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Fatty acid metabolism and modulation of human breast cancer cell survival

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RÉSUMÉ:

Récemment, l'intérêt pour l'étude du métabolisme des cellules cancéreuses et pour sa participation au développement tumoral s'est accru du fait de l'augmentation des risques de cancers chez les personnes obèses. En effet, plusieurs études épidémiologiques ont indiqué un lien entre obésité et cancers mais les mécanismes moléculaires impliqués sont largement inconnus. Chez les personnes présentant une résistance à l'insuline due à l'obésité, la métabolisation des acides gras libres (AGL) par les adipocytes devient insuffisante, causant une augmentation du taux sanguin en AGL. Peu de données permettent de comprendre comment les tissus et spécialement les tumeurs gèrent ce surplus de nutriments.

Ces dernières années, notre laboratoire a étudié les effets des AG alimentaires sur la prolifération et la mort de cellules issues de tumeurs mammaires humaines en culture. Le but de cette étude était de rechercher la base biochimique de l'action anti-apoptotique de l'oléate, un acide gras mono-insaturé à longue chaîne, sur des cellules provenant de cancer du sein humain. Nous nous sommes particulièrement concentrés sur le rôle de l'accumulation des triglycérides (TG) induite par le traitement à l'oléate sur l'apoptose provoquée par l'absence de sérum et de facteurs de croissance (FC) sur deux lignées cellulaires : les cellules MCF-10A, cellules immortalisées non-transformées épithéliales humaines et les cellules MDA-MB-231, lignée de cellules issues de cancer du sein humain. Les métabolismes des AG, des TG et du glucose, en parallèle avec la survie cellulaire à long terme en absence de sérum et de FC ont été étudiés sur des cellules traitées avec de l'oléate. L'effet de l'oléate a été étendu à plusieurs autres lignées cancéreuses mammaires MDA-MB-468, T-47D, MCF-7.

Nous avons montré qu'un traitement de 3 à 24 h avec l'oléate empêche l'apoptose induite par l'absence de sérum et de FC et favorise la survie cellulaire à long terme suite au traitement de 3 des 4 lignées cellulaires mammaires humaines. La survie de ces lignées cellulaires dans ces conditions a été associée à une

augmentation du stockage des TG. Nos résultats suggèrent que les stocks en TG dans les cellules tumorales telles que les MDA-MB-231 ne sont pas inertes et au contraire subissent un cycle constant et rapide de lipolyse des TG en AGL et re-estérification des AGL en TG (cycle TG/AGL). Ce cycle des TG/AGL qui a augmenté de manière dépendante de la dose d'oléate, demeure élevé durant plusieurs jours (8-10 jours) après le retrait de l'oléate et il est parallèle à la survie cellulaire en absence de sérum. Le métabolisme du glucose reste élevé dans les cellules MDA-MB-231 protégées de l'apoptose due à l'absence de sérum, fournissant une grande quantité de glycérol-3-phosphate nécessaire à l'estérification des AGL.

Ces résultats sont en accord avec l'interprétation que le cycle des TG/AGL joue un rôle dans l'effet anti-apoptotique de l'oléate. Nous proposons 2 mécanismes complémentaires pour expliquer comment un cycle élevé des TG/AGL pourrait favoriser la survie des cellules tumorales. Le premier impliquerait le récepteur membranaire aux acides gras couplé aux protéines G, GPR40, et le second est basé sur notre modèle de travail reliant l'augmentation du cycle des TG/AGL au maintien de rapport NAD^+/NADH intracellulaire nécessaire à la survie des cellules tumorales.

Mots clés : cancer sur sein, survie cellulaire, acides gras, triglycérides, lipolyse, cycle des TG/AGL, GPR40

ABSTRACT:

Recently, there has been a renewed interest in metabolism and its participation in the development of cancers, due to a major increase in obesity. Several epidemiological studies linking obesity with cancer have been published, but the molecular mechanisms involved are largely unknown. In the obesity-induced insulin resistant state, trapping of dietary free fatty acids (FFA) in adipocytes becomes inefficient, causing elevated blood FFA levels in the fed state, when they are not needed for energy production. Little is known how various tissues, especially tumors, deal with this surplus of fuels.

For the last several years, our laboratory has been studying the effects of common nutrient fatty acids on proliferation and death of human breast tumor cells in culture. The aim of the present study was to investigate the biochemical basis for the antiapoptotic action of long chain monounsaturated fatty acid oleate in human breast cancer cells. We focused specifically on the role of TG accumulation induced by treatment with oleate in the protection from apoptosis induced by serum and growth factor (GF) withdrawal in two cell lines: the MCF-10A non-transformed human breast epithelial cell strain and the human breast tumor cell line MDA-MB-231. The metabolism of FFA, TG and glucose, in parallel with long-term cell survival in the absence of serum and additional GF, was investigated in cells treated with exogenous oleate. The results were extended to a panel of human breast cancer cell lines: MDA-MB-468, T-47D, MCF-7.

We have shown that short-term (3-24 h) treatment with oleate prevents apoptosis and promotes long-term cell survival in the absence of serum, GF and exogenous oleate, in three out of four human breast tumor cell lines. The long-term serum-free survival in these cell lines was associated with a high capacity to store TG. Our data suggest that TG stores in tumor cells like MDA-MB-231 are not inert and instead undergo constant rapid turnover. This TG/FFA cycling which was found to be markedly up regulated in a dose dependent manner in response to short-term

oleate treatment remained stably elevated for many days and corresponded with long-term serum-free cell survival (8-10 days). Glucose metabolism remained high in serum starved MDA-MB-231 cells rescued from apoptosis by short-term treatment with oleate, providing glycerol-3-phosphate needed for FFA esterification.

The results are consistent with the interpretation that TG/FFA cycling plays a role in the antiapoptotic effect induced by treatment with oleate. We propose two possible, but not mutually exclusive explanations, as to how the elevated TG/FFA cycling could promote tumor cell survival. One may involve signaling via the cell-surface G protein coupled receptor GPR40 and the second one is based on our working model, which links up regulated TG/FFA cycling with the maintenance of intracellular NAD^+/NADH ratio needed for tumor cell survival.

Key words: breast cancer, cell survival, fatty acids, triacylglycerol, lipolysis, TG/FFA cycle, GPR40

TABLE OF CONTENTS:

LIST OF FIGURES:

CHAPTER I: INTRODUCTION

CHAPTER II: RESULTS

LIST OF TABLES:

CHAPTER III: RESULTS

LIST OF ABBREVIATIONS:

AA	amino acids
ACC:	acetyl CoA carboxylase
ACL:	ATP citrate lyase
ACS:	acyl-CoA synthetase
AGPAT	acylglycerol-phosphate acyltransferase
ALDA:	aldolase
AMPK:	AMP-activated protein kinase
AR	androgen receptor
ATGL	adipose triglyceride lipase
ATM	ataxia telangiectasia-mutated gene
BM:	basement membrane
BMI	body mass index
BRCA1	Breast Cancer 1 (mutation)
BRCA2	Breast Cancer 2 (mutation)
CHEK2	Checkpoint kinase 2
CL	cardiolipin
COX:	cyclooxygenses
CPT1:	carnitine palmitoyl transferase 1
CR	calorie restriction
CTC	circulating tumor cells
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DTC	disseminated tumor cells
4E-BP	eIF-4E binding protein 1
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF-4E	eukaryotic interaction factor 4E
EM	experimental medium

ENO1	enolase 1
ER	estrogen receptor
ERα	estrogen receptor α
FA-CoA	fatty acyl-CoA
FA	fatty acid
FAS	fatty acid synthase
FBS	fetal bovine serum
FFA:	free fatty acids
FH:	fumarate hydratese
FoxA2	Forkhead-box protein subclass A transcription factor
FOXO	Forkhead-box protein subclass O
G3P	glycerol-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GF	growth factor
GPAT	Glycerol-3-phosphate acyl transferase
GPI	glucose-6-phosphate isomerase
GPCR	G-protein-coupled receptor
HIF 1	hypoxia-inducible factor 1
Hif1α	hypoxia inducible factor α
HK	hexokinase
HSL	hormone sensitive lipase
IGF	insulin-like growth factor
IL-6	interleukin-6
LPA	lysophosphatidic acid
LD	lipid droplets
LDHA:	lactate dehydrogenase A
LPL	lipoprotein lipase
MG	monoacylglycerol
MGL	monoglyceride lipase
MRS	magnetic resonance spectroscopy
MS	mass spectroscopy

MUFA	monounsaturated fatty acids
mTOR	mammalian target of rapamacin
nGM	normal cells growth medium
NMR	nuclear magnetic resonance
OA-519	oncogenic antigen-519
OAA	oxaloacetate
PA	phosphatidic acid
PAP-1	phosphatidic acid phosphohydrolase-1
PCA	perchloric acid
PET:	positron emission tomography
PFK	phosphofructokinase
PFK-1	phosphofructokinase-1
PFKM:	phosphofructokinase M
PGK	phosphoglycerate kinase
PGM	phosphoglycerate mutase
PI3K	phosphatidylinositol 3-kinase
PKM	pyruvate kinase M
PL	phospholipid
PPARs	peroxisome proliferators-activated receptors
PPP	pentose phosphate pathway
PTEN	phosphatase and tensinhomolog deleted on chromosome 10
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
RTK	receptor tyrosine kinease
S6K	S6 kinase
SCD	succinate dehydrogense
SERM	selective estrogen receptor modulators
SFA	saturated fatty acids
Sir2	sirtuin 2
STM:	somatic mutation theory
SREBP 1c	sterol regulatory element binding protein 1c

TCA:	tricarboxylic acid
TG:	triacylglycerols
tGM	tumor cells growth medium
TLC	thin layer chromatography
TP53	tumor protein p53
TPI:	triose phosphate isomerase
TOFT:	tissue organization field theory
TORC1	TOR complex 1
TORC 2	TOR complex 2
TSC1/2	tuberous sclerosis 1/2
VDAC	voltage-dependent anion channel
VEGF:	vascular endothelial growth factor
VHL:	von-Hippel-Lindau
WHO	World Health Organisartion

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Moim drogim rodzicom w Polsce, ktorzy zaszczepili we mnie pasje do nauki i moim doroslym juz dzieciom, ktore zawsze byly dla mnie zrodlem inspiracji

To my dear parents in Poland who taught me the passion for science and to my grown-up children, who were always a source of inspiration to me

CHAPTER I

INTRODUCTION

1.1 Foreword

The development of cancer has been perceived as a microevolution during which cells acquire genetic changes and are subsequently selected for growth advantage. Evolution is shaped by environmental pressures as much as by genetic variations. Thus, the growing tumors and metastases are influenced by surrounding tissues and stroma as well as by many soluble factors, like hormones and metabolites. While the contribution of stroma to the development of cancers has been recently explored and the importance of hormones and growth factors is well established, the influence of metabolism on tumorigenesis in comparison has been relatively neglected. Recently, however, due to the major increase in obesity, studies focusing on the contribution of metabolism to the development of cancer have increased in number. Several epidemiological studies linking obesity with cancers have been published but the molecular mechanisms involved are largely unknown.

1.2 Cancer research in the post-genomic era

The development of cancer involves a complex sequence of events that usually occurs over many years. The last five decades of the 20th century were marked by the astonishing development of molecular biology, initiated by the discovery of the structure of DNA [1] and culminated with the sequencing of the human genome [2, 3]. The prevailing paradigm in cancer research during this time was the **somatic mutation theory (SMT)** of cancer. According to SMT, carcinogenesis takes place at the cellular level and the multi-step process of cancer development occurs through the gradual acquisition of genetic alterations in individual epithelial cell. The process of tumor development is thus perceived as analogous to Darwinian evolution, in which a succession of genetic changes and selection for growth advantage leads to clonal expansion of cancer cells [4, 5]. Studies of various mutations that occur during cancer development helped to identify a number of basic features of cancers, such as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [reviewed in 6].

Although SMT still remains the most widely accepted, alternative concepts are emerging. One of them is a the **tissue organization field theory (TOFT)** [reviewed in 7, 8]. According to TOFT, the gradual deterioration of tissue organization is the main cause of tumor progression. The concept of TOFT originated from 19th century research on the cellular pathology of cancer [9]. Interestingly, the abnormal appearance of cancer specimens observed with the light microscope is still the main criteria used in breast cancer diagnosis [reviewed in 10]. The tissue microenvironment or stroma which consists of fibroblasts, endothelial cells forming blood vessels, adipocytes and immune cells embedded in an extracellular matrix, together with the basement membrane (BM), which separates the epithelium from the stroma, have profound influence on epithelial tumor induction. In support of the TOFT theory are the observations that non-transformed, genetically normal cells can express a malignant phenotype when exposed to altered stroma. Thus, normal human breast epithelial organoids from reduction

mammoplasty can form tumors when grown as xenografts in immunocompromised mice, in the presence of altered human mammary fibroblasts [11, reviewed in 12]. On the other hand, pre-malignant breast epithelial cells undergo growth arrest and form polarized alveolar structures similar to normal epithelia in the presence of a reconstructed basement membrane [13]. The most compelling evidence supporting TOFT was provided by Maffini et al. [14]. They removed mammary epithelium from fat pads (stroma) of experimental rats and placed them in culture. One group of experimental animals and some epithelia from primary cultures were treated with carcinogen. Subsequently, the mammary tissue was reconstructed in treated and untreated rats using either carcinogen-treated or untreated mammary epithelial cells from primary culture. All animals which were treated with carcinogen and thus contained carcinogen-treated stroma, developed epithelial tumors, regardless of whether or not the mammary epithelium used to reconstruct their mammary tissue was exposed to carcinogen [14, reviewed in 15].

Another theory of cancer, which is becoming increasingly popular, is the **stem cell theory** of cancer. Originally inspired by the 19th century embryology, the theory is based on the fact that there are similarities in signaling pathways between embryonic and cancer cells. It suggests that cancers arise from stem cells, which are present in all tissues and are needed for tissue renewal [reviewed in 16, 17].

The molecular biology studies in the field of cancer over the past five decades revealed a remarkable complexity of the processes involved in tumor development. Recent reexamination of concepts alternative to SMT reflects the necessity to look at tumor progression from many angles and at many levels of cell and tissue organization.

Breast cancer

1.3.1 *Statistics*

Breast cancer is the most common cancer diagnosed among women (after non-melanoma skin cancer) and is the second leading cause of cancer death after lung cancer according to the National Cancer Institute [18]. An estimated number of 22,300 new cases will be diagnosed and over 5,300 death from this disease will occur in Canada in the year 2006 [19]. Based on current rates, 12.7 % of women born today (1 in 8) will be diagnosed with breast cancer and 30% of them will die of the disease. It is important to emphasize, however, that the risk of breast cancer increases sharply with age. Thus, a 35-year-old woman has a risk of 1 in 2,500, a 50-year-old woman has a risk of 1 in 50 and only at age 85 will the risk actually be 1 in 8. Breast cancer is the leading cause of death in American women between ages 50 and 55 [20].

Breast cancer incidence in U.S as well as in Canada has been rising steadily. Although better diagnosis and an aging of the population may be partially responsible for that, the increase seems to reflect a real trend and suggests possible involvement of environmental and life style factors in the development of the disease [20]. In spite of the increase in breast cancer incidence, in many countries mortality rates declined during the 1990s [19, 21] (Figure 1 and 2). This is encouraging and likely reflects improvements in managing the disease, such as early screening and broader use of appropriate treatments.

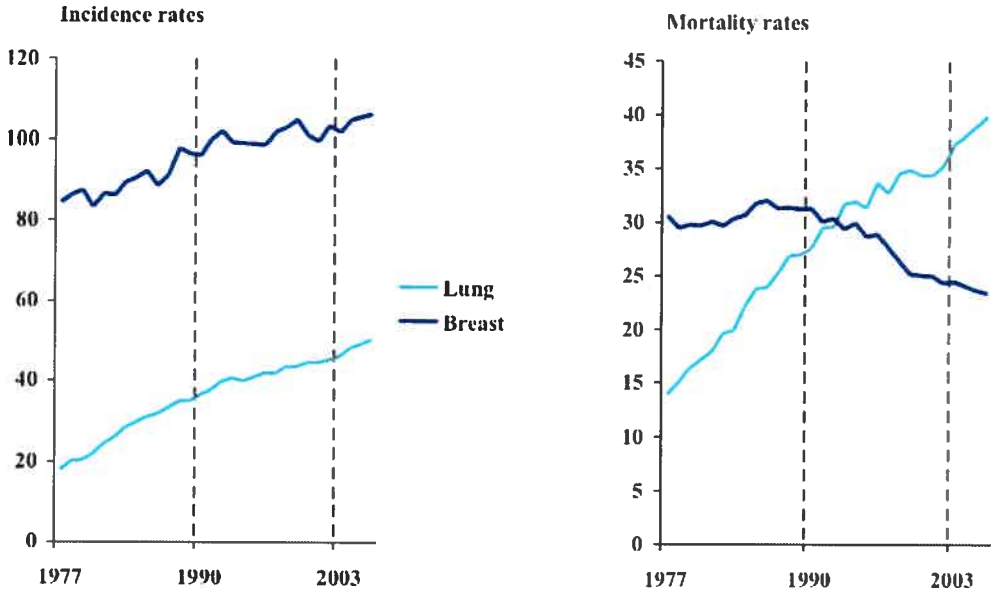


Figure 1. Trends since 1977 in age-standardized rates for breast and lung cancer among women in Canada.

