancer cells from reactive oxygen species (ROS), which are generated in the course of cell metabolism and by some chemotherapeutic drugs. Unsaturated fatty acids are more readily oxidized by ROS, so displacing these fatty acids with endogenously synthesized saturated fatty acids could protect the cancer cells from membrane peroxidation. A recent study by Liu et al. supports the role of FAS in protecting cancer cells from chemotherapy. They found that FAS inhibition sensitized MCF7 cells to chemotherapy but increasing FAS expression via transfection made the cells resistant (123).

Another possibility is that FAS is not over-expressed to produce more endogenous fatty acids, but instead to remove other metabolites in the cell that become cytotoxic at high levels. When FAS is inhibited, malonyl-CoA levels have been shown to increase rapidly (97, 124). A group led by Dr. Francis Kuhajda showed that inhibition of ACC, the enzyme that synthesizes malonyl-CoA, by 5-(tetradecyloxy)-2-furoic acid (TOFA) prevents this rise in malonyl-CoA and rescues MCF7 cells from FAS inhibition with C75 (97). In addition, TOFA was shown to decrease endogenous fatty acid synthesis and phospholipid biosynthesis in a similar manner to C75 but without inducing cell death by apoptosis. This suggests that increased malonyl-CoA, and not a decrease in endogenous fatty acid synthesis, is responsible for C75 cytotoxicity. Later, the same group supported these findings by showing that inhibition of malonyl-CoA decarboxylase (MCD), an enzyme that regulates malonyl-CoA levels, is cytotoxic to breast cancer cells but not to normal human fibroblasts (125). It is interesting to note that ACC activity is inhibited by fatty acids, so it is possible that palmitate and oleate rescue cancer cells from
FAS inhibition by preventing a rise in malonyl-CoA, and not by fulfilling the requirement for fatty acids (126).

It is not yet understood how malonyl-CoA has a cytotoxic effect in cancer cells. It was previously thought that malonyl-CoA initiates apoptosis via activation of CPT-1, which in turn stimulates autophagia and β-oxidation. CPT-1 inhibition, however, failed to rescue cancer cells from FAS inhibition (97, 127).

It is possible that another metabolite of fatty acid synthesis may be mediating the cytotoxic effects of FAS inhibition. As mentioned previously, aerobic glycolysis is another common feature of cancer cells. FAS and glycolysis seem to be connected in cancer cells as evinced by coordinated regulation of their gene expression (66, 128). Citrate and ATP, substrates of ACL, both feedback to inhibit phosphofructokinase-1 and decrease glycolysis (129, 130). Inhibition of glycolysis has been shown to initiate cell death in many types of human cancers, so it is possible that endogenous fatty acid synthesis serves to remove these metabolites so that aerobic glycolysis can continue (131). Clearly, more research is needed to understand why FAS is over-expressed in cancer.
1.3 Objectives and Hypothesis

The overall objective of my work was to better understand why FAS is over-expressed in cancer cells. Based on the observation that cancer cells maintain high FAS expression in the presence of exogenously supplied fatty acids and that FAS inhibition is cytotoxic in cancer cells, I hypothesized that cancer cells have a unique requirement for endogenously synthesized fatty acids. In my first study in Chapter 2, I set out to characterize the utilization endogenously synthesized fatty acids in breast cancer and non cancer cells. I found no unique role for endogenously synthesized fatty acids and showed that these fatty acids are excreted from cancer cells in measurable quantities, suggesting that FAS over-expression in cancer cannot be explained by a specific requirement for endogenously synthesized fatty acids. Based on these results, I hypothesized that the essential function of FAS over-expression in cancer cells may, instead, be to remove a cytotoxic metabolite. Because several substrates of FAS are involved in the regulation of glycolysis, in Chapter 3, I examined whether de novo lipogenesis is necessary to sustain high glycolytic activity in human breast cancer cells.
CHAPTER 2: The role of endogenously synthesized fatty acids in human breast cancer cells
2.1 Abstract

Fatty acid synthase (FAS) is over-expressed in many human cancers, even in the presence of pre-formed fatty acids supplied by the medium. Inhibition of FAS initiates apoptosis in cancer cells and decreases tumorigenesis in vivo, suggesting that FAS plays an essential role to cancer growth and survival. Based on this, we hypothesized that cancer cells utilize endogenously synthesized fatty acids differently from those supplied exogenously, and so have a specific requirement for endogenously synthesized fatty acids. To test this, two human breast cancer cell lines, MCF7 and MDA-MB-231, and non-transformed MCF10A human breast epithelial cells were treated with C14 labeled acetate or palmitate. Total lipids were extracted from the cells and culture medium and radioisotope incorporation into total lipids, cellular lipid classes, phospholipid classes and cholesterol was measured using standard chromatographic techniques and liquid scintillation counting (LSC). Radio-labeled fatty acids were identified using high-performance liquid chromatography (HPLC) and LSC. No difference in incorporation of endogenously synthesized and exogenously supplied fatty acids into lipid or phospholipid classes was detected in any of the cell lines. HPLC revealed that endogenously synthesized and exogenously supplied fatty acids are primarily palmitate, palmitoleate and stearate. Analysis of the culture medium revealed that the cancer cells secrete endogenously synthesized fatty acids at 3 fold higher levels than non-cancer cells. Comparison of cancer and non-cancer cells showed that cancer cells esterify proportionately less fatty acid to phospholipid and produce over 2 fold more choline glycerol phospholipid than non-cancer cells. These results suggests that FAS over-expression does not fulfill a requirement for endogenously synthesized fatty acids in cancer cells as these fatty acids are not used for unique functions within the cell. In addition, a portion of endogenously synthesized fatty acids are excreted, suggesting that they are produced at higher levels than needed to support proliferation.
2.2 Introduction

FAS over-expression has been detected in many types of cancer, both in established tumors and pre-malignant lesions (40). This over-expression appears to confer a survival advantage to cancer cells as it is associated with advanced cancer stage, metastasis and poor prognosis, however, it is not yet understood how this occurs (85, 94, 104, 132).