Rates were standardized to the age distribution of the 1991 Canadian population. Data from Surveillance Division, CCDPC, Public Health Agency of Canada.

Source: Canadian Cancer Statistics. Canadian Cancer Society/ National Cancer Institute of Canada, www.cancer.ca

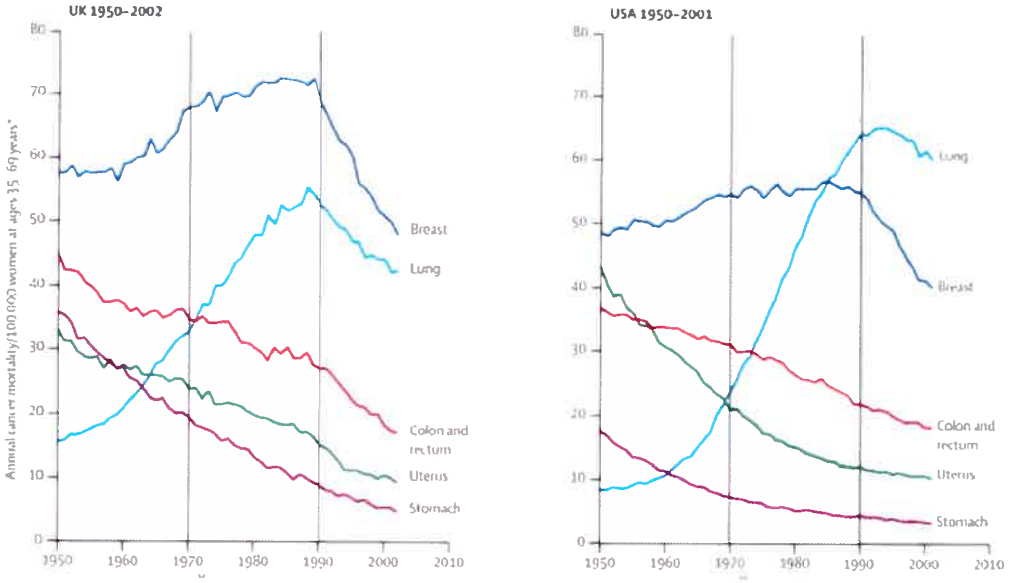


Figure 2. Trends since 1950 in age-standardized (25-69 years) death rates*, comparing breast and selected other types of cancer, among women in UK and USA.

*The age-standardized rate is the mean of the seven separate rates in the 5-year age ranges 35-39 up to 65-69. Data from WHO statistics on death and on population estimates.

Source: Early Breast cancer Trialists' Collaborative Group (2005) Lancet 365:1687-1717

1.3.2 *Breast cancer as a genetic disease*

One of the main risk factors for breast cancer is a family history, which suggests an inherited component in the development of the disease. Around 5–10% of all cases and 25–40% of cases in younger patients (under the age of 35 years) have a hereditary origin [22]. Two major susceptibility genes, which were identified about ten years ago, are BRCA1 and BRCA2 [23, 24]. Carriers of mutations in these genes are also at increased risk for the development of ovarian cancer [25 reviewed in 26]. Both mutations are highly penetrant. The chromosomal location of BRCA1 and BRCA2 genes and the structures of proteins coded by the genes are known, but their functions are still being investigated. They are both tumor suppressors involved in the maintenance of genome stability. BRCA1 is implicated in DNA repair, transcriptional regulation, cell-cycle progression and meiotic sex chromosome inactivation while BRCA2 is an essential component of the complex responsible for homologous recombination [27]. Both mutations account for substantial proportions (20 % each) of all familiar cases [28]. Additional known familiar susceptibilities to breast cancer include mutations in other tumor suppressors such as CHEK2, a gene encoding the protein kinase required for DNA repair and replication, which accounts for 5% of all familiar cases, and TP53 gene encoding the p53 protein responsible for cell cycle arrest during DNA damage and involved in the regulation of apoptosis. Mutations in TP53 (Li-Fraumeni syndrome) are responsible for about 1% of all familiar cases. A small fraction of other cases are related to mutations in PTEN (Cowden's syndrome), LKB1 (Peutz-Jeghers syndrome) or ATM (ataxia telangiectasia-mutated gene) [28]. ATM encodes yet another protein kinase that acts as a tumor suppressor. Activated via damage to DNA, ATM stimulates DNA repair and blocks cell cycle progression. One mechanism through which this occurs is ATM dependent phosphorylation of p53. Despite the evident progress in understanding the genetic causes of breast cancer, the genetic bases of the majority (54 %) of all familiar predispositions are still not known [29].

It should be noted that while the majority of known mutations predisposing to breast cancer are implicated in the maintenance of genome integrity, two of the

predisposing factors for breast cancer (PTEN and LKB1) are also linked to nutrient uptake and the regulation of metabolism. Their place in the network of nutrient sensing and signaling for survival is shown in Figure 3.

1.3.3 *Non-genetic factors involved in breast cancer development - estrogens*

Breast cancer is a heterogeneous disease in its clinical, genetic and biochemical profile. It arises from the epithelium of the mammary gland (milk producing lobules and ducts, which transport milk to the nipple). Malignant transformation of the stromal components (fibroblasts, endothelial cells forming blood vessels and adipocytes) is very rare and it is not included in this category [20]. There is evidence that estrogens, the steroid hormones essential for development and function of a normal mammary gland, play an important role in the development of breast cancer. Thus, the risk of the malignancy is related to the cumulative exposure to endogenous and exogenous estrogens and includes: early menarche, late age of menopause and hormonal therapy after menopause.

Estrogens are steroid hormones produced primarily by ovaries, with some contribution from the placenta, adipocytes and adrenal glands. They act mainly through two nuclear receptors ERα and ERβ, which are ligand-inducible transcription factors and are both expressed in breast tissue [30]. Two thirds of all breast cancers express ERα, which is believed to be responsible for tumor cells proliferation [31]. Curiously, however, the proliferating cells in a normal mammary gland rarely express steroid hormone receptors [31,32]. This apparent paradox is still the subject of debate [33, 34]. Nevertheless, antiestrogen therapies are being developed and have been found to be very effective for treatment of this malignancy as well as successful in chemoprevntion for high-risk patients, such as those showing abnormal breast histology or carriers of a genetic predisposition [21, 35]. Current antiestrogene strategies include: (a) antiestrogens, such as tamoxifen, that inhibit estrogen binding to its main target, ERα, (b) aromatase inhibitors, that prevent synthesis of endogenous estrogens, and (c) pure antiestrogens, such as fulvestrant, that block the action of estrogens [36].

Although very successful, endocrine therapies are still the subject of intensive research for at least two reasons:

(1) They have to be designed in such a way so that they do not interfere with other aspects of women's health. This is especially true for long-term chemopreventive therapies. Estrogens influence physiology of reproductive and cardiovascular systems, metabolism of bones and the integrity of the central nervous system. The new category of therapeutic agents currently being developed for breast cancer treatment, selective estrogen receptor modulators (SERMs), may be suitable for endocrine therapy [36, 37]. These are nonsteroidal agonists/antagonists of estrogens action. It is anticipated that the effects of these drugs may vary depending on the target tissue. Indeed, tamoxifen, a widely used breast cancer therapeutic drug, is the first of this type. It acts as an antagonist in the breast to prevent breast cancer progression and as an agonist in the bone to preserve bone density.

(2) The second challenge facing endocrine therapies is the development of resistance. Some ER-positive breast cancers do not respond to antiestrogen therapy (intrinsic resistance), while others stop responding after long-term therapy (acquired resistance). Interestingly, tumors that acquire resistance (30 to 50 % of treated ERα positive tumors) retain the expression of the receptor. Resistance to endocrine therapies is a complex phenomenon, which may involve many different mechanisms [38]. For example, the direct interaction between signaling through ERα and several other transduction pathways can convert the inhibitory effects of the tamoxifen-ERα complex into a stimulatory effect. Cross-talk between signaling from ERα and HER2/neu (EGFR type receptor and the common oncogene in breast cancer), might be involved in drug resistance of tumors overexpressing this cell surface receptor [36, 39] . In addition, ERα activity could be regulated by phosphorylation mediated directly by Akt [40]. The signaling from HER2/neu involves the PI3K/Akt pathway activation as well. As discussed later in this chapter, this pathway is implicated in the upregulation of glucose uptake and the stimulation of lipogensis in many tumor cells. Interestingly, there are indications that interfering with lipogensis by inhibiting fatty acid synthase (FAS), the final enzyme responsible for the synthesis of fatty acids (FA), can alter upstream signaling form ERα. This suggests that lipid metabolism

itself may be an important factor involved in the estrogen response of breast cancer cell [41].

1.3.4. *Breast cancer as a chronic disease – cancer dormancy*

The risk of recurrence for cancer patients is the highest within five years after diagnosis and more then half of the patients develop metastasis during this time. Breast cancer belongs to the small group of cancers that have a relatively unusual risk of late recurrence, even 20 years after diagnosis. The other cancers belonging to this group are melanoma, non-Hodgkin's lymphoma and renal carcinoma [42]. The early relapse and late relapse seem to occur through different mechanisms. The characteristic feature of a late relapse is that it occurs in two stages, an early stage, when there is no expansion of tumor cells and the late stage characterize by exponential tumor growth. The early phase of relative tumor quiescence followed by late recurrence has been called cancer or tumor dormancy[42]. Cancer dormancy is not well understood and it is extremely difficult to study due to the very low numbers of dormant cancer cells. By applying sensitive immunocytochemistry, one can detect disseminated tumor cells (DTC; one cell per bone marrow aspirate) in the bone marrow of 20-40% of cancer patients that do not show signs of metastasis [43].

Other very sensitive methods can identify circulating tumor cells (CTC) from the blood. They allow the detection of 1 cell per 20 ml of blood in about 36 % of cancer patients that do not show clinical signs of disease [42]. The diagnostic value of DTC and CTC detection is not yet clear. Considering that about 20 % of patients will relapse after long-term remission, the phenomenon is worth serious consideration. The late relapse has a stochastic characteristic and currently there are no tests that can predict which patients are at risk for recurrence. Persistence of DTC or CTC in the body of cancer patients suggests that cancer may be considered as a chronic disease.

We have already mentioned that environmental and life-style factors may be involved in breast cancer development. Considering the possible chronic character of the disease, prevention may be an important therapeutic option. Consistent with this

view, the life-style factors, such as obesity, diet and exercise, were shown to markedly affect breast cancer risk and survival after diagnosis.

1.3.5 *Obesity and the risk of developing cancer*

Epidemiological studies suggest that obesity is a metabolic disorder that affects the development of many different types of tumors, including colon, breast (postmenopausal), endometrium, kidney (renal cell), oesophagus (adeno-carcinoma), gastric cancer, pancreatic, gallbladder, liver, non-Hodgkin's lymphoma, leukemia, multiple myeloma, rectum, ovary and prostate [reviewed in 44, 45]

Although the molecular mechanisms linking cancer promotion with obesity are still not understood, the association is strong. Thus, obese individuals with a body mass index above 30 kg/m^2 (BMI, defined as weight in kilograms divided by height in meters squared) have an overall increased risk of developing many types of cancer of approximately 1.5 to 2 fold and an over 3 fold increased risk of developing cancers of the endometrium and oesophagus. Overweight postmenopausal women have a 1.66 fold increased risk of developing breast cancer [45]. The parameters indicating metabolic dysfunction, such as insulin resistance, visceral adiposity, hypertriglyceridemia and hyperglycemia, were all shown to correlate with an increased risk of developing breast cancer [46-49]. Overweight women have not only an elevated risk of developing breast cancer, but also an increased chance of cancer recurrence (1.8-1.9 fold) and increased mortality (1.4-1.6 fold) [50 reviewed in 51]. Obesity appears to be strongly related to mortality in women with estrogen receptor-negative breast cancers, for which there exist fewer therapeutic options [52].

1.3.6 *Excessive adiposity and its contribution to cancer*

Fat deposits in different anatomical sites of obese individuals are unequal. Upper-body fat, including visceral and abdominal subcutaneous deposits, strongly correlates with increased risk for the development of insulin resistance, diabetes and cancer [47]. The accumulation of fat in the upper body is controlled by various factors, including heredity (about 50%) and sex (more common in males than in

females), and is closely associated with glucose intolerance, hyperinsulinemia, hypertriglyceridemia and other features of what is called metabolic syndrome or syndrome X [53]. A causal link between visceral obesity and various disorders has been difficult to determine. There is evidence that visceral adipose tissue is more dynamic and has a higher lipolytic activity. The increased release of free fatty acids (FFA) from visceral adipose tissue to the portal vein, which drains directly into the liver, could cause liver dysfunction. However, it is also likely that visceral obesity is not harmful by itself but is a sign of an underlying metabolic phenotype, which manifests itself in an altered adipose tissue distribution [reviewed in 53].

It has been recognized that the storage and release of fat is not the only function of adipocytes. These cells also participate in physiological homeostasis through the production of hormones and cytokines, which can act in autocrine, paracrine or endocrine fashion (for examples: leptin, resistin, adiponectin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6) and apolipoproteins [54]. The endocrine function of adipocytes in obese individuals may well be one of the factors contributing to the development of neoplasia [44]. This is particularly true for breast cancer in postmenopausal women, since their adipose tissue is also the main source of estrogen [55]. Adipocytes are major components of human breast tissue, making up about 90 % of their volume. It has been shown that in postmenopausal women, local estrogen levels in breast tumors could be as much as 10 times higher than in the circulation [56]. Obesity-related breast cancers are also more often ER-positive [57]. It was proposed that various signaling pathways activated by adipocytes may crosstalk with each other and with signaling from the ER and synergistically promote tumorigenesis [55].

Obesity is also independently positively linked to elevated blood FFA levels, which have been implicated in the development of pathologies such as insulin resistance and tissue lipotoxicity [58 reviewed in 59, 60, 61].

1.3.7 *Metabolic interventions (diet and exercise) can improve survival in breast cancer patients*

Metabolism can affect the progression of cancer. The recent studies showed that dietary interventions such as a decrease in dietary fat or the promotion of energy expenditure by means of an increase in physical exercise can improve survival in postmenopausal women who have been treated for early-stage breast cancer. Remarkably, only a few hours of exercise a week (3 to 5 hours of walking) can reduce the risk of death from breast cancer by up to 50% [62] and decreased dietary fat (to 20%, from about 40% of total calories from fat, which reflects the typical Western diet), can reduce the risk of tumor recurrence for all breast cancer patients by 24%, and for patients with estrogen receptor-negative breast cancer by 42% [63]. The efficacy of these interventions is comparable to that of established adjuvant therapies. Thus, the risk reduction of recurrence after hormone therapy is estimated at about 50% for estrogen receptor-positive cancers [64] and treatment with Trastuzumab, the monoclonal antibody inhibiting the activity of HER2/neu tyrosine kinase receptor (often amplified in breast cancers) reduces mortality by 33% [65].

1.4 Metabolism and the control of cell survival

1.4.1 *Growth factors regulate cell survival by inhibiting apoptosis and by directly controlling cell access to nutrients*

The nutritional environment of most cells in the body of healthy individuals is highly regulated. The individual cells within the body are usually not limited for growth by the extracellular concentration of glucose, fatty acids (FA) and other nutrient substrates. Thus, cell survival, growth and proliferation are mainly controlled by the signals from exogenous growth factors (GF) rather than nutrient substrates [66]. Cells within multicellular organisms are constantly exposed to numerous signals from their surroundings, including soluble factors, signals from the extracellular matrix or the signals from neighboring cells. GF signaling is responsible for the balance between cell accumulation and cell death within tissues.

Individual cells require GF signaling to maintain their survival and in the absence of those permissive signals they undergo apoptosis. They also require GF to initiate cell division. During tumorigenesis, individual cells acquire mutations in the signaling pathways that allow them to avoid apoptosis and proliferate in the absence of GF [6]. Recently, Thompson et al. showed that GF also control the access to extracellular nutrients in individual cells. They showed that when cells are withdrawn from GF, the rates of uptake of glucose and amino acids decrease and the transporters for iron (transferrin receptor), as well as cholesterol (LDL receptor), are also down regulated [67, 68]. Finally, they demonstrated that when Rab7 (which regulates endocytic membrane traffic and mediates the internalization and degradation of nutrient transporters) was inhibited, GF-deprived cells displayed prolonged, growth factor-independent cell survival. Therefore, GF regulate cell survival by inhibiting apoptosis as well as by directly controlling cell access to nutrients [69].

1.4.2 *Signaling via Akt serine/threonine kinases regulates cell survival by several mechanisms*

Thompson et al. hypothesized that cancer cells have to acquire autonomy for uptake of nutrients in order to become fully transformed. They showed that activation of the Akt family of serine/threonine kinases, often triggered by oncogenic alterations in GF signaling, promotes increased nutrient uptake [70, 71 reviewed in 72]. Thus, they were the first to reveal that Akt can control cell survival by controlling cellular metabolism.

Akt serine/threonine kinases are activated in many types of cancers and this activation leads to enhanced resistance to apoptosis [73]. Signaling via Akt can induce cell growth and division in addition to survival. Some of the survival signaling pathways overlaps with the mitogenic pathways, but some are distinct. A recent review on cell cycle regulation by Akt was published by Brazil et al [74] and reviews on regulation of cell survival by Akt signaling can be found in Hammerman et al. and in Mc Cormick [75, 76]. Figure 3 briefly summarize the current knowledge regarding Akt induced regulation of cell survival, and can be described as follows. Akt can be activated by signals from various tyrosine kinase receptors in breast

cancers, such as the HER2 oncogene. Activated Akt increases cell survival by inhibiting various proapoptotic targets, such as TSC1/2, IKKα, FOXO and Bad [76]. In addition, Akt activation promotes cell survival by stimulating cellular glucose and fatty acid metabolism. Akt activates glycolysis, presumably by stabilizing HIF-1 transcription factor [77], which in turn is responsible for the upregulation of all the glycolytic enzymes (for detailed discussion see section 1.5.2). In addition to this, Akt may directly activate the expression of several enzymes involved in metabolism of glucose, including glucose transporters, hexokinase [78] and phosphofructokinase 1 and 2 [79]. The intracellular metabolism of glucose and FA are tightly interconnected (for detailed discussion see section 1.6.3). Akt activition of glycolysis will result in the production of more substrate for FA synthesis. Thus, Akt was shown to activate enzymes involved in FA synthesis via SREBP-1 [80] and it can directly phosphorylate ACL, the enzyme catalyzing the first step in conversion of glycolytically-derived citrate to cytosolic acetyl CoA (a precursor of FA, cholesterol and isoprenoid synthesis) [81]. There is evidence that upregulation of FA synthesis itself could be important for cancer cell survival but the mechanisms are not yet understood [82].

1.4.3 *Cells can sense changes in the levels of many nutrients and can adapt their metabolism*

As discussed above, the cellular metabolism of glucose and fatty acids may be directly up regulated by growth factors to promote the survival of proliferating cells. However, it has long been recognized that individual cells are also able to sense their metabolic status independent of GF. Such homeostatic responses are evolutionarily conserved [83]. For example, a decline in the cellular ATP/ADP ratio results in activation of AMPK, which turns on ATP-generating catabolic pathways while turning off ATP-consuming processes (cell growth) (Figure 3).

Activated AMPK can adjust the activities of various metabolic enzymes by phosphorylation and may also modulate intracellular signaling for cell growth and division [84]. A recent discovery is that cells may also sense their NAD^+/NADH ratio. It was demonstrated that the yeast longevity protein Sir2 (sirtuin) is a NAD-

dependent histone deacetylase [85]. Remarkably, sirtuins are highly conserved in evolution, and are implicated in the control of metabolism and lifespan in yeast, worms and flies, particularly in the effects of calorie restriction (CR) on longevity [86]. Mammalian sirtuin (SIRT1) inhibits apoptosis by direct deacetylation of FOXO, p53 or Bax-binding partner Ku-70 [87,88]. The exact mechanism of regulation of sirtuins in humans is not well understood and its connection to NAD^+/NADH ratio still needs to be clarified.

Another protein involved in sensing and communicating the metabolic status of cells is mTOR (Figure 3), the mammalian kinase which is a target of rapamacin. mTOR is located downstream of Akt. It is known to respond to amino acid starvation and regulates protein translation. However, mTOR also integrates signals regarding cellular metabolism and energy status by communication with AMPK and by directly responding to ATP levels. In addition, mTOR controls aspects of glucose homeostasis and recent studies suggest that it may also control fat metabolism [reviewed in 89]. The physiological function of mTOR is the control of cellular growth (increase in cell mass). mTOR is also involved in the response to starvation by controlling degradation of cellular content, including organelles, to ensure survival under nutrient-depleted conditions, a process known as macroauthophagy. The yeast TOR protein can form two types of complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 is sensitive to rapamycin and it regulates the timing of cell growth. TORC2 is insensitive to rapamycin and regulates spatial growth (where cell will grow). One interesting aspect of mTOR signaling is the recently discovered existence of a negative feedback loop from nutrient sensing to the insulin-responsive Akt signaling pathways. Thus, mTOR is emerging as a very important factor in the control of not only cell growth but also cell survival and metabolism [89].

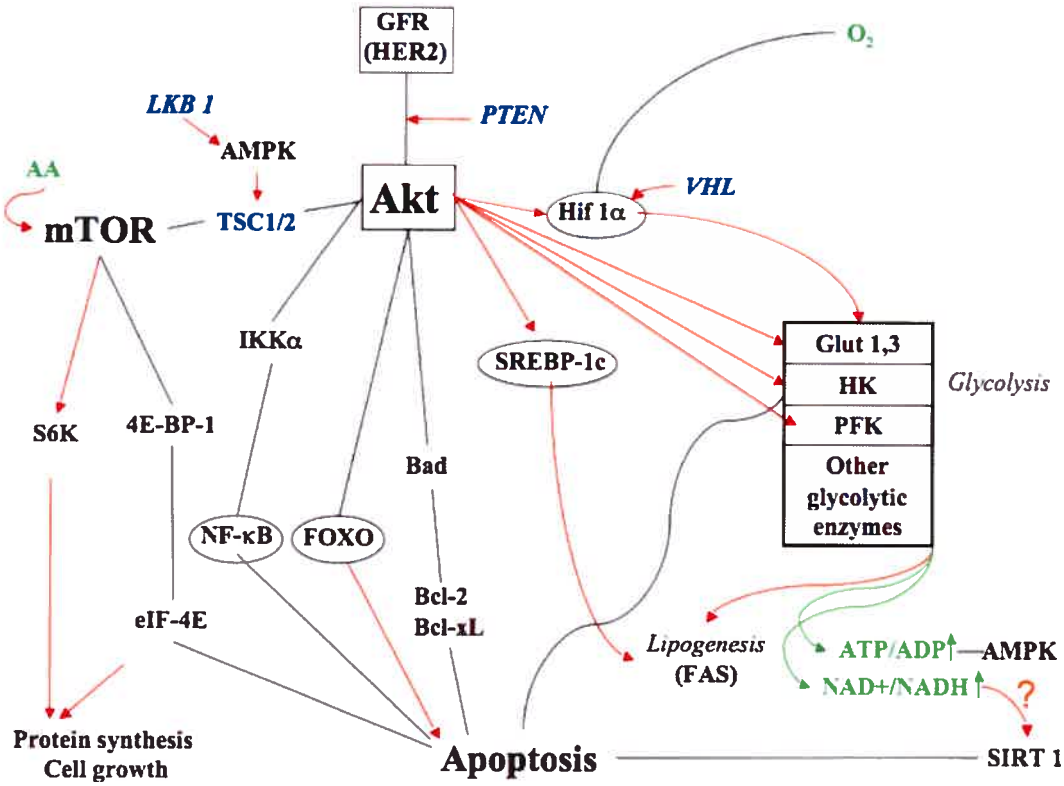


Figure 3. Akt signaling for cell survival

Akt inactivates by phosphorylating proteins, which are directly or indirectly involved in the induction of apoptosis (Bad, IKKα, FOXO). Akt signals through the mammalian target of rapamycin (mTOR) by inactivating its repressor, tuberous sclerosis complex (TSC1/2). TSC1/2 is a tumor suppressor mutated or deleted in several cancers [90]. Interestingly AMPK can activate the complex having an antagonistic effect on mTOR function. Akt activates directly or indirectly glycolysis and lipogenesis. This results in changes in the levels of important metabolites (AMP, NAD+), which can signal back via proteins sensitive to their levels (AMPK and SIRT1 respectively) to modulate metabolism and/or cell survival. Hexokinase (HK) directly prevents apoptosis by binding to the mitochondrial outer membrane. Black lines indicate inhibition and red arrows indicate stimulation. Transcription factors are encircled, metabolites are green and tumor suppressors are in blue.

1.4.4 *Some metabolic enzymes directly participate in signaling for cell survival*

The interesting aspect of upregulation of glycolysis in the context of the control of cell survival is that glycolytic enzymes themselves can directly participate in antiapoptotic effects. The best-known example of this is hexokinase (HK) (Figure 3), which binds with high affinity to the outer mitochondrial membrane and interacts with other membrane-associated proteins, including VDAC to inhibit apoptosis [91, 92]. In fact, many enzymes involved in glycolysis have additional, nonglycolytic functions, which include transcriptional regulation, stimulation of cell motility, apoptosis [reviewed in 93], and even DNA repair [94]. Glycolysis is one of the most ancient metabolic pathways, and these recently discovered nonglycolytic functions of glycolytic enzymes may reflect an evolutionary strategy, designed to coordinate metabolism with other cellular functions.

1.5 New concepts regarding the role of glucose metabolism in tumorigenesis

1.5.1 *Glucose metabolism is up regulated in tumor cells: the Warburg effect*

Cancer cells show major changes in the metabolism of glucose. While the majority of non-transformed cells use oxidative phosphorylation in mitochondria for energy production and switch to glycolysis only upon oxygen deprivation (hypoxia), tumor cells show high glycolytic rates and rely on glycolysis for energy production even in the presence of oxygen. Up-regulation of both glucose uptake and glycolysis are common features of many cancers and have application in noninvasive cancer imaging used for diagnosis and staging of tumors by positron emission tomography (PET) [95]. This test uses radiolabeled glucose analogs to detect differential glucose uptake by fast-growing tumors and metastases.

The reason for the metabolic switch to glycolysis in the presence of oxygen in tumors, the so-called "Warburg effect", is still not clear. Over 70 years ago Warburg proposed that the effect is a result of mitochondrial dysfunction [96] and developed a cancer theory based on altered metabolism [97]. However, according to the generally accepted views, alterations in tumor cell metabolism are symptoms of

transformation or response to the tumor's microenvironment rather than a cause of cancer. Thus, the Warburg's theory was never accepted.

1.5.2 *The transcription factor HIF-1 coordinately regulates an integrated response to low oxygen and a switch to high glycolysis*

Growing tumors are often hypoxic due to insufficient blood supply. Thus, up-regulation of glycolysis could occur in response to hypoxia and could be an adaptation to hostile tumor environment. The cellular response to oxygen deprivation is controlled by the transcription factor: hypoxia-inducible factor 1 (HIF-1), which regulates a multiplicity of genes, including those coding for all of the glycolytic enzymes, and others encoding paracrine growth factors such as vascular endothelial growth factor (VEGF). Activation of these genes assures an integrated response to low oxygen, including a switch to high glycolysis and recruitment of new blood vessels [98].

1.5.3 *Tumor suppressor genes and oncogenes are involved in the upregulation of glycolysis: the Warburg effect re-examined*

HIF-1 is degraded in the presence of oxygen. Disruption of HIF-1's oxygen dependent degradation leads to its constitutive activation. Interestingly, recent data shows that HIF-1 could be activated by factors other then tumor environment and that it may possibly play a crucial role in the process of transformation [99]. First of all, it was demonstrated that genes involved in the control of HIF-1 degradation function as classical tumor suppressors (a germline mutation in one allele predisposes carriers to develop tumors in specific organs). Three genes of this type were identified: the von-Hippel-Lindau (VHL) gene, which predisposes carriers of germline mutations to kidney, blood vessel and adrenal tumors, and two genes encoding enzymes of the tricarboxylic acid cycle: succinate dehydrogenase (SCD) and fumarate hydratase (FH), which predispose carriers of germline mutations to hereditary paraganglioma and leiomyomatosis/renal cell cancer syndromes, respectively [reviewed in 100, 101]. Moreover, HIF-1 could be detected in non-hypoxic areas of tumors [102] being stabilized in response to stimulation by a

growth factor, such as insulin-like growth factor (IGF), or in cells transformed by oncogenes including v-Src, c-Src, ras or HER 2 [reviewed in 103].

Stabilization of HIF-1 is not the only way in which cells can acquire high glycolytic rates. Some oncogenes can directly activate glycolytic enzymes. So far two examples are known: MYC and Akt oncogenes. In the case of MYC, it was shown that several key glycolytic genes have highly conserved Myc binding sites. Myc directly binds to their promoters and transactivates them in non-hypoxic conditions [104]. The exact mechanism involved in activation of glycolysis by Akt is not known, but it appears to be independent of HIF-1 [70, 105]. Akt is involved in redirecting cellular metabolism in response to growth factors stimulation or oncogenic alterations to support cell growth and proliferation [reviewed in 72].

The present views on up-regulation of glycolysis in cancer cells are summarized in Figure 4 [103]. Thus, it seems clear that the up-regulation of glycolysis that is so often observed in cancer cells may not be just an adaptive phenomenon, since it could be autonomously acquired during tumor progression. Its contribution to tumorigenesis, however, is still controversial and not widely accepted [106].

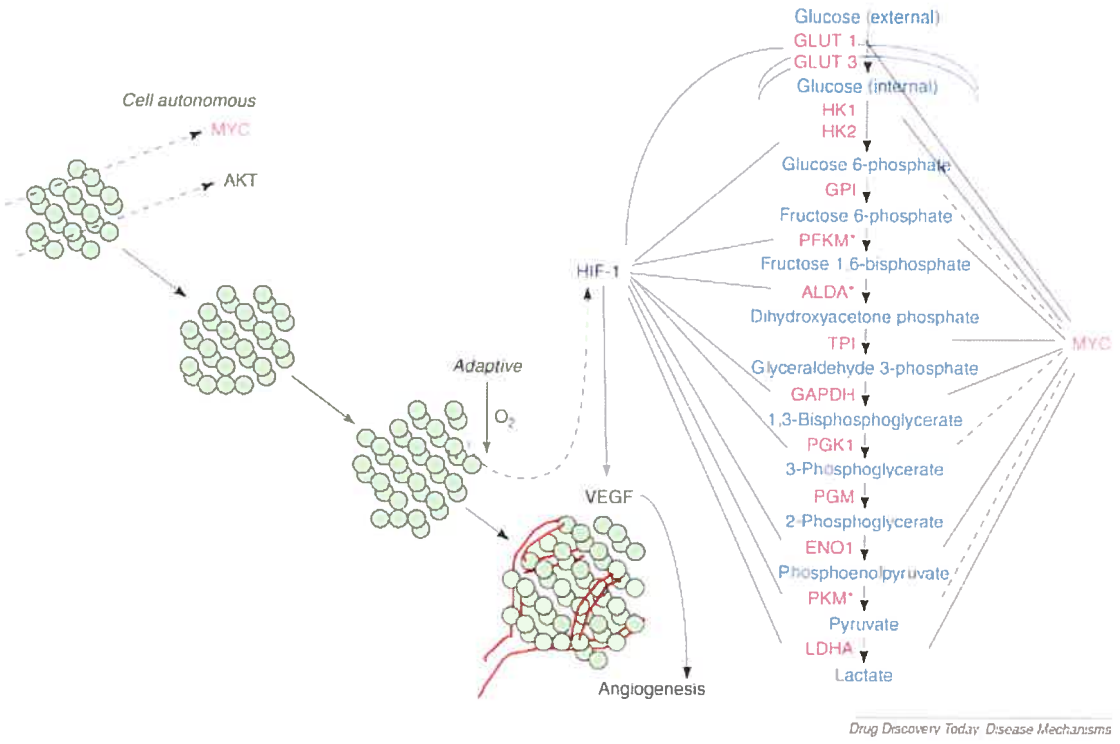


Figure 4. Cell autonomous oncogenic alterations and adaptation to hypoxia contribute to tumor aerobic glycolysis

The figure denotes a tumor mass that could have sustained genetic alterations that activate either AKT or MYC, resulting in cell autonomous activation of aerobic glycolysis. In the case of MYC, which encodes a transcription factor, glycolytic genes are directly activated. As the tumor mass continues to enlarge, diffusion limitation causes local hypoxia that induces the HIF-1. HIF-1, in turn, activates the glycolytic genes as well as factors such as VEGF, which induces angiogenesis. The glycolytic pathway is shown on the right. Genes affected by either HIF-1 or MYC are indicated by lines. For MYC, the thickness represents the level of direct binding of Myc to glycolytic genes.

Source: Kim, J.W., Gardner, L.B. and Dang, C.V. (2005) Drug Discovery Today: Disease Mechanisms 2 (2): 233-238.

1.6 Overview of FFA metabolism and its regulation

1.6.1 *Control of FFA levels in the blood*

The level of FFA (those which are not components of circulating triacylglycerols, Figure 5) in the blood is highly controlled between 0.1 - 0.8 mM [107]. After a meal, FFA released from dietary fat are absorbed by enterocytes that line the small intestine [108]. Inside the enterocytes, they are esterified to form triacylglycerols (TG) and then are exported into the circulation as chylomicrons. They are released from chylomicrons after hydrolysis mediated by lipoprotein lipases (LPL), which are produced by adipose tissue and muscles, and then secreted to the surface of capillary endothelial cells nearby. In fed state, the majority of FFA released from chylomicrons will be taken up by adipocytes and immediately re-esterified to form storage TG inside the cell. Thus, the multistep process of trapping dietary FFA inside adipocytes consists of sequential cycles of esterification and lipolysis. In normal individuals, this complex process is almost 100% efficient 1 h after a meal and the efficiency then decreases to 10-30% by 6 h [109]. This allows for a gradual release of FFA into the blood at longer times after the meal. Thus, the FFA levels in the blood are highest in a fasted state (over-night fast) when they are needed as a fuel in various tissues. Lipolysis in adipocytes is carried out by the highly regulated enzyme hormone sensitive lipase (HSL) and other associated lipases [110, 111].