FAS seems to play a different role in cancer cells and non-cancer cells. In most non-cancer cells, FAS is expressed at very low levels since cells derive their lipids from exogenous sources (31). In contrast, endogenous fatty acid synthesis is thought to be the major contributor to the lipid requirements of cancer cells, although the actual uptake of exogenous fatty acids in cancer has not been thoroughly studied (133, 134). FAS inhibition causes cell cycle arrest and initiates apoptosis in cancer cells, but does not affect the growth and survival of non-cancer cells (96, 125, 135). Further, unlike in normal cells, FAS expression in cancer is not subject to control by nutritional signals, such as insulin and exogenous fatty acids (136) (95) (137). It is clear that FAS plays an essential and unique role in cancer cells, but it is not yet known why it is up-regulated.

In the present study, we have investigated how human breast cancer cells and untransformed breast epithelial cells utilize fatty acids synthesized endogenously by FAS compared to those derived from exogenous sources. By making this direct comparison, we sought to identify unique features of endogenous fatty acid utilization to better understand the role of FAS up-regulation in cancer cells. We hypothesized that breast cancer cells would utilize endogenously synthesized fatty acids differently from those derived exogenously, and that cancer and non-cancer cells would differ in their utilization of endogenous fatty acids. We discovered, however, that fatty acids synthesized by FAS are not used for unique functions in cancer cells and are produced in excess of the quantitative requirement of the cells. Fatty acid synthesis may instead play a role in the regulation of other metabolic processes in cancer.
2.3 Materials and Methods

**Cell Culture**

MCF-7 and MDA-MB-231 human breast cancer cells and MCF-10A non-cancerous human breast epithelial cells were obtained from the American Type Culture Collection (ATCC, Manassass, VA, USA). Experiments were conducted in Dulbecco’s Modified Eagle’s Medium (DMEM) F12 supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO, Life Technologies, Grand Island, NY, USA). Cells were maintained at 37°C in a 5% CO₂ atmosphere and only cells that were passaged <20 times were used for experiments.

**Western blot analysis**

Western blots were performed as described in detail by Lu et al (138). Briefly, cells were lysed in RIPA buffer and equal amounts of protein were separated by 10% SDS-PAGE. Anti-FAS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Anti-β-actin (Sigma Aldrich, Oakville, ON) were used for Western analysis. Bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) and a Flurochem digital analyzer (ProteinSimple, Santa Clara, CA, USA).

**Cell treatments and lipid extraction**

All cells were plated in triplicate (1 x 10⁵ cells/well) in 24 well plates and allowed to adhere and grow for 48 h prior to treatment. [2-¹⁴C]-Acetic acid (53 mCi/mmol) and [1-¹⁴C]-palmitic acid (55.4 mCi/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Each radiochemical was first mixed with FBS before being added to the cell culture medium to a final activity of 0.4uCi/ml. Cells were incubated in the ¹⁴C palmitate or acetate treatment media for 4 or 6 h respectively. After incubation, medium was harvested and stored at -20°C prior to further analysis. The cells were washed in medium plus FBS, then harvested by trypsinization and counted using a hemocytometer. All results are reported per 10⁵ cells.
Lipids were extracted from cells and culture media using a method adapted from Bligh and Dyer (139). Briefly, following preliminary extraction with chloroform : methanol (1:2 v/v), additional chloroform and 1M NaCl were added to yield chloroform : methanol : NaCl (2:2:1 v/v). Samples were vortexed for 30 sec then centrifuged for 10 min to separate phases. The aqueous phase was re-extracted with chloroform to maximize lipid recovery. Extracts were stored under nitrogen at -80 °C for further analysis.

**Thin layer chromatography**

Total lipids were separated into neutral lipid and phospholipid classes via TLC. All TLC plates were washed with chloroform : methanol (2:1) prior to use and activated by heating at 100°C for 1 h. Neutral lipids were separated on TLC G plates (EMD Chemical, Gibbstown, NJ, USA) in heptane : diethylether : acetic acid (60:40:2), and phospholipids on TLC H plates (Analtech, Newark, DE, USA) in chloroform : methanol : 2-propanol : 0.25% KCl : triethylamine (30:9:25:6:18). Neutral lipid and phospholipid classes were identified by comparison to authentic standards visualized under UV light after spraying with 0.1% 8-anilino-1-naphthalene sulfonic acid.

**Preparation of Fatty Acid Methyl-Esters and High Performance Liquid Chromatography (HPLC)**

Solvents were removed from lipid extracts under nitrogen and samples were reconstituted in 2ml of hexane and 2ml of 14% boron trifluoride in methanol. Samples were incubated for 1 h at 100°C to convert fatty acids to their methylesters (FAMEs). Two ml of water were then added and samples were centrifuged to separate the phases. The top layer containing the FAMEs was collected and samples was dried down under nitrogen and reconstituted in acetonitrile. FAMEs were separated by HPLC (Waters 2690, Boston, MA, USA) under conditions described in detail by Chen *et al* (140).
Liquid scintillation counting

Radioactivity in neutral lipids, phospholipids, cholesterol and HPLC fractions was quantified using a Packard TRI-CARB2900TR liquid scintillation counter (Packard, Meriden, CT, USA) with a detector efficiency of 46.4%. Radioactivity was expressed as nCi/10^5 cells.

Data analysis

All statistics were performed using SPSS version 18 (IBM, Armonk, NY, USA). The three cell lines were compared using a 1 way ANOVA with a Bonferroni post-hoc correction. Utilization of endogenous and exogenous fatty acids was compared using Student’s t-test. Data are expressed as means ± SEM of 3-5 separate experiments conducted in triplicate with significance set at p<0.05.

2.4 Results

Esterification of exogenous and endogenous fatty acids into cellular lipids

We first measured the total incorporation of exogenous and endogenous fatty acids into the lipids of our three cell lines; MCF10A, non-cancerous breast epithelial cells, and MCF7 and MDA-MB-231 breast cancer cells. Incorporation of endogenously synthesized fatty acids into cellular lipids was assessed by treating the cells with [1-14C] acetate. Acetyl-CoA is used as a substrate by FAS in the synthesis of endogenous fatty acids, so measuring the incorporation of 14C-acetate into the cell lipids allows the utilization of endogenous fatty acids to be assessed. Incorporation of exogenous fatty acids was measured by treating cells with [1-C14]-palmitic acid, extracting the lipids and measuring the radioactivity using LSC. Palmitate is the end product of FAS and is present in FBS that is commonly added to culture media, so incorporation of the 14C-palmitate into the cell lipids reflects the usage of exogenous fatty acids. Preliminary experiments showed that incorporation was linear and detectable in all three cell
lines at 4 hours for $^{14}$C palmitate and 6 hours for $^{14}$C acetate, so these treatments times were used for all the experiments.

Figure 8a shows that the cancer cells incorporated 2-3 fold more radioactive acetate into their total lipids than the non-cancer cells, reflecting a higher rate of endogenous fatty acid synthesis. This was expected based on the higher expression of FAS in the breast cancer cell lines compared to the untransformed cells reported by others and confirmed by us via Western analysis (Fig. 8c) (94). All three cell lines incorporated similar levels of palmitate into total lipids (Fig. 8b).

Incorporation of radioactivity from $^{14}$C acetate and palmitate into neutral lipid and phospholipid classes

To compare the utilization of endogenous and exogenous fatty acids in the three cell lines, total lipids were fractionated by TLC into the major lipid classes: PL, DAG, FFA, TAG and CE. No differences in the incorporation of endogenous and exogenous fatty acids into the neutral lipid classes was detected in any of the three cell lines (Fig. 9). Comparison between the cell lines revealed that the MCF-10A cells incorporated proportionately more exogenous palmitate into phospholipids than the two cancer cell lines. A similar pattern of incorporation was seen with endogenously synthesized fatty acids (Fig. 9).

Phospholipids were then separated by TLC into their constituent phospholipid classes: SM, PC, PS, PI and PE. Cancer cells incorporated significantly more exogenous than endogenous fatty acid into PC and the non-cancer cells incorporated significantly more exogenous than endogenous fatty acid into S (Fig. 10). Furthermore, the cancer cells esterified approximately 3-fold more endogenous and exogenous fatty acids to PC than the non-cancer cells. The cancer cells also produced proportionately less SM and PS from the $^{14}$C-acetate and $^{14}$C-palmitate and less PE from $^{14}$C-palmitate than the non-cancer cells.
**Fatty acids from exogenous and endogenous sources are incorporated into the total cell lipids predominately as palmitate and stearate**

Radiolabeled fatty acids in the total cell lipid extracts were identified using HPLC and LSC. Radioactivity from both $^{14}$C-acetate and $^{14}$C-palmitate was mostly in palmitate (16:0) and stearate (18:0) in all three cell lines (Fig. 11).

**Excretion of endogenously synthesized fatty acids**

In order to determine whether all endogenously synthesized fatty acids are retained by the cells, total lipids were extracted from the $^{14}$C-acetate treatment medium and the radioactivity in the total lipids and neutral lipid classes was measured. We detected radioactivity in the extracts from all 3 cell lines, but the cancer cells had over 2-fold higher levels than the non-cancer cells, reflecting higher levels of fatty acid excretion (p<0.05, Fig. 12a). In the cancer cells, this represents approximately 10% of the $^{14}$C-acetate incorporated into total cell lipids (Fig. 8A). Figure 12b shows the radioactivity in the total lipid extract was primarily in free fatty acids. HPLC analysis revealed that the free fatty acids were primarily palmitate and palmitoleate (Fig. 12c).
Cancer cells incorporate more $^{14}$C acetate into total cell lipids than non-cancer cells. (A,B) Cells were treated with $^{14}$C labeled acetate or palmitate for 6 or 4 hours respectively. After incubation, total lipids were harvested and radioactivity was measured. Treated cells were plated in tandem with untreated cells which were counted for normalization. Different letters denote bars that are significantly different $p<0.05$. (C) Western blot of FAS expression in the three cell lines. Bars represent the mean of 3-5 independent experiments, completed in triplicate.
Figure 9: Incorporation of $^{14}$C from acetate and palmitate into neutral lipid classes

No significant difference was detected in the relative esterification of endogenous and exogenous fatty acids into the main neutral lipid classes. Comparison between the three cell lines showed that the MCF10As incorporated a significantly greater proportion of exogenous fatty acids into the PL class than the two cancer cell lines ($p<0.05$). Cells were treated with $^{14}$C acetate or $^{14}$C palmitate to assess the usage of endogenous and exogenous fatty acids respectively. Total lipids were extracted, lipid classes were separated using TLC and radioactivity was quantified using LSC. Phospholipid (PL), diacylglyceride (DAG), free fatty acid (FFA), Triacylglyceride (TAG) and cholesterylester (CE). Bars represent the mean of 3-5 independent experiments, completed in triplicate.
Figure 10: Incorporation of $^{14}$C from acetate and palmitate into phospholipid classes