Obesity is often linked to insulin resistance, which is not well understood, and manifests itself in an inability of various tissues to respond to insulin signaling [reviewed in 112]. In the obesity-induced insulin resistant state, trapping of dietary FFA in adipocytes becomes inefficient, causing elevated blood FFA levels even in the fed state. In this situation, higher than normal amounts of FFA are available to many tissues and organs, even though they do not need them for energy production [54]. Little is known how these tissues deal with the surplus of FFA fuel. For human tissues other than adipocytes, liver, skeletal muscles, heart and pancreatic β-cells, there is little or no data describing the metabolism of FFA, their rate of oxidation,

esterification, formation of lipid stores or lipolysis in the healthy or obese states. Considering the profound influence of obesity, diet and life-style on the development of breast and other types of cancer, as well as development of metabolic syndrome and diabetes, more knowledge is needed regarding FFA metabolism in different normal and tumor tissues.

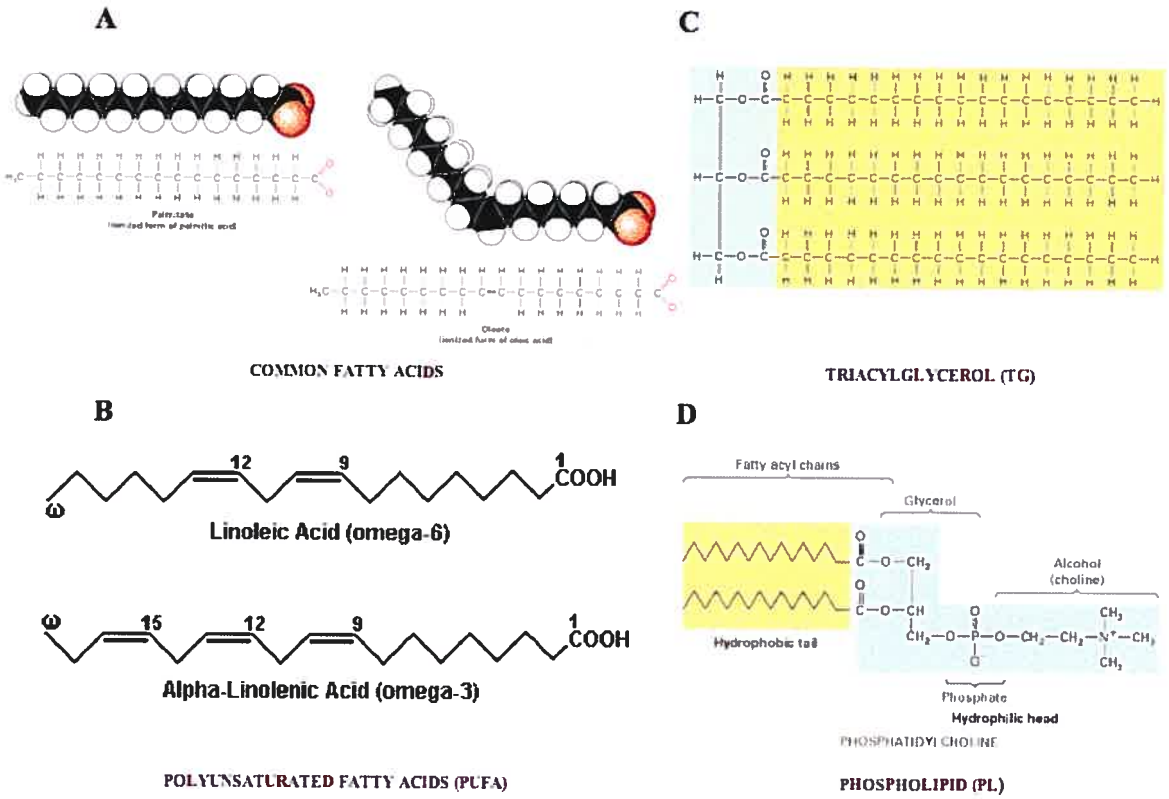


Figure 5. Structure of selected fatty acids and common complex lipids

A, Model molecules and chemical structure of common FA: palmitate (C16:0) and oleate (C18:1) ***B***, Structure of most common polyunsaturated fatty acids: linoleic acid (C18:2) and linolenic acid (C18:3) ***C***, Structure of triacylglycerol (TG) ***D***, Structure of phosphatidylcholine, a common membrane glycerophopholipid.

Source: Lodish H, Berk A, Zipursky L, Matsudaira P, Baltimore D, Darnell J: Molecular Cell Biology. Fourth edition. W.H. Freeman and Company (2000)

1.6.2 *Intracellular metabolism of exogenous FFA: introduction*

FA are the simplest lipids, consisting of long alkyl chains with a terminal carboxyl group. Most FA in human body exist in complex form, as storage TG as well as structural phospholipids (PL) (Figure 5). Circulating FFA (those which are not components of complex lipids) are bound to serum albumin and their level in the blood is tightly controlled [107]. The transport of FFA into cells is believed to occur via passive diffusion as well as by protein mediated transmembrane transport [113, 114]. Once inside the cell, FFA are activated to the corresponding fatty acyl-CoA (FA-CoA) by acyl-CoA synthetase (ACS). The fate of FA-CoA subsequently varies between tissues and depends on the overall metabolic state of the body as well as on the individual needs of different specialized cells [115]. When esterified with glycerol-3-phosphate (G3P), FA-CoA are preserved in the form of storage TG or can be channeled to form phospholipids. Enzymes, which initiate the esterification process, are called G3P acyl transferases (GPAT) [116]. Alternatively, FA-CoA can be transported to mitochondria by carnitine palmitoyl transferase 1 (CPT 1) and oxidized, generating ATP and CO_2 [117]. Thus, FA, like glucose, are fuel substrates.

1.6.3 *Coordinate regulation of glucose and FFA metabolism in the body and within an individual cell*

The metabolic pathways of the fuel substrates: FFA and glucose are interdependent and reciprocally regulated. Utilization of glucose and FFA by different tissues within the body is largely coordinated by insulin but the fine-tuning is brought about by various intracellular mechanisms. Hence, elevated glucose concentration stimulates pancreatic β-cells to secrete insulin, which suppresses lipolysis in adipocytes, preventing release of FFA. This eliminates competition for fuel substrates in peripheral tissues (muscles). Thus, in healthy individuals, FFA become the major fuel substrate only when glucose and insulin concentrations are low [107, 118, 119]. Fine-tuning mechanisms, which allow individual cells to sense the availability of fuels and adjust their metabolic pathways accordingly, include the inhibitory effect of elevated FFA concentrations on glucose metabolism described in

skeletal muscles by Randle et al [120], and the inhibitory effect of high glucose concentration on fatty acid oxidation discovered by McGarry and Foster [121]. The McGarry's effect described in hepatocytes is briefly illustrated in Figure 6. When glucose is abundant, cells in various tissues, in particular liver cells, have an elevated glycolytic rate and produce large amounts of pyruvate, which enters the tricarboxylic acid (TCA) cycle in mitochondria. TCA cycle intermediates are replenished and the surplus is transported from the mitochondrial matrix back to the cytoplasm in the form of citrate. Citrate is then metabolized by a sequence of cytoplasmic enzymes: ATP citrate lyase (ACL), acetyl CoA carboxylase (ACC) and finally fatty acid synthase (FAS) to produce endogenous fatty acids. Malonyl-CoA, which is an intermediate in this process, acts as an allosteric inhibitor of CPT I. Therefore, the presence of malonyl-CoA signals the abundance of glucose in liver cells that begin to produce endogenous FA from glucose and shuts off oxidation of the newly formed FFA, promoting their esterification and storage. Inhibition of CPT I by malonyl-CoA will also prevent oxidation of exogenous FFA if they are available.

An additional level of complexity to this intracellular metabolic regulation is added by signaling from AMP-activated protein kinase (AMPK). AMPK is an evolutionarily conserved sensor and regulator of energy balance in cells. It becomes activated by phosphorylation when levels of AMP increase, indicating a reduced ATP/ADP ratio. Activated AMPK turns on ATP-generating catabolic pathways while turning off ATP-consuming processes (in particular cell growth) in response to an energy crisis. Thus, it will induce oxidation of FFA when glucose is not providing enough energy or in the situation of high energy consumption (muscle contraction) [122, 123]. Interestingly, the upstream kinase responsible for AMPK activation is a tumor suppressor LKB1, responsible for the development of benign intestinal tumors (Peutz-Jeghers syndrome) and predisposing carriers to malignant cancers in other tissues (including a breast) [124]. The deregulation of the AMPK/malonyl-CoA fuel sensing and signaling network has been proposed to be involved in the development of the metabolic syndrome, which predisposes to several chronic disorders, including obesity, diabetes, hypertension and premature atherosclerosis [125].

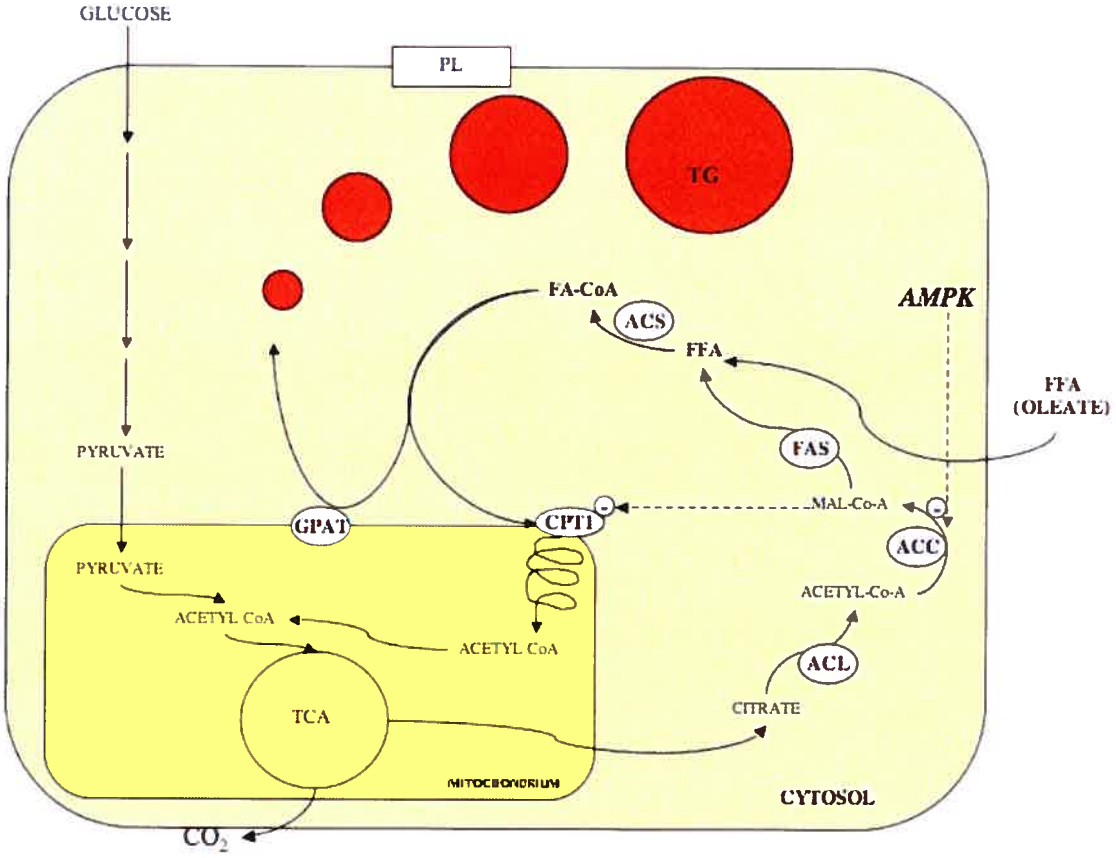


Figure 6. Intracellular metabolism of glucose and FA are interdependent

Exogenous FFA become activated inside the cell by ACS to form FA-CoA, which can be further metabolized in mitochondria to produce ATP. CPT-1 catalyzes the first rate-limiting step of its transport to mitochondria. Alternatively, FA-CoA can be esterified to glycerol-3 phosphate to form storage TG or PL. When glucose is available, it is metabolized via glycolysis and the TCA cycle to produce ATP. At high glycolytic rates, the TCA cycle will be replenished and the surplus will be transported from the mitochondrial matrix back to the cytoplasm in the form of citrate. Citrate is a precursor for *de novo* FFA synthesis. Malony-CoA, an intermediary metabolite between citrate and FFA, is an allosteric inhibitor of FFA oxidation. Its levels can also be controlled by AMPK, which inactivates ACC by phosphorylation.

1.6.4 *Storage of FFA and TG, lipolysis and TG/FFA cycling*

Although most cells in the body are able to esterify FFA to produce TG, surplus dietary FFA are stored almost exclusively in lipid droplets of adipocytes in the form of TG. Liver cells and adipocytes are specialized in the synthesis of TG, which can be hydrolyzed when FFA are needed as fuel (between meals). In healthy individuals, some storage of TG occurs in skeletal and cardiac muscles in addition to adipocytes and liver. However, this is only for local needs [126].

TG stored in lipid droplets can be hydrolyzed to produce FFA and glycerol. This process (lipolysis) has been mostly studied in adipocytes, where it is carried out by the highly regulated enzyme, hormone sensitive lipase (HSL) in addition to other less studied lipolytic enzymes in particular, adipose TG lipase (ATGL) [reviewed in 111]. FFA that are released from TG by lipolysis can be immediately re-esterified within the cell to form TG again. Lipolysis and re-esterification are two opposite processes involved in what is referred to as intracellular TG/FFA cycling [127, 128]. TG in lipid droplets of adipocytes are constantly turning over with an average half-life of a few days [126]. Cellular TG/FFA cycling can occur in all tissues containing TG stores. It is a multi-step, complex cycle involving many enzymes. As shown in Figure 7, at least one enzyme activates FFA to form FA-CoA [129], then at least five others are involved in the sequential esterification of FA-CoA onto the glycerol backbone [116], and subsequently at least three different lipases are involved in the sequential hydrolysis of the esterification products: triacylglycerols (TG), diacylglycerols (DAG) and monoacylglycerols (MG) [111]. TG/FFA cycling is considered to be a "futile cycle", since it's main outcome is the consumption of energy (production of 1 molecule of TG consumes 6 molecules of ATP). However, this process allows preservation of intracellular FA pools when they are not oxidized for energy production, and has been linked to thermogenesis in brown adipose tissue [130].

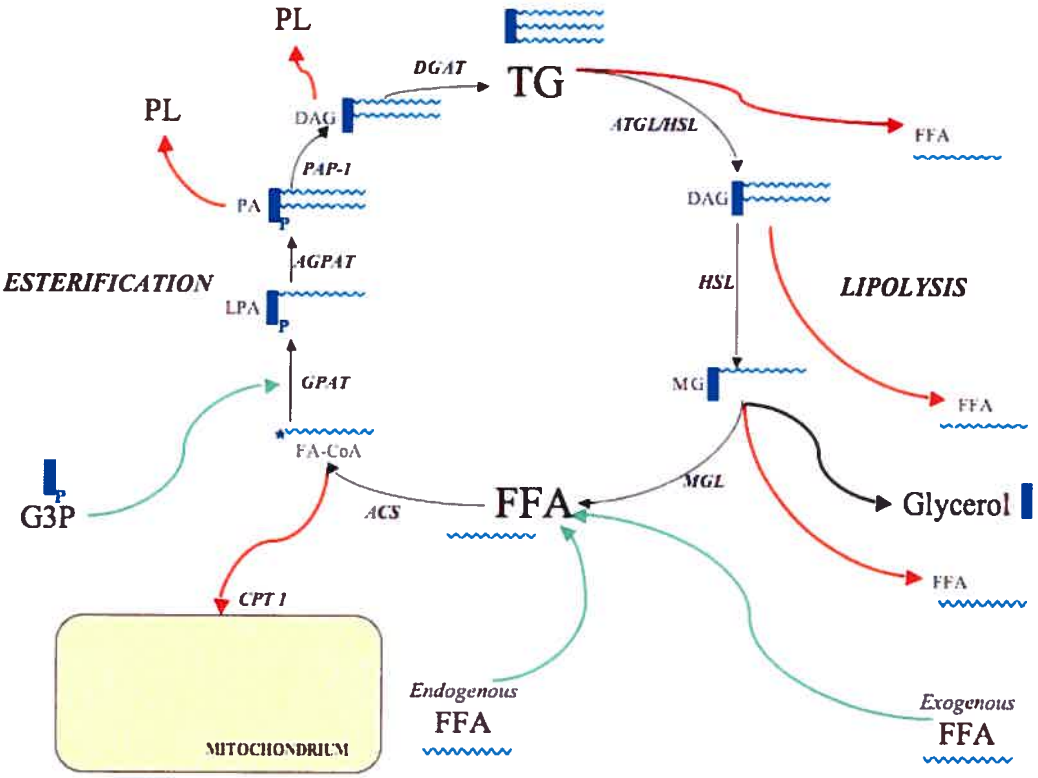


Figure 7. Schematic illustration of the intracellular TG/FFA cycle

TG/FFA cycling consists of sequential reactions of esterification (left) and hydrolysis (lipolysis shown to the right). The cycle is supplied by free fatty acids (FFA) from exogenous and/or endogenous sources and by glycerol-3-phosphate (G3P) (green arrows). The cycle can be depleted at several steps (red arrows). Thus, fatty acyl-CoA (FA-CoA) can be channeled to oxidation in mitochondria and intermediates in triacylglycerol (TG) synthesis may be channeled to the production of phospholipids (PL). FFA released by lipolysis can be secreted outside the cell or immediately re-esterified.