Cancer cells esterify a significantly greater proportion of exogenous fatty acids to PC than endogenous fatty acids (p<0.05 vs. endogenous FAs, denoted by *). Comparison between the three cell lines showed that the cancer cells incorporate significantly more of both endogenous and exogenous fatty acids into PC than non-cancer cells (p<0.05). Cells were incubated with $^{14}$C acetate or $^{14}$C palmitate, and total lipids were extracted and analyzed chromatographically. Sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE).
The main products of endogenous and exogenous fatty acids in both cancer and non-cancer cells are palmitate and stearate. Incorporation of radio-labeled treatments into the fatty acids was measured via HPLC and LSC.
Cancer cells excrete endogenously synthesized fatty acids in greater quantities than non-cancer cells. Total lipids were extracted from the $^{14}$C acetate treatment medium and radioactivity was measured in the total lipids (A) and neutral lipid classes (B). Radioactive fatty acids were identified using high performance liquid chromatography (C). Different letters denote bars that are significantly different p<0.05.

**Figure 12:** Excretion of endogenous fatty acids
2.5: Discussion

Here we compared the utilization of endogenously synthesized and exogenously supplied fatty acids in cancer and non-cancer cells from the human breast. It has been reported in several cancer types that most endogenously synthesized fatty acids are incorporated into phospholipids, and that incorporation diminishes when FAS is inhibited (99, 121, 122, 128). This is taken as evidence that a major reason for FAS over-expression in cancers is to synthesize fatty acids for incorporation into phospholipid membranes to support cell proliferation. Our work shows, however, that exogenously supplied fatty acids are also predominately incorporated into phospholipids in breast cancer cells, so phospholipid synthesis does not depend only on endogenously synthesized fatty acids. Furthermore, we show that non-cancer cells also incorporate both endogenously synthesized and exogenously supplied fatty acids into phospholipids, and indeed, incorporate greater percentages of both types of fatty acid into phospholipids than cancer cells.

Since we found that most endogenously synthesized fatty acids are esterified to phospholipids, we examined the distribution of fatty acids into the main phospholipid classes. We found that the two cancer cell lines incorporated a significantly higher proportion of exogenously supplied fatty acids into PC than endogenously synthesized fatty acids. Since PC is the major component of cell membranes, our results support the notion that endogenously synthesized fatty acids are not specifically directed towards membrane synthesis in cancer cells (26).

Our results show that cancer cells esterify proportionately more of both endogenous and exogenous fatty acids to PC than non-cancer cells, confirming previous studies (141). We also found that cancer cells esterify proportionately less fatty acid to other phospholipid classes than non-cancer cells. These results are of interest because some of these phospholipid classes are known to be involved in pathways that regulate cell growth and survival. PI is produced at proportionately lower levels in the cancer cells than in the non-cancer cells and is
involved in a wide variety of cell signaling pathways, notably the PI3K/Akt pathway which is mutated in >70% of breast cancers and is implicated in cell cycle progression, cell survival, and chemotherapeutic resistance (142). Indeed, increased growth factor signaling and activation of SREBP1 via this pathway has been suggested as a potential explanation for FAS overexpression in cancer (143). SM is part of the ceramide pathways and is also produced at proportionately lower levels in cancer than non-cancer cells. Recently, γ-radiation and chemotherapeutic drugs have been shown to act through this pathway to produce cytotoxicity in cancer (144). Thus, our findings suggest that changes in the phospholipid composition of membranes could play a role in growth, proliferation and chemoresistance in cancer cells.

Fatty acids can be modified by enzymes such as elongases and desaturases to produce different fatty acids with diverse functions (145, 146). Therefore, we used HPLC analysis to determine whether unique species of fatty acids are produced within cancer cells. However, both endogenously synthesized and exogenously supplied fatty acids were identified as palmitate or stearate in both breast cancer and untransformed cells. Taken together with our other findings, these results suggest that endogenously synthesized fatty acids do not fulfill a unique function in cancer cells since they are not used to produce different fatty acids, they are esterified to the same lipid and phospholipid classes in the same proportions as those derived exogenously, and their distribution within neutral lipids in cancer cells is not different from untransformed breast epithelial cells.

Analysis of the medium following culture of the cells in the presence of 14C acetate showed that the breast cancer cell lines excrete endogenously synthesized fatty acids, and do so at significantly higher levels than the non-cancer cells. The excreted fatty acids account for approximately 10% of the 14C-acetate incorporated into the total cell lipids. While it is possible that a small proportion of these fatty acids result from cell death, this cannot explain all our observations. TLC of the lipids extracted from the culture medium showed that the fatty acids were predominately in their free form. However, only about 10% of the endogenous fatty acids
extracted from the cells were free fatty acids while phospholipids accounted for 50-60%. Thus, if the fatty acids in the culture medium resulted from cell death, they would be present mostly as phospholipids, and not free fatty acids. These results suggest that the cancer cell lines have up-regulated activity or expression of fatty acid transport proteins. Furthermore, our results suggest that FAS over-expression does not fulfill a quantitative requirement for fatty acids in cancer cells since these fatty acids are not entirely retained by the cells.

Attempts to rescue cancer cells from FAS inhibitor-induced cell death with exogenous fatty acids have yielded mixed results, with incomplete rescue being achieved only at supra-physiological concentrations of fatty acids. In one study, the authors noted that complete rescue could not be achieved because the required concentrations of palmitate were high enough to inhibit growth, independent of the FAS inhibitor (37). They suggested that another mechanism may be involved in cell death induced by inhibiting FAS that does not rely on limiting the supply of endogenously synthesized fatty acids. The results of the present study showing that both cancer cells and non-transformed cells excrete endogenously synthesized fatty acids supports the notion that tumor cells up-regulate FAS for reasons other than membrane biosynthesis.