A comprehensive literature search revealed that there are no publications describing data on lipolysis or on TG/FFA cycling in cancer cells. TG are stored within cytoplasmic lipid droplets (LD). LD are macromolecular lipid assemblies consisting of hydrophobic core of neutral lipids (TG, DAG, and sterol esters) surrounded by a monolayer of phospholipids. Their presence in mammalian cells has been associated with the storage and transport of energy (FA). Thus, they are present in adipocytes, liver and muscle cells. However, most, if not all mammalian cells can form LD in some circumstances, such as exposure to exogenous oleate [131]. Recently, it became apparent that some proteins are tightly associated with LD in various cell types. These proteins include those involved in LD biogenesis, trafficking and mobilization (such as lipases) as well as caveolin and others, which are not directly involved in metabolism [132]. Formation of LD results in redistribution of caveolin from the plasma membrane to the surface of LD. Thus, it appears that LD are not just drops of fat, but instead are dynamic organelle-like structures that might be involved in modulating signal transduction from cell surface receptors [reviewed in 132, 133, 134].

1.7 Alteration of lipid metabolism in cancer cells

1.7.1 *Overexpression of fatty acid synthase (FAS) and upregulation of lipogenesis*

Many cancers are able to synthesize FA endogenously and express high FAS activity. Although the complex interrelations between glucose and FFA metabolism are well known (described above), the research on FAS in cancers has largely been performed independently from investigations in glucose metabolism. It was shown in 1953 that neoplastic tissues have high levels of fat synthesis *in vitro* [135]. However, interest in this phenomenon was only generated in 1994, when it was discovered that the oncogenic antigen-519 (OA-519) that is linked to poor prognosis in breast cancer patients is actually the FAS enzyme [136]. Subsequently, FAS was found to be overexpressed in other types of tumors, including prostate, colon, ovarian,

endometrial and thyroid, and to be a marker of a more advanced disease [reviewed in 82].

The mechanism involved in overexpression of FAS in cancers is not well understood. There is evidence that the whole pathway of fatty acid synthesis is up regulated and that all its key enzymes, including ACL and ACC (see Figure 6), are also overexpressed [137]. Several studies demonstrated that the sterol regulatory element binding protein 1c (SREBP 1c) might participate in upregulation of this pathway [80,138-140]. SREBP 1c is a member of a family of transcription factors, which play an important role in normal cell physiology as they co-ordinate lipogenic gene expression and cellular lipid homeostasis [reviewed. in 141, 142]. Overexpression of FAS correlates with elevation in levels of SREBP 1c in various cells in culture that are stimulated with androgen, EGF or transformed with H-ras, suggesting that the activation of signaling pathways leading to proliferation induces lipogenesis via SREBP 1c [80, 137, 138].

Interestingly, high FAS activity is linked to proliferation not only in tumors. FAS is essential in embryonic development [143] and it is highly expressed in rapidly proliferating tissues like the endometrium [144]. The signaling upstream of SREBP 1c in tumor cells may involve the PI3K/Akt pathway, which was shown to be implicated in the upregulation of FAS in different cells and tumors [145-147]. The central role of Akt in the coordinated stimulation of glucose and fatty acid metabolism is illustrated in Figure 8, which shows an integrated view of the cancer cell metabolism [72].

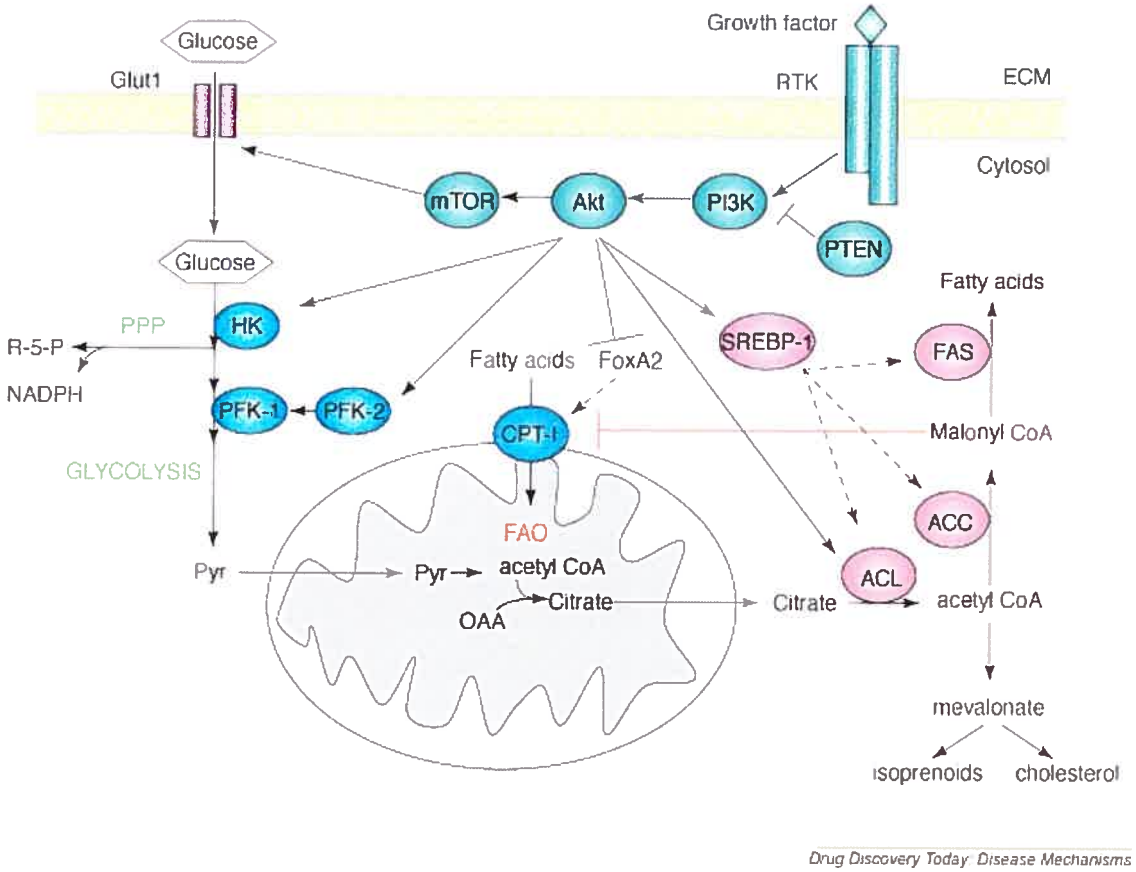


Figure 8. Akt signaling coordinately stimulates glucose and fatty acid metabolism

Akt activation downstream of growth receptor tyrosine kinase (RTK) signaling upregulates glycolysis to fuel FA synthesis and inhibits FA oxidation. Increased glucose uptake through the Glut 1 transporter and increased glucose catabolism through activation of the glycolytic enzymes hexokinase (HK), phosphofructokinase-1 (PFK-1) and PFK-2, lead to a high flux of glucose carbons into the mitochondria as pyruvate (pyr). This leads to the formation of citrate, which is then transported to the cytosol and cleaved by ACL to generate cytosolic acetyl CoA, the precursor of FA, cholesterol and isoprenoid syntheses. ACL is an Akt phosphorylation target and its levels are increased downstream of Akt activation coordinately with those of lipogenic enzymes, including acetyl ACC and FAS. This increase can be attributed to increases in the activity of SREBP-1. The NADPH essential for fatty acid and cholesterol synthesis can be produced from the pentose phosphate pathway (PPP) downstream of glucose-6-phosphate generation by HK. High activities of ACL and ACC, coupled with high levels of citrate, lead to high amounts of malonyl CoA,

which is an allosteric inhibitor of CPT-1, the enzyme responsible for the translocation of FA into the mitochondria for FA oxidation (FAO). In addition, Akt phosphorylates and inactivates the transcription factor F-box protein subclass A FoxA2, which normally activates CPT-1 to stimulate FAO. Overall, Akt favors anabolic processes (glycolysis and fatty acid synthesis) and inhibits catabolic processes (fatty acid oxidation). ECM: extracellular matrix; OOA, oxaloacetate.

Source: Hatzivassiliou G et al. (2005) Drug Discovery Today: Disease Mechanism 2(2): 255-262.

1.7.2 *Positive feedback regulation between lipogenesis and signaling from various receptors*

As discussed above, stimulation of cell proliferation by signaling initiated from cell surface receptors and hormones causes up regulation of FAS activity. A common oncogene in breast cancer cells involved in the stimulation of cell proliferation is HER2 (erbB2/neu), an EGFR type receptor. Transcription of the FAS gene is directly downstream of HER2/neu signaling, as it was shown in an immortalized human mammary epithelial cell line H16N2 overexpressing HER2 gene [148]. Interestingly, inhibition of FAS activity markedly reduces expression of this oncogene in breast and ovarian cancer cell lines (SK-Br3, BT-474, MDA-MB-453 and SK-Ov3) [149]. At least two other reports described a similar response to the inhibition of FAS. Thus, depletion of FAS protein by RNAi results in loss of ERα in the human endometrial adenocarcinoma cell line (Ishikawa cells) [150] and chemical inhibition of FAS down-regulates phospho-Akt in ovarian carcinoma cells (SK-Ov3) [146]. These may suggest the existence of a positive feedback regulation between FAS activity and the expression of cell surface receptors. The mechanism responsible for this feedback regulation is presently unknown.

1.7.3 *Imaging of lipids and their metabolites in cancer cells*

Lipids and other metabolites within the body and in cultured cells are detectable with noninvasive techniques like magnetic resonance spectroscopy

(MRS). This technique, particularly clinical ^{1}H MRS imaging, repeatedly shows important differences in choline phospholipid metabolites of malignant versus benign breast tissues [151]. Recent evidence suggests that increased activity of choline kinase and phospholipase C in breast cancer cells could contribute to the effect [152]. Although, the molecular basis for the effect is still not well understood, choline metabolism is already considered as a potential target for anticancer therapy [153, 154].

1.7.4 *Dietary fatty acids and cancer*

Human cells are able to synthesize most of their FA *de novo* from glucose, but to a large extent dietary supply determines the composition of FA in body lipids. Many animal studies have been conducted to determine how different types of dietary fat affect the risk of developing cancer. They clearly demonstrated that not only quantity but also the type of fat is an important modulator of mammary tumorigenesis. A particularly important role in this process was assigned to the essential FA, those that cannot be synthesized by humans. These are omega ω-3 and ω-6 polyunsaturated fatty acids (PUFA), which contain a double bond near the methyl end of the molecule (Figure 5). The animal studies indicated that PUFA inhibit carcinogenesis, especially ω-3 fatty acids present in fish. However, the epidemiological studies in humans on the relationship between fish in the diet and cancer risk are not very consistent, so the effect of PUFA on cancer prevention in humans remains unclear [reviewed in 155]. All FA can be oxidized in mitochondria or peroxisomes to produce energy, but PUFA can also be metabolized by other specialized enzymes, such as cyclooxygenases (COX), lipooxygenases and cytochrome P450 monoxygenases, to produce eicosanoids, which are short-lived, hormone-like lipids (eg. prostaglandins, leukotrienes, thromboxanes and docosanoids). They have a wide array of biological activities, from modulation of inflammatory and immunological responses to effects on cell growth and differentiation. Dietary PUFA and their metabolites are also natural ligands of the peroxisome proliferator-activated receptors (PPARs), which act in a similar manner to other nuclear hormone receptors and transcription factors [reviewed in 156, 157].

The PPARs influence the expression of genes involved in the control of cell growth and differentiation. Thus PUFA could affect tumorigenesis in many different ways [reviewed in 158, 159]. However, more epidemiological studies are needed to clarify their effects on tumorigenesis.

Epidemiological studies on the effect of individual FA on breast carcinogenesis are extremely difficult to do in humans. The assessment of FA intake from reported dietary intake is very complex since converting food items into their FA content may be complicated by the fact that same foods may vary in their FA composition over the years due to different methods of food production and processing. One way to overcome this type of problems is to use biomarkers of dietary FA intake such as FA composition of adipose tissue, erythrocyte membranes, serum and plasma. Meta-analysis of published results correlating the composition of FA in biological samples with breast cancer risk suggest that ω-3 PUFA protect while monounsaturated FA (MUFA) and saturated FA (SFA) increase the risk of developing breast cancer [160].

1.7.5 *Effects of common nutrient fatty acids on breast cancer cells in culture*

For the last several years our laboratory has studied the effects of exogenous FFA on human breast tumor cells in culture. We have studied the effects of the long chain saturated FA palmitate (C16:0) and the monounsaturated FA oleate (C18:1) on cell proliferation and cell death. These are the most common dietary FA and are also the main FA in all tissues (for example mouse adipose tissue consists of 48% oleate and 18% palmitate). It is important to emphasize that these two FA are not the direct substrates for the above-mentioned oxidative enzymes which metabolize PUFA, neither are they good ligands for PPARs, although oleate was shown to activate PPARα [161].

The focus of our research has been on the effects of these FA as fuel substrates on breast cancer cell survival and proliferation. We have shown that they have profound effects on human breast cancer cells in culture. Palmitate induces apoptosis in a number of breast cancer cell lines and oleate has the opposite effect; it promotes cell proliferation and prevents apoptosis induced by palmitate [162].

Studies on the toxicity of palmitate in various cell systems have suggested that the most likely reason for its proapoptotic action is the stimulation of ceramide synthesis. Examples include hematopoietic cells [163], β-cells [164], cardiomyocytes [165] and astrocytes [166]. The results published from our laboratory have shown that ceramides may not be responsible for the apoptotic effect of palmitate on breast cancer cells. The effect is most likely related to a deficiency in mitochondrial cardiolipin (CL) brought about by an inadequate supply of unsaturated FFA and increased CL turnover during exposure to exogenous palmitate. We have found that palmitate and oleate are channeled to different metabolic pathways, and oleate protects against lipotoxicity induced by palmitate by channeling this saturated FA into intracellular lipid storage pools and by sustaining the synthesis of CL for mitochondrial membrane integrity [167].

1.7.6 *Fatty acids may exert their effects by binding to G-protein-coupled receptors*

It was recently demonstrated that FFA could act as signaling molecules by activating G-protein-coupled receptors (GPCR). Medium and long chain FFA are ligands for GPR40 and short chain fatty acids are ligands for GPR41 and GPR43 [reviewed in 168]. Another receptor (GPR120), for long chain unsaturated fatty acids, was recently discovered in the intestine [169]. GPR40 is expressed mainly in the human pancreas and possibly also in the brain. The GPCRs responding to other lipids [reviewed in 170] and various metabolites, including nicotinic acid and TCA cycle intermediates, such as succinate (GPR91) and α-ketoglutarate (GPR99), form a growing family of tissue specific receptors which might be involved in the coordination of the metabolism between different parts of the body [171, 172].

We have demonstrated expression of GPR40 in human breast cancer cells. Our data suggest that the effects of oleate on human breast cancer cells may involve signaling via this cell-surface G protein coupled receptor and activation of the PI3K-AKT survival pathway [173].

1.7.7 *Accumulation of TG induced by oleate may have more general significance in cell death/survival pathways*

We already mentioned that palmitate and oleate are channeled to different metabolic pathways, and that oleate is a particularly good precursor for TG formation. It is not clear why the metabolism of these two common FA would be so different, but it appears that this is a general phenomenon. Thus, the observations regarding the accumulation of lipids induced by oleate, the toxicity of palmitate, and the protective role of oleate versus palmitate were previously reported in other cell types: cardiomyocytes [174], human skin fibroblasts [175] and lymphocytes [176]. However, these studies did not deal with the mechanism and biological significance of this protection.

Evidence that TG rather then exogenous oleate, protect against cell death induced by saturated FA, comes from studies on overexpression of stearoyl-CoA desaturase, the enzyme responsible for synthesis of unsaturated FA from endogenous saturated precursors. The studies showed that elevated endogenous levels of monounsaturated FA result in the resistance to palmitate-induced lipotoxicity. In the same study it was also demonstrated that impaired of TG synthetic activity sensitize to lipotoxicity induced by both saturated and monounsaturated FA [177]. Therefore, the accumulation of TG promoted by exogenous and/or endogenous oleate may have an adaptive function and protect tissues against lipotoxicity.

We as well as other investigators have observed that monounsaturated fatty acids may also be protective against apoptosis induced by factors other than palmitate. For example, oleate prevents apoptosis induced by serum withdrawal in MDA-MB-231 human breast cancer cells [162] and palmitoleate (C16:1) protects against apoptosis induced by serum withdrawal and cytokines in rat β-cells [178]. This may suggest that accumulation of TG induced by oleate may have more general significance in cell death/survival pathways.

The link between TG accumulation and cell death/survival pathways is circumstantial at the moment. In the above mentioned studies (including our own) TG were produced by addition of oleate, but oleate was always present in the medium. Thus, one can argue that the antiapoptotic effect observed was exerted by

exogenous oleate itself, for example by signaling via the cell-surface G protein coupled receptor and activation of the PI3K-AKT survival pathway.

1.8 The aim of the present study

In the last decade, there has been a renewed interest in the metabolism of cancer cells. The exciting new findings obtained recently can be summarized as follows: 1) signaling pathways induced by GF affect metabolism, and just as importantly, metabolic pathways such as lipogenesis can modulate the expression/function of GF; 2) changes in metabolism observed in tumor cells are not just passive homeostatic adjustments to transformation or adaptation to the tumor microenvironment, they are induced by oncogenic mutations and can actively contribute to tumor progression; 3) some of the regulators of metabolism are tumor suppressors, which can promote the development of tumors and 4) metabolism of glucose and fatty acids contributes in various ways to the antiapoptotic effects of oncogenes. Clearly, cell metabolism is becoming an exciting area of research and the elucidation of the links between basic metabolic pathways and survival pathways may offer new therapies for cancer.

Breast cancer is one of those cancers for which life-style factors, such as obesity, diet and exercise, were shown to influence the risk of cancer development and have an impact on survival after diagnosis, but the molecular mechanisms involved are largely unknown. The endocrine function of adipocytes may well be one of the factors contributing to the development of breast cancer in obese postmenopausal women, since their adipose tissue is the main source of estrogen and can directly affect breast cancer progression. However, obesity is also independently positively linked to elevated blood FFA levels, which have been implicated in the development of various pathologies. The most common dietary FA and the main FA in our tissues are oleate and palmitate. We have evidence that oleate is a particularly good precursor for TG formation and it protects breast tumor cells against apoptosis induced by palmitate or by serum withdrawal.

The aim of the present study was to address the following hypothesis: **FA (especially oleate) present in excess in the blood of breast cancer patients suffering from metabolic conditions such as obesity or metabolic syndrome, contribute to the progression of breast cancer by promoting accumulation of TG in cancer cells, which help them to survive in unfavorable conditions, such as in the absence of appropriate GF.**

As it was mentioned above, some of the effects of oleate and palmitate that we observed in breast tumor cell lines were already described in different cell types, including cells not derived from tumors (neonatal cardiomyocytes, human fetal skin fibroblasts and mouse lymphocytes). We needed to determine if the effects that we investigated were specific to cancer cells or were more general and common to both cancer and non-cancer cell types. Thus, it was of interest to include as a control in our study, the MCF-10A cell strain, which is derived from normal human breast epithelial tissue. Therefore, our model system consisted of two cell lines: the MCF-10A non-transformed human breast epithelial cell strain, and the human breast tumor cell line MDA-MB-231. The studies were further extended to a panel of well-characterized human breast tumor cell lines: MDA-MB-468, T-47D and MCF-7. The survival and proliferation of MCF-10A cells in culture is controlled by several GF, including epidermal growth factor (EGF), hydrocortisone, insulin, in addition to other serum factors present in 5% fetal bovine serum (FBS). In contrast, MDA-MB-231 cells (and other breast tumor cells) can grow without the presence of the growth factors (EGF, hydrocortisone and insulin), but still require the unknown serum factors present in 5 % FBS to survive and proliferate *in vitro*. Thus, MDA-MB-231 cells are more autonomous, consistent with their origin from advanced, highly metastatic breast tumor.

Using these model cells we addressed directly the question of TG being involved in cell survival pathways. TG were produced inside the cells by exposure to exogenous oleate and then oleate was removed from the medium. This simple manipulation allowed comparison between cells containing elevated TG levels, easily detectable under microscope as lipid droplets, and the control, untreated cells.

Since cell survival is governed mainly by GF it seems reasonable to address the protective role of TG against death induced by withdrawal of serum and exogenous GF. Our model system was used to address the following specific questions:

#1: Could treatment with oleate and elevated TG content protect non-transformed, less autonomous MCF-10A cells against cell death induced by serum and GF withdrawal?

#2: Is elevated intracellular TG content in human breast cancer cells responsible for protection against serum withdrawal induced apoptosis and which mechanism(s) might be involved in this protection?

Here, we present evidence from this model system, which suggests that cell survival in GF-dependent "normal" cells cannot be altered by oleate, while survival of more autonomous tumor cells may be modulated by this fuel/nutrient. Our data *in vitro* is consistent with the proposed hypothesis about possible contribution of metabolism to the progression of breast cancer.

CHAPTER II
METHODOLOGY

Materials

Fatty acid sodium salts were purchased from Nu-Check, Prep (Elysian, MN). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA) and was heat inactivated at 56°C for 30 min. Fatty acid-free BSA (Fraction V, 96% essential FFA free), was obtained from Sigma (St-Louis, MO). [1-^{14}C] palmitic acid (55 mCi/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). [1-^{14}C] oleic acid (60 mCi/mmol), D-[U-^{14}C] glucose (317 mCi/mmol), and D-[5-^{3}H] glucose (17 Ci/mmol) were obtained from Amersham Biosciences (Baie d'Urfé, Quebec, Canada). [1,2^{14}C] acetic acid, sodium salt (113mCi/mmol) was obtained from ICN (Costa Mesa, California) and (+)-Etomoxir, Na-salt was obtained from HHAC Labor Dr. Heusler GmbH (Stutensee, Germany).

Special Solutions

5% BSA sterile solution was prepared by dissolving BSA (fatty acid-free) in Krebs-Ringer bicarbonate buffer containing 10 mM HEPES/pH 7.4 (KRBH). The pH was adjusted as necessary and the solution was filtered through a 0.22-μm filter. The solution was stored at 4°C for a week or at -20°C for 2-3 months.

Unlabeled albumin-bound fatty acids (oleate and palmitate) were prepared as follows: fatty acids sodium salts (≥99% purity) were stirred with 5% BSA (fatty acid-free) at 37°C for 16 h, then the suspension was filtered through a 0.22-μm filter, and the fatty acid concentration was measured using a NEFA C kit (Wako Chemicals USA, Inc. Richmond, VA). Concentration of stock solutions was adjusted to 4 mM, using 5% BSA. FFA stocks were stored frozen in aliquots and thawed only once.

Cell Lines and Culture Conditions

Tumorigenic human breast cancer cell lines MDA-MB-231, MDA-MB-468, T-47D and MCF-7, and the non-tumorigenic cell strain derived from normal human epithelial cells MCF-10A, were obtained from the American Type Culture

Collection (Manassas, VA). Cells were routinely cultured at 37°C in a humidified atmosphere with 5% CO_2 in a 50/50 mixture of Dulbecco's modified Eagle's Medium and Ham's F-12 (DMEM/F12; Wisent; St-Bruno, Quebec) containing 2 mM glutamine and 5% heat-inactivated FBS. For MCF-10A cells, DMEM/F12 was supplemented with insulin 10 µg/ml, hydrocortisone 0.5 µg/ml (both from Sigma, St-Louis, MO) and recombinant human epidermal growth factor (EGF) 20 ng/ml (Invitrogen).

All experiments were performed in minimal essential media (MEM) (phenol red-free; Sigma-Aldrich, Oakville, ON, Canada) as described below. Thus, MDA-MB-231 cells were cultured in MEM medium containing 5% fetal bovine serum (FBS), which will be referred to in this thesis as "tumor cell Growth Medium" (tGM) and MCF-10A cells were cultured in MEM medium containing 5% fetal bovine serum (FBS) and also the above-mentioned defined growth factors (insulin, hydrocortisone and EGF). This medium will be referred to in this thesis as "normal cell Growth Medium" (nGM). Apoptosis was induced in both cell types by placing them in "Experimental Medium" (EM), which was a MEM medium without serum or added growth factors

Treatment Protocols

Treatment protocols used are indicated in figure legends. When two cell types were compared, they were treated with the same protocol.

The majority of experiments with MDA-MB-231 cells were performed as follows: cells were plated in triplicate in 25 cm^2 flasks at a low density of 1 x 10^5 cells per flask in a growth medium (tGM). MCF-10A cells, when used for comparison, were plated at 2.5 x 10^5 cells/flask in nGM. After two days of growth, cells were washed with PBS and the medium was replaced with EM for 18 h. After this period of GF starvation, the medium was replaced with fresh EM containing albumin-bound oleate at concentrations ranging from 0 to 400 µM completed with 0.5% BSA. The final concentration of fatty acid free BSA was adjusted to 0.5%. For long-term survival

experiments, MDA-MB-231 cells were cultured in EM, after exposure to oleate for 24 h, and media were changed daily.

The majority of experiments with MCF-10A cells (sections 4.1.2 and 4.1.3) were performed as follows: cells were plated in triplicate, in 25 cm^2 flasks at a density of 2.5 x 10^5 cells/flask in a growth medium (nGM). MDA-MB-231 cells when used for comparison were plated at 1 x 10^5 cells/flask in tGM. After 24 h growth, the media was replaced with the fresh growth media containing albumin-bound oleate at concentrations ranging from 0 to 400 μM completed with 0.5% BSA. The final concentration of fatty acid free BSA was adjusted to 0.5%. Then cells were washed with PBS and subjected to GF starvation in fresh EM.

Cell Counting

To determine the number of adherent cells, culture dishes were washed with PBS and treated with trypsin for 2 min. 1ml of 0.05% solution of trypsin containing 0.53 mM EDTA (Wisent; St. Bruno, Quebec) was used per 25 cm^2 flask. The cells were collected with additional 2 ml of MEM containing 0.1% BSA and immediately counted using a hemocytometer. Cell number was calculated from a mean value obtained after counting cells in five individual squares. The number of cells in each square was usually between 50-100.

Cell Protein Assay

Cells were grown in individual 25 cm^2 flasks, washed once with PBS at room temperature (RT) and scraped using a plastic cell scraper in 3 ml PBS at RT. The cells were collected by centrifugation at 1,500 rpm (400 x g) for 5 min at RT and the cell pellet was lysed with 100 μl of lysis buffer containing 6.25 mM Tris pH 6.8 and 2% SDS and 6 M urea. The solution was sonicated on ice, 2 x 10 sec, using a sonicator at maximum setting (Vibra Cell; Sonics & Materials Inc., Danbury, CT). Protein concentration was determined using a BCA Protein Assay kit from Pierce (Rockford, IL) with albumin as a standard.

Caspase 3 Assay

Caspase 3 activity as an index of apoptosis was determined in cell lysates prepared from individual cultures using the protocol described in CaspACE™ Fluoremetric assay system (Promega, Madison, WI) with Ac-DEVD-AFC as the caspase-3 substrate (BIOMOL; Plymouth Meeting, PA). Briefly, after treatment both adherent and unattached cells were harvested and combined. After sedimentation at 500 x g for 10 min, the cells were washed twice with ice-cold PBS, lysed for 10 min on ice with a cell lysis buffer containing: 10 mM Tris-HCl/pH 7.5; 10 mM $NaH_2PO_4/NaHPO_4$; 130 mM NaCl; 1% Triton x-100; 10 mM sodium pyrophosphate and centrifuged (10 min, 15,000 x *g*, 4° C) to remove nuclei and debris. Fifty µg of proteins were incubated with 50 µM Ac-DEVD-AFC at 30° C. Fluorescence was analyzed using a FluoStar-Optima microplate reader (BMG Lab Technologies, Offenburg, Germany) in fluorescence mode using an excitation wavelength of 380 nm and an emission wavelength of 505 nm. The reaction was allowed to proceed for 30 min with a reading every minute. Caspase-3 activities were determined by calculating the slope of the reaction over 30 min.

Fatty Acid and Glucose Oxidation

Fatty acid oxidation was determined by measuring the amount of $^{14}CO_2$ liberated from samples incubated with [1-^{14}C] palmitic acid or [1-^{14}C] oleic acid using a modified procedure described in reference [179]. Briefly, cells were grown according to experimental procedure in duplicate 25 cm^2 flasks. Then, media were discarded and replaced with 0.9 ml of fresh MEM containing 0.1% BSA and incubated at 37°C for 30 min in a CO_2 incubator. Subsequently, 100 µl of the 10X reaction mix (prepared freshly 2 h in advance and containing 10 mM carnitine, 1 mM palmitate, 4% BSA and 0.5 µCi per culture flask of labeled fatty acid) was added to each flask. The flasks were immediately sealed with rubber serum vial stoppers. The stoppers were fitted with plastic tubes containing folded glass fibre

filter paper (Whatman GF/B) saturated with 0.15 ml of 5% KOH. The sealed flasks were incubated for 1 h at 37°C. Control blank flasks contained all reagents without cells. The reaction was stopped by injecting 0.3 ml of 40% perchloric acid through the serum stopper into each flask with a syringe and the flasks were then shaken gently for 24 h at RT. Filters were then removed and placed into scintillation vials containing scintillation liquid. Radioactivity was counted 24 h later, using a liquid scintillation counter (Tri-Carb 2100TR). Results were expressed as nanomoles of FFA released/h/mg of cell protein.

Glucose oxidation was measured by determining the release of $^{14}CO_2$ from [U-^{14}C] glucose. The experimental setup to capture $^{14}CO_2$ was as described above for FFA oxidation. Cells were preincubated in 0.9 ml MEM medium (with or without serum or with 0.1% BSA) for 30 min at 37°C in a CO_2 incubator (5% CO_2 and 95% air) after which 100 µl aliquot of D-[U-^{14}C] glucose diluted in MEM was added to each flask (0.5 µCi/flask). The flasks were then sealed and incubated for 1 h at 37°C. The reaction was stopped and the radioactivity captured by the filters was measured as described above. The results were expressed as nmol of glucose oxidized/h/mg of protein.

Glucose Utilization

Glucose utilization was measured as described in [180]. Briefly, cells were grown in duplicate 25 cm^2 flasks according to experimental procedures. The media were discarded and cells were preincubated with 0.9 ml of MEM media for 30 min at 37°C under CO_2, then 100 µl of D-[5-^{3}H] glucose in MEM media (1 mCi/flask) was added per flask and the cells incubated for 1 hr at 37°C. Adding 0.3 ml of 40% perchloric acid (PCA) to each flask stopped the reaction. 300 µl of cell culture supernatants were then spotted onto glass fiber filters (Whatman GF/B) and placed inside the caps of scintillation vials. The vials containing 600 µl of water were closed and left at RT for 3 days. Radioactive water evaporated from the filter samples and equilibrated with the water in the vials. Radioactivity was determined as described above after addition of 5 ml of scintillation fluid to each flask and closing

the flask with new caps. The results were expressed as cpm/h/mg of protein. Protein concentration was measured in cell extracts obtained from separate flasks treated in parallel but without addition of radioactive glucose or PCA (see cell protein assay).

Lipogenesis

Cells were grown according to experimental procedure in duplicate 25 cm^2 flasks. Then media were discarded and replaced with 1 ml of fresh MEM containing 0.1% BSA and incubated at 37°C for 1 h in a CO_2 incubator. Subsequently, 5 µl of the 5 times diluted [1,2 ^{14}C] acetic acid (1mCi/flask) was added to each flask and the cells were incubated for 4 hr at 37°C in a CO_2 incubator. Cells were then harvested on ice by scraping, washed twice with cold PBS and lipids were extracted as described below. The extracted lipids were dried under N_2, dissolved in a small volume of chloroform and analyzed by TLC as described below.

Lipid Extraction

Lipids were extracted from cells as described in reference [181]. Briefly, cells were harvested on ice by scraping, transferred to glass tubes and washed twice with 3 ml of cold PBS. After centrifugation at 500 x g for 5 min at 4°C, cell pellets were resuspended in 3 ml of methanol/chloroform mixture (2:1), vortexed and left at 4°C for 16 h. After this period of extraction, 0.8 ml of PBS, 1ml of 1 M NaCl and 1 ml of chloroform were added to each tube. Tubes were vortexed again and centrifuged at 500 x g for 5 min at 4°C to separate water the phase from the organic phase. The organic phase (at the bottom of the tube) was aspirated using glass Pasteur pipette and transferred to clean glass tube. The organic phase was then washed once with a mixture of 1 M NaCl/methanol (9:1) and collected as described above. The organic phase containing total lipids was stored at -20°C in closed tubes, under N_2.

Thin Layer Chromatography (TLC) of Neutral Lipids

The extracted lipids were dried under N_2, dissolved in 30 µl of chloroform and 15 µl was spotted on silica gel TLC plate (Whatman). The plate was then developed in a closed chamber containing mixture of petroleum ether: diethyl ether: glacial acetic acid (70:30:1). Lipids were visualized by staining with iodine vapors and classes of lipids were identified by comparison with standards (Sigma-Aldrich Corp., St. Louis, MO). Radioactively labeled lipids were visualized by autoradiography.

Triacylglycerol Assay

Cellular TG content was determined using the GPO-Trinder kit (Sigma Diagnostics, St-Louis, MO) and samples were prepared as described in reference [182]. MDA-MB-231 cells were plated in 75 cm^2 flasks at $3x10^5$ cells/flask. Three to four flasks were prepared to obtain cell numbers needed for an assay (two million cells were required for each sample). Cells were harvested on ice by scraping, washed twice with cold PBS and lipids were extracted as described above. The extracted lipids were dried under N_2, dissolved in 100 µl of 2% v/v Thesit detergent (Sigma, St-Louis, MO) in chloroform, dried again under N_2, and resuspended in 50 µl of water. The samples were then vortexed and sonicated in a water bath sonicator (Crest Tru-Sweep (50/60 Hz), Trenton, NJ) for 15 min at RT. Two hundred µl of GPO-Trinder kit reagent was added directly to each sample tube, mixed gently and incubated for 5 min at 37°C. The reaction mixture was transferred to a 1.5 ml microfuge tube and centrifuged at 10,000 rpm at 4°C for 5 min. The supernatants were transferred to a 96 well plate and the OD measured at 540 nm using a microplate reader (Bio-Rad 3550). Triolein (glyceryl trioleate; Sigma-Aldrich) was used as a standard.