On the basis of observations that FAS inhibitors induced apoptosis whereas ACC inhibitors did not, Pizer et al suggested that accumulation of toxic levels of malonyl-CoA, not depletion of fatty acids, was likely the cause of cancer cell death (124). Most recently, Zhou et al have shown that inhibition of malonyl-CoA decarboxylase (MCD) by either RNAi interference or pharmacological approaches, leads to increased malonyl-Co-A levels in cancer cells and potentiates cell death induced by pharmacological FAS inhibition (125). Malonyl-CoA acts as a feedback regulator of several metabolic processes including β-oxidation of fatty acids via CPT-1 and glycolysis via phosphofructokinase-1 (27). This suggests that FAS may be involved in the regulation of other metabolic processes via removal of inhibitory molecules. Our results support the notion that the role of FAS over-expression in cancer is the removal of a cytotoxic
metabolite, such as malonyl-CoA, rather than fulfilling a requirement for endogenous fatty acid synthesis.

Swinnen et al have shown that glycolytic and lipogenic enzymes are coordinately regulated in cancer, both by PI3K/PkB and SREBP1 and by AMPK (128, 147-149). In contrast to the findings of Zhou and Pizer, Swinnen's group showed that ACC inhibition decreases proliferation and induces cell death in prostate cancer cells, at odds with the notion the connection between FAS activity and other metabolic processes is malonyl-CoA (150). However, these groups used different ACC inhibitors in their studies, and thus the differences in their findings may be due to off target effects.

In summary, we have found that breast cancer cells do not have a specific qualitative or quantitative requirement for endogenously synthesized fatty acids. They are used in the same ways as those derived exogenously by cancer cells, and their distribution among the cellular lipid classes does not differ between cancer and non-cancer cells. Further, endogenously synthesized fatty acids are excreted from cancer cells, indicating that they are produced in excess of the quantitative requirements of the cells. Our results suggest that FAS plays another role in cancer cells in addition to the provision of fatty acids for membrane biosynthesis, possibly the regulation of other metabolic processes such as glycolysis.
CHAPTER 3: Increased lipogenic activity is not required to support increased glycolytic activity in breast cancer
3.1 Abstract

Fatty acid synthase (FAS) is over-expressed in many human cancers and its activity seems to be required for cancer cell survival. Recently, it has been suggested that the essential function of FAS in cancer cells is not the synthesis of fatty acids, but rather the removal of a cytotoxic or inhibitory molecule, and that FAS may therefore be involved in the regulation of other metabolic pathways in cancer. In this study, we tested the hypothesis that the high lipogenic activity of cancer cells supports their increased glycolytic rates. MCF7 and MDA-MB-231 breast cancer cells and MCF10A non-transformed breast epithelial cells were cultured under anoxic conditions to stimulate glycolysis. Despite a significant increase in glycolytic activity in all three cell lines under anoxic compared to normoxic conditions, no concomitant increase in lipogenic activity was detected. Increased de novo lipogenesis is therefore not required to sustain an increase in glycolysis under anoxia.

3.2 Introduction

Experiments from our lab and others, suggest that FAS over-expression in cancer serves a purpose other than the synthesis of endogenous fatty acids. Cancer cells are only partially rescued from FAS inhibitor cytotoxicity by the addition of physiologic concentrations of exogenous fatty acids (37, 94, 115, 116). We have shown that cancer cells utilize endogenously synthesized fatty acids in the same ways as those derived exogenously. Furthermore, we have shown that cancer cells excrete a portion of their endogenously synthesized fatty acids, suggesting that these fatty acids are produced in excess of requirements.

Here, we tested the hypothesis that high lipogenic activity in cancer is associated with the maintenance of glycolysis. Aerobic glycolysis is a common feature of most types of cancer and its inhibition causes cancer cell death (131). In normal cells, glycolysis is inhibited by its end products, including ATP, citrate and malonyl-CoA (129, 130). FAS and other lipogenic
enzymes utilize these molecules in the production of palmitate or cholesterol which could prevent negative feedback inhibition of glycolysis. This notion is supported by work by a group led by Francis Kuhajda showing that levels of malonyl-CoA increase rapidly following FAS inhibition, and that cancer cells may be rescued from FAS inhibitor cytotoxicity by inhibiting the production of malonyl-CoA (124, 125, 151). FAS also oxidizes NADPH, which can alter the redox status of the cell, reducing the NADH/NAD+ and NADPH/NADP+ ratios. Several groups have suggested that this may further stimulate glycolytic activity, though this has yet to be tested in cancer (152-154). Finally, Swinnen et al showed that lipogenic and glycolytic gene expression seem to be coordinately regulated by androgens in prostate cancer, which suggests that their functions may be interconnected (147).

To test our hypothesis, we compared the glycolytic and lipogenic activities of MCF7 and MDA-MB-231 breast cancer cells and MCF-10A non-cancer cells cultured under anoxic and normoxic culture conditions. Previous experiments have shown that anoxia increases glycolytic activity in breast cancer cells, so this design allowed us to determine whether de novo lipogenesis increases concomitantly with glycolytic activity (155). Surprisingly, our results after either 3 h or 12 h of anoxia showed that lipogenic activity decreased in anoxic conditions though glycolytic activity increased, indicating that increased lipogenic activity is not needed to support high glycolytic activity in cancer cells.

3.3 Materials and Methods

Cell culture and treatments

MCF10A, MDA-MB-231 and MCF-7 cells were cultured and maintained as described in detail in section 2.2 of this thesis. For all experiments and treatments, cells were plated in triplicate (1 x 10^6 cells/well) in 24 well plates and allowed attach for 48 h before treatment. [5-^3H] Glucose (20Ci/mmol) for measuring glycolysis was purchased from American Radiolabeled
Chemicals (BC, Canada). [2-^{14}C]-Acetic acid (53 mCi/mmol) for measuring FAS activity was purchased from Moravek Biochemicals Inc. (Brea, CA, USA).