Lipid Droplets Staining

Lipid droplets were visualized by staining with Oil Red O. The stock solution was prepared by dissolving 300 mg Oil Red O (Allied Chemical) in 100 ml of 99% 2-propanol. The working solution was prepared freshly by diluting the stock solution with water (3:2), kept at RT for 10 min and filtered through a 0.22 µm filter (Corning PES filter). Cells grown in Petri dishes (60 mm) were washed twice with 5 ml PBS and incubated with 3 ml of Oil Red O working solution for 15 min at RT with gentle shaking. The staining solution was removed and the cells were then washed once with PBS, and fixed with 10% formalin (Anachemia) for 25 min. The cells were then washed again with PBS and stained for 5 min with Harris Hematoxilin (Sigma-Aldrich). 3 ml of 10% glycerol in PBS was added to each dish to prevent drying. Photomicrographs were taken from a representative field using an inverted microscope (Nikon Eclipse TE300) at 400X magnification and a digital camera (Nikon CoolPix 990).

Lipolysis

MDA-MB-231 cells were plated in 75 cm^2 flasks at 3 x 10^5 cells/flask. Three to four flasks were prepared to obtain cell numbers needed for an assay (two million cells were required for each sample). Cells were trypsinized, pooled, resuspended in serum-free MEM medium containing 0.1% BSA and counted. The cells were then resuspended in fresh MEM medium containing 0.5% BSA at a density 2 x 10^6 cells/100 µl and incubated in 48 well plates (100 µl/well) for 3 h at 37°C in a CO_2 incubator. Samples were then transferred to Eppendorf tubes and centrifuged at 1,500 rpm for 5 min at 4°C. The supernatants were collected and frozen at -80°C. The rate of lipolysis was measured using two complementary assays. Glycerol released from the cells to culture media was measured in triplicate using the Triglyceride GPO-Trinder kit (Sigma Diagnostics, St-Louis, MO) and the amount of

FFA released was measured in duplicate using the Wako NEFA test kit (Wako Chemicals USA, Inc. Richmond, VA). Results were expressed as nmol glycerol or FFA released/h/10^6 cells.

FACS Analysis

DNA staining for FACS analysis was performed as described in reference [183]. Briefly, cells were harvested by trypsinization, washed with PBS containing 0.1% BSA at 4°C and fixed with 70% ethanol for 10 min at -20°C. They were then collected by centrifugation (1,000 rpm per 5 min) and resuspended in staining buffer prepared by adding NP40 (0.6% v/v) and 36 µg/ml RNase to a 1 x dilution of a 20 x stock (1.17 g of sodium chloride, 2.13 g of sodium citrate, and 0.10 g of propidium iodide in 100 ml of water, pH 7.6 adjusted with acetic acid). Stained cells (10,000) were analyzed with a flow cytometer (FACS SCAN, Beckton Dickinson) and cell cycle analysis was performed using Cell Quest Pro software.

Statistical Analysis

Statistical significance was calculated with the Student's t-test. A P-value of <0.01 was considered significant.

CHAPTER III
RESULTS

3.1 Elevated intracellular TG content is associated with resistance to apoptosis in MDA-MB-231 breast cancer cells but not in MCF-10A non-transformed cells

3.1.1 *Serum-free survival of MDA-MB-231 breast cancer cells after treatment with oleate correlates with the formation of intracellular lipid droplets*

Serum withdrawal is known to induce apoptosis in cells that require GF for survival. We have shown that exogenous oleate added to culture media promotes the accumulation of triacylglycerols (TG) [167] and protects MDA-MB-231 cells from apoptosis induced by serum withdrawal [162]. To determine if treatment with oleate and elevated TG content could protect non-transformed, less autonomous cells against cell death induced by serum and GF withdrawal, we performed experiments with human non-transformed breast epithelial cells MCF-10A. These cells require both uncharacterized growth factors present in 5% FBS, as well as defined growth factor supplements, including EGF, hydrocortisone, and insulin, to promote cell growth. They are therefore cultured in MEM medium containing 5% FBS and also the above-mentioned growth factors. This medium will be referred to in this thesis as normal cell Growth Medium (nGM) as opposed to tumor cell Growth Medium (tGM) used for culturing MDA-MB-231 cells and containing 5% FBS as the only supplement.

Figure 9A shows that treatment of MDA-MB-231 cells with oleate for 24 h, following 24 h of serum starvation, prevents apoptosis (caspase-3 activation) that occur after placing these cells in serum-free conditions. In contrast, the presence of oleate has no effect on survival of MCF-10A cells. They enter apoptosis after being transferred to serum and GF-free medium regardless of the presence of oleate (Figure 9B). We therefore performed metabolic studies to try to understand how oleate protected MDA-MB-231 cells and not MCF-10A cells from apoptosis induced by withdrawal of GF and serum. For the sake of simplicity, MEM medium without any additives (no serum and no defined GF) will be called Experimental medium (EM).

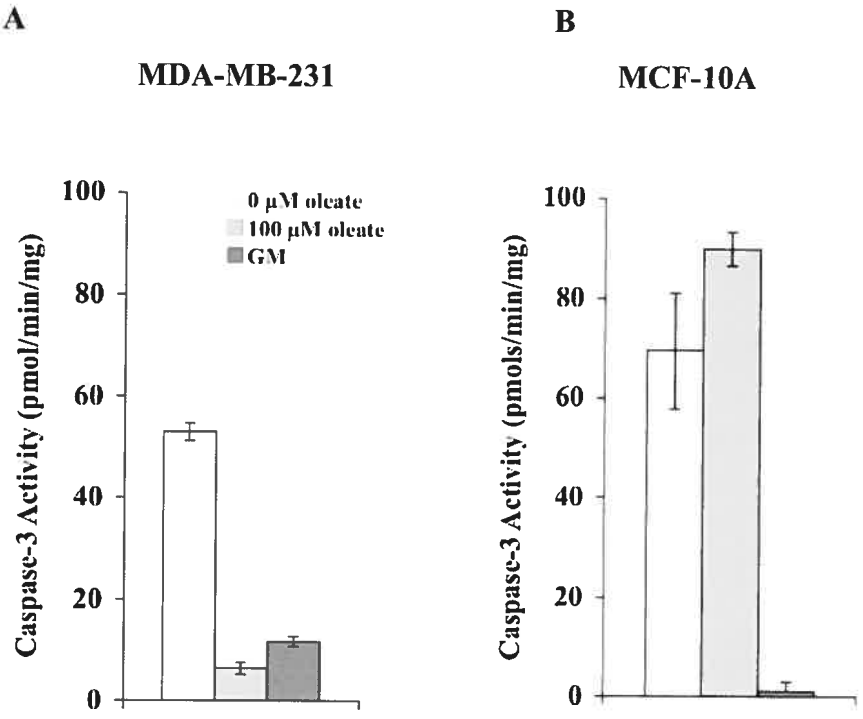


Figure 9. Effect of treatment with 100 μM oleate on apoptosis induced by serum and GF withdrawal in MDA-MB-231 and MCF-10A cells

MDA-MB-231 human breast tumor cells (*A*) and MCF-10A human breast epithelial cells (*B*) were grown in corresponding growth media (GM); tumor cell Growth Medium (tGM) and normal cell Growth Medium (nGM), respectively, as described in Methods. Cells were placed in Experimental Media (EM) for 24 h to starve for serum and GF, and then were treated with 100 μM oleate (bound to 0.5% BSA) for 24 h in fresh EM (light grey bars). Controls included 0 μM oleate (0.5% BSA only) (white bars) and corresponding growth media (dark grey bars). Caspase-3 activity was assessed in cell lysates prepared from individual cultures at the end of experiment. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

In various tissues, exogenous oleate, once taken up by the cell, can be channeled to oxidation or esterification, depending on the overall metabolic state of the body and the needs of an individual cell. Figure 10A shows that MDA-MB-231 cells have an almost 30-fold lower rate of oleate oxidation compared to MCF-10A cells. On the other hand, exposure to 100 μM oleate causes the formation of lipid droplets in MDA-MB-231 (Figure 10B) but not in MCF-10A cells (Figure 10C). Since the main components of lipid droplets are storage TG, the appearance of lipid droplets is a marker of oleate esterification and formation of TG. Thus, our results show that MDA-MB-231 breast tumor cells, which have a relatively low rate of fatty acid oxidation and a relatively high capacity for esterification and for storage of TG in lipid droplets, are protected by oleate from apoptosis induced by withdrawal of serum. In contrast, MCF-10A non-transformed breast epithelial cells, which have a relatively high rate of fatty acid oxidation and a relatively low capacity for esterifciation and storage of TG, are not protected by oleate from apoptosis induced by withdrawal of serum and GF.

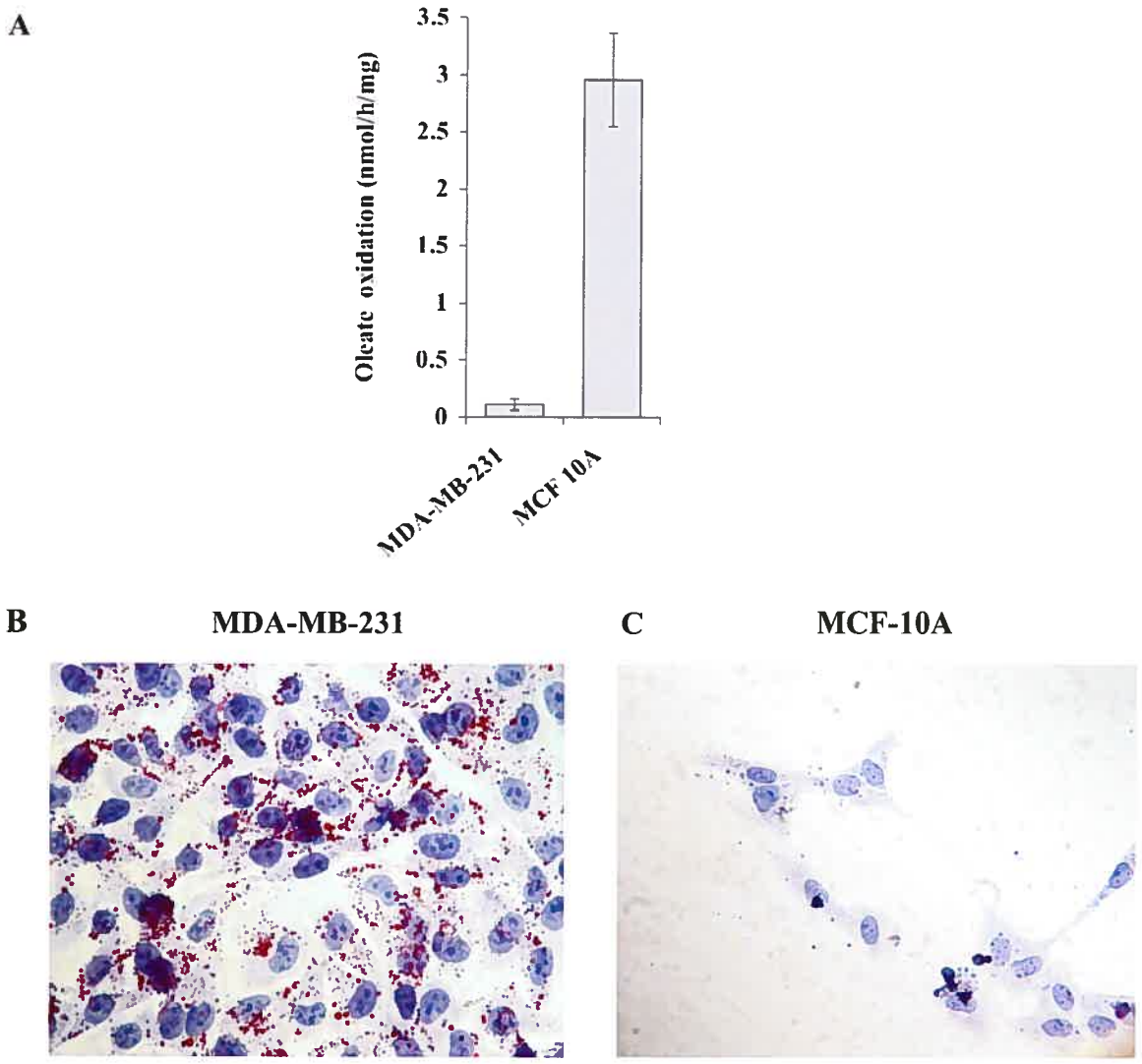


Figure 10. Differential oleate metabolism in two cell types: MDA-MB-231 and MCF-10A

Cells were grown and treated as in Figure 7 legend. ***A***, Oleate oxidation was determined by measuring the release of radioactive CO_2 from [1-^{14}C] labeled oleate at the end of 24 h serum and GF starvation period. The data represent the mean and SE for two independent experiments performed in duplicate (n=4). ***B***, ***C***, Representative photomicrographs (400X) of MDA-MB-231 (***B***) and MCF-10A (***C***) cells stained with Oil Red O to visualize neutral lipids after 24 h treatment with 100 μM oleate.

3.1.2 *Lipid droplet formation does not protect non-transformed MCF-10A cells from apoptosis induced by serum and GF withdrawal*

We tested whether MCF-10A cells would produce lipid droplets when treated with elevated concentrations of oleate. The cells were grown in nGM and then treated with a range of physiological oleate concentrations (100 μM and 400 μM) for 24 h in nGM. Figure 9 shows that 400 μM oleate induced formation of lipid droplets in MCF-10A cells, suggesting that these cells are able to esterify fatty acids and store TG in lipid droplets when treated with sufficiently high oleate concentrations. For comparison, MDA-MB-231 cells formed lipid droplets when treated with both 100 μM and 400 μM oleate (Figure 11). Furthermore, the size of the lipid droplets increased with increasing oleate concentrations. After the treatment with oleate, cells were washed with PBS and shifted to Experimental Medium (EM), which contains no serum or added growth factors for 48 h. MDA-MB-231 cells containing droplets were completely protected from apoptosis induced by 48 h incubation in EM (Figure 12A). It is important to point out that this protection occurred in the absence of exogenous oleate (48 h after treatment with oleate). In contrast, MCF-10A cells, even though they initially contained lipid droplets (after treatment with 400 uM oleate) were not protected against apoptosis when placed in EM for 48 h (Figure 12B).

Thus, MDA-MB-231 cells when treated with oleate formed lipid droplets and become resistant to apoptosis. The formation of lipid droplets however was not associated with the resistance to apoptosis in non-transformed MCF-10A human breast epithelial cells.

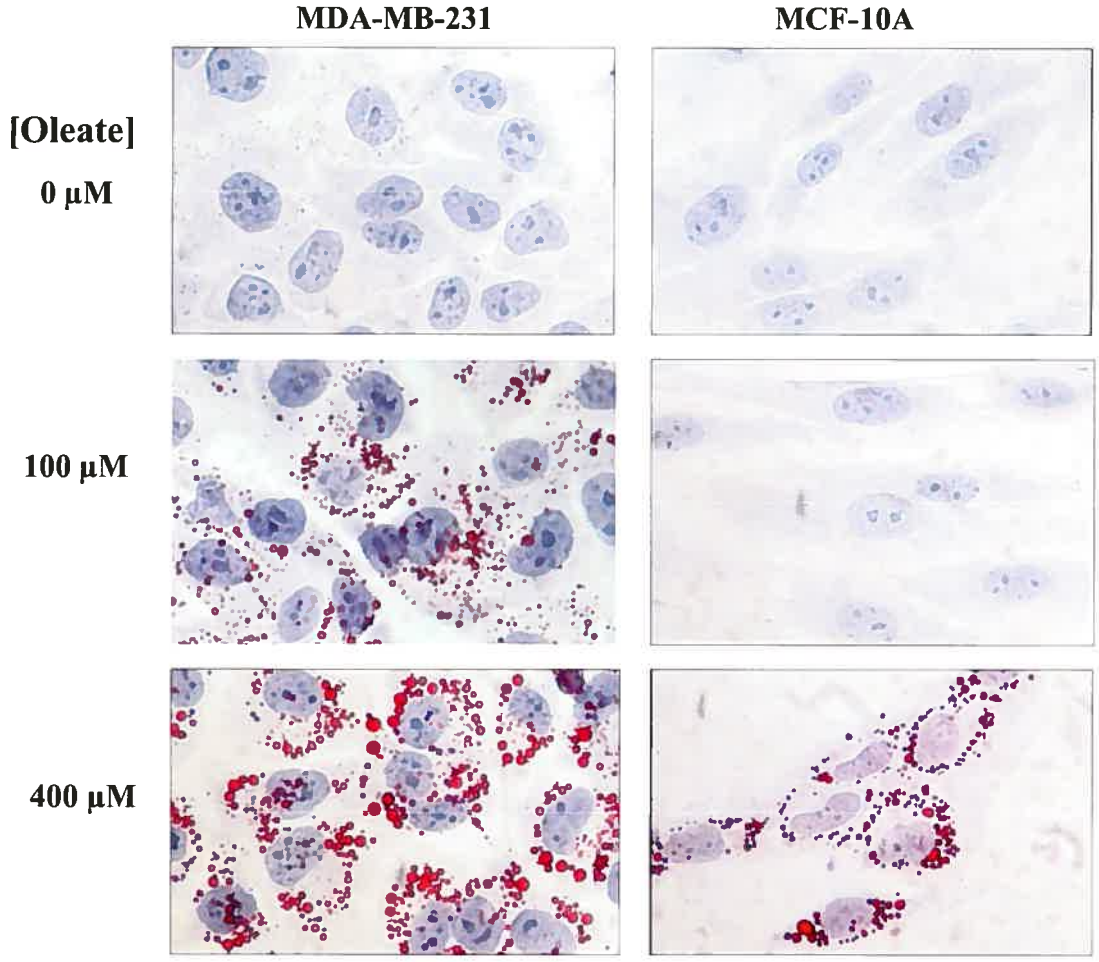


Figure 11. Effect of treatment with 100 µM and 400 µM oleate on lipid droplet formation in two cell types: MDA-MB-231 and MCF-10A

Cells were grown in corresponding growth media as described in Methods. The cells were treated with the indicated concentration of oleate in growth media for 24 h and representative photomicrographs (400X) of MDA-MB-231 and MCF-10A cells stained with Oil Red O were taken.