Cells were treated with $^{3}$H-glucose or $^{14}$C-acetate diluted to a final activity of 1 uCi/well or 0.5 uCi/well respectively in glucose-free RPMI 1640 medium supplemented with 25 mM D-glucose. The cells were then incubated at 37°C for 3 or 12 h under either normoxic or anoxic conditions. Normoxic conditions were defined as the normal culture conditions for the cells, 5% CO$_2$. Anoxic conditions were obtained by placing the 24-well culture plates in resealable bags and pumping in nitrogen under positive pressure. Anoxia was confirmed by a Dry Anaerobic Indicator Strip (BD Biosciences, ON, Canada) that changes colour at oxygen concentrations of <0.05%. Unlabelled cells were plated and harvested in tandem with the treated cells so results could be normalized to viable cell number. The number and viability of these cells was assessed via trypan blue exclusion with a hemocytometer.

**Glycolytic activity**

The method for measuring glycolysis was adapted from a protocol kindly provided by Cho et al (156). After the 3 or 12 h incubation under normoxic or anoxic conditions, triplicate 50 μl media samples from each $^{3}$H-glucose treated well were harvested into 200 μl microfuge tubes and mixed with an equal volume of 0.2 N HCl. The microfuge tubes were then transferred to scintillation vials containing 500 μl of doubled distilled water, which were then capped and sealed with parafilm. The vials were incubated at room temperature for 48 hours, a length of time shown by Cho et al to produce equilibrium between the $^{3}$H$_2$O generated from the $^{3}$H-glucose treatment at the enolase step in glycolysis and the H$_2$O in the scintillation vial. The diffused $^{3}$H in the 500 μl of water and the undiffused $^{3}$H in the microfuge tube, representing unmetabolised $^{3}$H-glucose, were measured via liquid scintillation counting.

Glycolytic activity was calculated as a ratio of the diffused to undiffused counts (sample$^{\text{diff}}$), normalized for the diffusion rate of $^{3}$H$_2$O (frac$^{\text{diff}}$), the background diffusion of $^{3}$H-glucose (frac$^{\text{bkgd}}$) and the amount of unlabelled glucose in the culture medium (156). The frac$^{\text{diff}}$
is the ratio of diffused to undiffused counts of a cell free sample of $^3\text{H}_2\text{O}$ while the $\text{frac}^{\text{bkgd}}$ is the ratio of diffused to undiffused counts from a cell free sample of $^3\text{H}$-glucose treatment medium. The equation for this calculation is:

$$\frac{\text{sample}^{\text{diff}} - \text{frac}^{\text{diff}}}{\text{frac}^{\text{diff}}} \times \text{nmol glucose/well} = \text{glycolytic activity}$$

**De novo lipogenesis**

Total lipids were extracted from the $^{14}\text{C}$-acetate treated cells as described in section 2.2 of this thesis and radioactivity from $^{14}\text{C}$-acetate was measured via LSC. The total lipids contained neutral lipids (PL, DAG, FFA, TAG, CE) as well as cholesterol. Previous work in our lab showed that over 80% of endogenously synthesized fatty acids are neutral lipid, with less than 20% of $^{14}\text{C}$-acetate used to synthesize cholesterol.

**Data Analysis**

All statistics were performed using SPSS version 18 (IBM, Armonk, NY, USA). Glycolytic and FAS activity under normal or anoxic conditions was compared using Student’s t-test. Data are expressed as means ± SEM of 3 separate experiments with significance set at $p<0.05$.

**3.4 Results**

*Anoxia increases glycolytic but not lipogenic activity*

Incubation under anoxic conditions for 3 h increased the glycolytic activities of MCF10A and MCF7 cells 2-3 fold compared to normoxia, though the glycolytic activity of the MDA-MB-231 breast cancer cells did not change significantly (Fig 15A). FAS activity, however, was
significantly lower under anoxic than normoxic conditions in MCF10A and MDA-MB-231 cells but did not change significantly in the MCF7 cells (Fig 15B). We reasoned that 3 h may not have been sufficient time for FAS activity to change in response to anoxia, so we repeated the experiment, incubating the cells under anoxia for 12 h. Glycolysis increased significantly in all three cell lines compared to normoxia (Fig 16A), however, there was no concomitant increase in FAS activity under anoxia.
Figure 13: Glycolysis and *de novo* lipogenesis at 3 hours

A) Glycolytic activity increases under anoxia compared to normal conditions in MCF10As and MCF7s (p<0.05) but not significantly in MDA-MB-231s (p=0.07). B) *De novo* lipogenesis decreases under hypoxia compared to normal conditions in MCF10A and MDA-MB-231s (p<0.05) but not significantly in MCF7s (p=0.1).
Figure 14: Glycolytic and lipogenic activities at 12 hours

A) Glycolytic activity increases in all three cell lines during 12 hours of culture in anoxia compared to normal conditions (p<0.05). B) Lipogenic activity does not change significantly after 12 hours culture under anoxia compared to normal conditions.
3.5 Discussion

Increased glycolytic activity is a major metabolic hallmark of cancer. In normal cells, glycolysis is up-regulated most commonly in hypoxic conditions, where decreased oxygen concentrations limit the availability of O₂ as the final electron acceptor in the electron transport chain and prevent the complete oxidation of glucose (157). As a result, glycolysis is the main provider of ATP in hypoxia, and glycolytic activity is therefore essential to cell survival under these conditions. For glycolysis to continue, its end-products must be rapidly removed to avoid feedback inhibition. Thus, lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate, which is known to be formed as a part of the cellular response to hypoxia. For example, in hypoxic areas of the normal prostate gland, approximately 75% of glucose is converted to lactate (152). The remaining glucose is metabolized to citrate and acetyl-CoA which can either be excreted or used as substrates by ACC, FAS or HMG-CoA synthase (152). Citrate, malonyl-CoA and ATP are all known to inhibit glycolysis, so their metabolism to palmitate via FAS and other lipogenic enzymes may help to sustain a high glycolytic rate. Glycolysis also reduces NAD⁺ to NADH, which in turn can reduce NADP⁺ to NADPH. Studies in the ischemic heart have shown that an increased NADH/NAD⁺ ratio has inhibitory effects on glycolysis (154). De novo lipogenesis may therefore play a role in maintaining a redox state that is supports glycolysis by oxidizing NADPH to NADP⁺ which in turn depletes stores of NADH.

Because de novo lipogenesis may be involved in the maintenance of high rates of glycolysis during hypoxia in normal cells, we hypothesized that it would play a similar role in cancer, where glycolysis occurs at high rates in aerobic conditions. In support of this hypothesis, Zhou et al showed that malonyl-CoA levels increase when FAS is inhibited and seem to mediate cytotoxicity induced by FAS inhibition (125). Furthermore, Swinnen et al previously demonstrated coordinate regulation of FAS and glycolytic genes in prostate cancer.
In our study, though glycolytic rates were increased by 2-3 fold in breast cancer and non-cancer cells by hypoxia, there was no concomitant increase in *de novo* lipogenesis. Instead, lipogenesis was either decreased or unaltered in all three cell lines after 3 or 12 h of culture in anoxia.

Recently, Jung *et al* measured FAS protein and gene expression in HepG2 liver carcinoma cells under normoxic and anoxic conditions (158). They showed a marked decrease in both FAS mRNA and protein expression under hypoxic conditions, supporting our findings. In contrast, Furuta *et al* showed that FAS mRNA and protein expression were increased by hypoxia in breast cancer cells *in vitro* and *in vivo* (159). Jung *et al* showed that high concentrations of glucose could restore FAS expression in hypoxia, so it is possible that differences in glucose availability between these two studies could explain their opposite results. In our study, however, glucose utilization calculated from the measured glycolytic activities of the cells showed that less than 10% of the available glucose (25 mM) would have been metabolized during the treatment period. Thus, glucose availability was not a limiting factor in our experiments.

Neither of the two previous studies examined lipogenic or glycolytic activity, but rather measured gene and protein expression. FAS is known to be regulated post-translationally by ubiquitination and degradation, so it is possible that the increases of FAS mRNA and protein expression observed by Furuta *et al* did not translate into increases in FAS activity (40). Further, FAS gene expression is regulated by Akt and mTORC, which also regulate glycolytic genes, so the observed increase in FAS expression could be a byproduct of the increase in glycolytic gene expression induced by hypoxia (160).

FAS may still play a role in the maintenance of aerobic glycolysis in cancer cells. A limitation of our study is that we increased glycolytic activity by culturing the cells in anoxic conditions. It is possible that anoxia down-regulates lipogenic activity via a mechanism that is independent of glycolysis, or that different mechanisms are involved in sustaining glycolysis.
under aerobic conditions than in anoxia. However, our study clearly shows that an increase in lipogenic activity is not needed to support an increase in glycolytic activity in cancer. Further studies are required to understand why FAS is over-expressed in cancer cells.
CHAPTER 4: General Discussion
4.1: Overview and Conclusions

The objective of this thesis was to explore the role of endogenously synthesized fatty acids in breast cancer cells. FAS is over-expressed in many types of human cancers, even in the presence of high concentrations of fatty acids supplied exogenously. Its inhibition causes cell cycle arrest and initiates apoptosis, which indicates that FAS over-expression plays an essential role in cancer cells. Our lab and others, however, have shown that these cells cannot be completely rescued from the cytotoxic effects of FAS inhibitors by the addition of exogenous fatty acids (37, 115, 116). This suggests that the function of FAS in cancer is more complex than fulfilling a quantitative requirement for fatty acids. Fatty acid synthesis is energetically expensive, so its over-expression must provide a survival advantage to cancer cells, otherwise this characteristic would be selected against in the evolution of a tumor, rather than being a common feature of the cancer phenotype. We reasoned that cancer cells may instead have a qualitative requirement for endogenously synthesized fatty acids - that they use these fatty acids for specific functions that are not fulfilled by exogenous fatty acid. This would explain why cancer cells invest energy and reducing equivalents into fatty acid synthesis when exogenous fatty acids are available in excess.

We explored this idea in our first study (Chapter 2) by comparing the utilization of endogenously synthesized and exogenously supplied fatty acids in two breast cancer cell lines and one non-cancer breast epithelial cell line. We showed that:

- Endogenously synthesized fatty acids are not directed into different neutral lipid or phospholipid classes from those supplied exogenously in cancer or non-cancer cells
- Endogenously synthesized fatty acids are used to produce the same fatty acids (palmitate and stearate) as those derived exogenously in cancer or non-cancer cells
- Cancer cells have a different composition of phospholipids compared to non-cancer cells, but this does not seem to be mediated by endogenously synthesized fatty acids
- Cancer cells excrete endogenously synthesized fatty acids
Based on these results, we concluded that FAS over-expression in cancer cells fulfills neither a qualitative nor a quantitative requirement for endogenously synthesized fatty acids, because these fatty acids are not used for specific functions or even retained in the cells. As the end product of FAS does not play an essential role in cancer, yet FAS activity is known to be required for cancer cell survival, this suggests that FAS may instead play a role in removing toxic or growth inhibitory molecules in cancer cells. In examining the substrates of FAS and its associated enzymes, ACC and ACL, we found that several of them are involved in the negative feedback regulation of glycolysis. Cancer cells are known to exhibit high levels of glycolytic activity even under aerobic conditions, so in our second study, we set out to determine whether de novo lipogenesis was involved in the maintenance of a high level of glycolytic activity. To do this, we increased the glycolytic rates of our cells by culturing them under anoxic conditions and measured de novo lipogenesis. We found that:

- \textit{De novo} lipogenesis does not increase concomitantly with glycolytic activity in hypoxia
- There is a trend towards decreased \textit{de novo} lipogenesis in cancer cells cultured in anoxia

We concluded that \textit{de novo} lipogenesis is not required to sustain a high glycolytic rate in cancer cells, at least under anoxic conditions. While FAS may be involved in removing toxic or inhibitory molecules from cancer cells, this does not appear to be related to glycolysis.
4.2: Limitations and Future Directions

An obvious limitation of our work is that it was conducted in a cell culture model and thus it is not known whether our results are relevant in vivo. Commercial cancer cell lines are propagated in culture for decades, and though genotypic and morphological comparisons can be drawn with cancer cells in vivo, differences may still exist. Further, the environment of cancer cells in culture is very different than that in vivo in terms of oxygen and nutrient availability, growth factor and cell death signaling and immune system interaction, all of which could impact our results. As an exploratory study of cancer cell biology, our use of cell culture is justified and supported by many studies in the literature using the same models, however, the weight of our conclusions should not be overstated. Animal or human work comparing the utilization of endogenously synthesized and exogenously supplied fatty acids in tumors using stable isotopes would be a useful next step to understand the role of FAS in cancer cells.

A limitation of our first study is that we did not compare the quantitative utilization of endogenously synthesized to exogenously supplied fatty acids in our cell lines to determine what proportion of cell lipids are derived endogenously vs. exogenously. Comparing the contributions of endogenously synthesized to exogenously supplied fatty acids to cancer cell lipids was not an original objective of our study, however, if we had shown that endogenous fatty acid synthesis is an insignificant contributor to cancer cell lipids, it could have supported our other findings.

It is widely stated in the literature that cancer cells derive most of their cellular lipids from endogenous fatty acid synthesis. This perception seems to be based almost entirely on early reviews of FAS in cancer that cited a paper by Ookhtens et al from 1984 measuring the incorporation of radio-labeled glucose into the lipids of Ehrlich ascites tumors in mice (94, 148, 161); (134). They found that fatty acid synthesis in the liver contributed less than 1% of the radioactivity in the tumor lipids, the adipose contributed 6% and the tumor itself synthesized 93% of the radio-labeled lipids. It was concluded that endogenous fatty acid synthesis provides
the majority of the lipids in cancer cells. This is almost certainly an over-estimation, however, since the mice used in the study were maintained on fat free diets, so the potential contribution of dietary fatty acids to the tumor was not measured. Further, the study used mice fed ad libitum and given a glucose meal (fed-refed) for all experiments. Under these conditions in normal cells, insulin stimulates glucose uptake and suppresses fatty acid uptake and β-oxidation (27). It is possible that these conditions would decrease tumor fatty acid uptake from the circulation as well. Certainly, these conditions would suppress the release of endogenous fatty acids from the adipose tissue to the circulation and would therefore limit their availability to the tumor cells (162). So while endogenous fatty acid synthesis undoubtedly contributes to the total lipids of the cell, its percent contribution is almost certainly lower than 93%. Despite this, the Ookhtens article and the 93% figure have been cited by several influential reviews on FAS in cancer, which may have led to misconceptions in the literature about the role of endogenously synthesized fatty acids in cancer cell growth (40, 161). New research comparing the contributions of endogenously synthesized and exogenously supplied fatty acids to cancer cell lipids is needed to fully understand the role of FAS in cancer.

In our second study, a limitation is that the effect of hypoxia on de novo lipogenesis is not well understood, either in cancer or non-cancer cells. We induced a 3 fold increase in glycolytic rate by culturing our cells in anoxic conditions. These conditions did not stimulate increased de novo lipogenesis in any of our cells lines, which led us to conclude that an increase in de novo lipogenesis is not needed to support an increase in glycolytic rate in anoxic conditions. It is possible, however, that the cellular mechanisms for sustaining an increased glycolytic rate during hypoxia may by different than the mechanism by which a high glycolytic rate is sustained under aerobic conditions in cancer. Future studies that measure de novo lipogenesis when glycolysis is stimulated by another mechanism would clarify this point.
4.2: Significance

Aberrant cellular metabolism is increasingly considered an important part of the cancer phenotype. Our study is important because it supports the notion that FAS over-expression in cancer is more complicated than providing bulk fatty acids for membrane biosynthesis. By showing that the essential function of FAS in breast cancer is likely not the provision of endogenous fatty acids, our work directs future researchers to look upstream of FAS, to its substrates and their functions, to understand this metabolic abnormality. We began this search with our second study, and though we did not find a link between de novo lipogenesis and glycolysis, there are many other possibilities left to explore. When the reason for FAS over-expression and other metabolic abnormalities in cancer becomes better understood, new pathways and processes may be identified as targets for novel cancer therapies. Treatments targeting the rapid proliferative rates of cancer cells have been the mainstay of cancer treatment in the 20th century, but this approach produces many side-effects and ultimately does not cure the disease. It is possible that with more research, new therapies targeting cancer metabolism may provide an alternative or a supplement to traditional therapies, resulting in better outcomes for patients.
CHAPTER 5: References


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