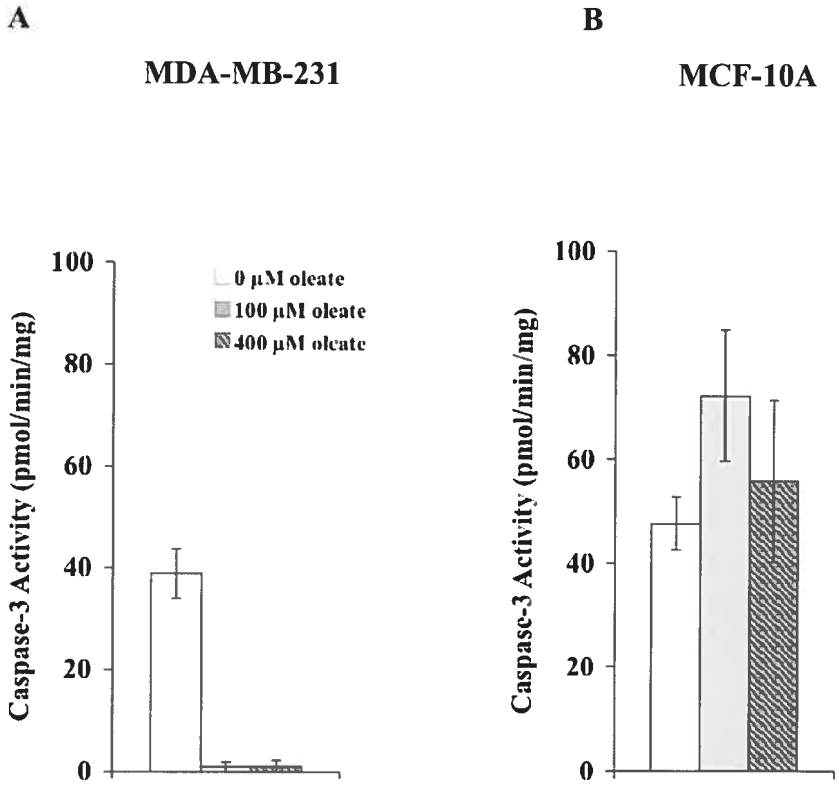


Figure 12. Effect of treatment with 100 µM and 400 µM oleate on apoptosis in two cell types: MDA-MB-231 and MCF-10A

Cells were grown and treated as in Figure 9 legend. Then, MDA-MB-231 (*A*) and MCF-10A cells (*B*) were washed in PBS and incubated for 48 h in EM (lacking serum and GF) to induce apoptosis. After this incubation period, caspase-3 activity was assayed in cell lysates. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

3.1.3 *Inhibition of fat oxidation in MCF-10A cells does not improve their survival in GF-free conditions*

The resistance to apoptosis in cells treated with oleate appeared to be inversely correlated with the rate of fatty acid oxidation in our model cell lines (see Figure 10). Therefore, we asked whether the inhibition of fatty acid oxidation could affect the survival of MCF-10A cells after they formed lipid droplets with elevated oleate concentration. Figure 13A shows that 200 nM etomoxir, an irreversible inhibitor of CPT I, the enzyme that catalyzes the rate limiting step in fatty acid oxidation, does not prevent apoptosis induced by GF withdrawal in MCF-10A cells treated with 400 μM oleate. Etomoxir reduced fatty acid oxidation by more than 90 % in these cells (Figure 13B). Thus, although the rate of fatty acid oxidation in etomoxir treated MCF-10A cells was similar to that of MDA-MB-231 cells, this did not render them resistant to apoptosis induced by serum and GF withdrawal.

3.1.4 *Glucose metabolism is GF dependent in MCF-10 A cells and GF independent in MDA-MB-231 cells*

Fatty acids and glucose are both fuel substrates and their metabolic pathways are interdependent. The rate of glucose uptake, and in consequence glucose metabolism is down regulated in many cell types when GF are withdrawn [67,68]. We decided to verify this in both cell lines by determining the effect of the Experimental Medium on the rate of glycolysis (glucose utilization) and the rate of glucose oxidation. Figure 14A and B shows that in MCF-10A cells glucose metabolism (both utilization and oxidation) is down regulated by about 65%, 24 h after serum and GF withdrawal. In contrast, glucose metabolism in MDA-MB-231 cells is not altered in EM (Figure 14C and D). MDA-MB-231 cells have a high rate of glucose metabolism, which is typical for cancer cells. At basal conditions (in corresponding growth media) glucose utilization and glucose oxidation were respectively 1.8 fold and 2.7 fold higher in MDA-MB-231 cells compared to MCF-10A cells.

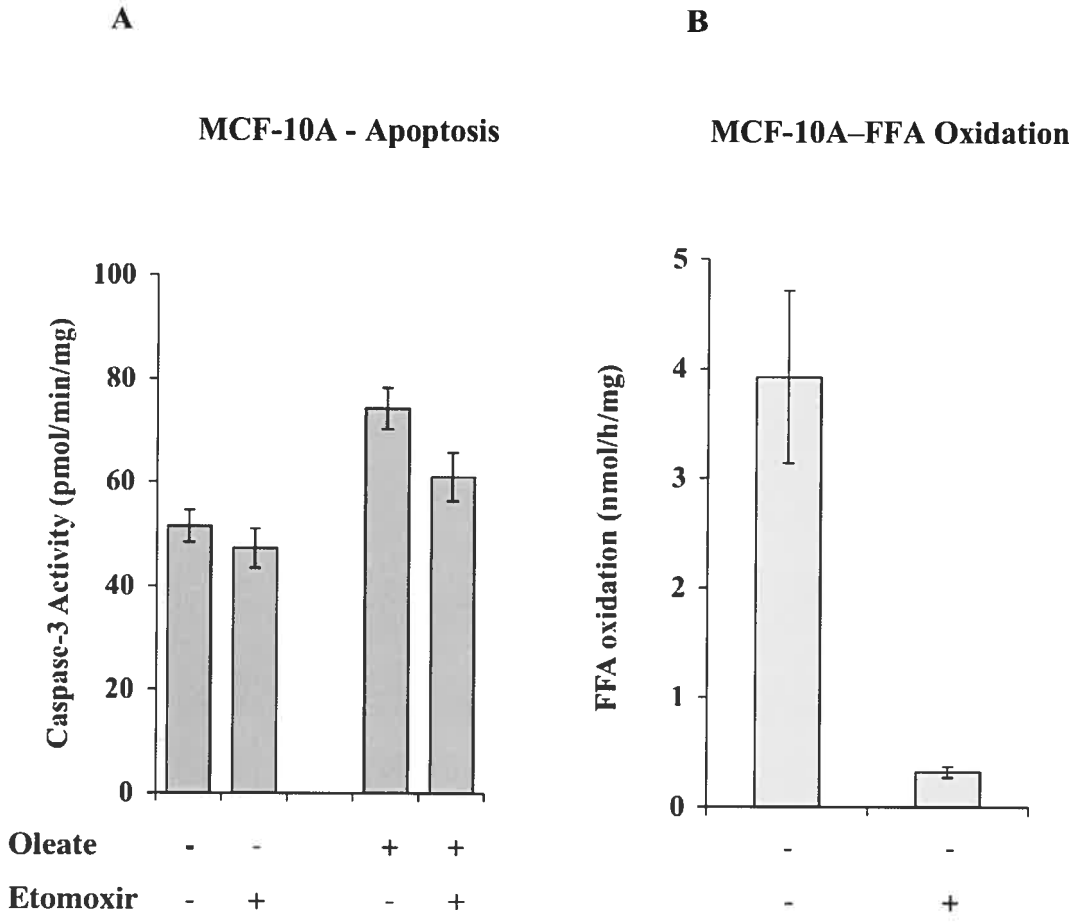


Figure 13. Effect of etomoxir, an inhibitor of fat oxidation, on apoptosis induced by serum and GF withdrawal in MCF-10A cells

A, Cells were grown in a growth medium (nGM) for 24 h and incubated without or with 400 μM oleate in fresh growth media for another 24. Then cells were washed with PBS, and exposed to EM, which did not contain oleate, with or without 200 nM etomoxir for 48 h. After this incubation period, caspase-3 activity was measured in cell lysates prepared from individual cultures. ***B***, Fatty acid oxidation was determined in control conditions (no oleate) by measuring the release of radioactive CO_2 from [1-^{14}C] labeled palmitate 2 h after the addition of etomoxir. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

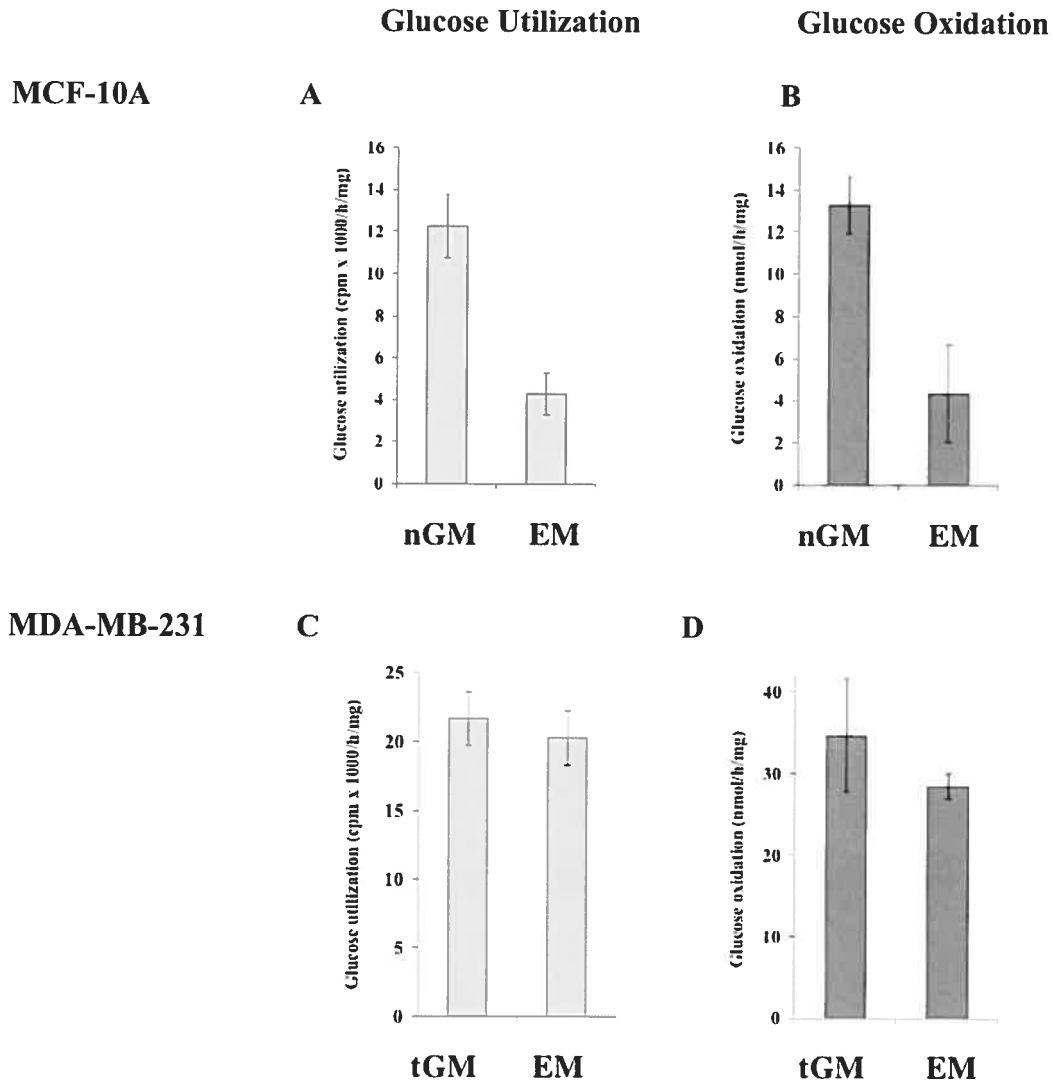


Figure 14. Effect of growth media vs. experimental media on glucose metabolism in MCF-10A and MDA-MB-213 cells

MCF-10A cells (***A, B***) and MDA-MB-213 cells (***C, D***) were grown in a corresponding growth medium (nGM or tGM respectively) for 24 h, washed with PBS, and incubated in EM for another 24 h. Glucose utilization (release of tritiated water from D-[5-^{3}H]-glucose (***A, C***) and glucose oxidation (release of labeled CO_2 from D-[U-^{14}C] glucose (***B, D***) were determined as described in Methods. Measurements were performed in GM before transferring cells to EM and then in EM, 24 h later. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

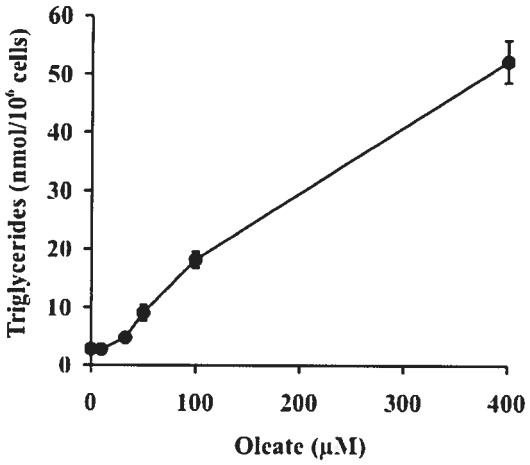
3.2 Upregulation of TG/FFA cycling by oleate is associated with long-term growth factor independent survival of MDA-MB-231 cells.

3.2.1 *MDA-MB-231 cells show high rates of TG/FFA cycling in serum-free conditions*

The ability of MDA-MB-231 cells to efficiently esterify oleate for storage as TG is illustrated in Figure 15A. Total intracellular TG content of the cells was found to increase in a dose dependent manner in response to 24 h treatment with increasing concentrations of oleate. TG stored in lipid droplets can be hydrolyzed to produce FFA and glycerol. This process (lipolysis) has been mostly studied in adipocytes, where it is carried out by the highly regulated enzyme, hormone sensitive lipase (HSL) in addition to other less studied lipolytic enzymes such as ATGL [111]. FFA that are released from TG by lipolysis can be immediately re-esterified within the cell to form TG again. Lipolysis and re-esterification are two opposite processes involved in what is referred to as intracellular TG/FFA cycling [127,128].

Lipolysis in MDA-MB-231 cells was studied using two complementary assays, in which the amounts of glycerol and FFA released into the cell media from the breakdown of TG were compared (Figure 15B and Table I). Figure 15B shows that MDA-MB-231 cells have a high rate of lipolysis as measured by glycerol released relative to their total TG content. Moreover, their rate of lipolysis increased dramatically with exposure to increasing concentrations of exogenous oleate. Following a 24 h incubation with the highest concentration of exogenous oleate tested (400 μM), cellular TG content was determined to be 52 nmol/10^6 cells and glycerol released during 1 hr was 35 nmol/10^6 cells. The results presented in Table I and Figure 15B suggest that MDA-MB-231 cells must re-esterify FFA very efficiently and have very active TG/FFA cycling.

A MDA-MB-231 cells – TG Content



B MDA-MB-231 cells – Lipolysis

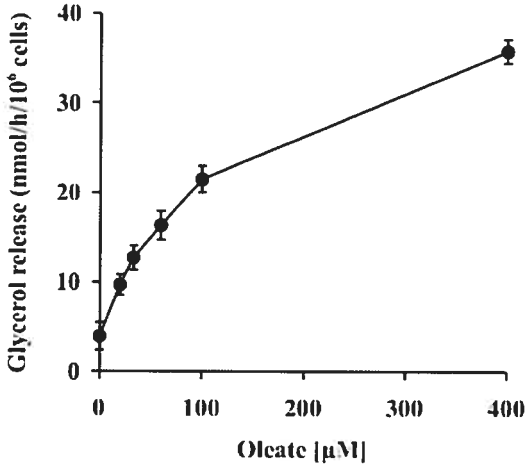


Figure 15. Effect of oleate concentration on cellular TG content and rate of lipolysis in MDA-MB-231 cells

Cells were grown in a growth medium (tGM), washed with PBS and placed in Experimental Medium (EM) for 24 h to starve for growth factors as described in Methods. Then the cells were treated with the indicated range of oleate concentrations (bound to 0.5% BSA) for 24 h in fresh EM. At the end of the treatment period, TG content (***A***) and lipolysis rate (***B***) were measured as described in Methods. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

Table I: Comparison between the cellular TG content after treatment with different oleate concentrations and the rate of lipolysis of MDA-MB-231 cells measured by two complementary methods (glycerol release and FFA release)

	TG content (nmols/10^6 cells)	Rate of Lipolysis	
[Oleate] (μM)		Glycerol released (nmols/h/10^6 cells)	FFA released (nmols/h/10^6 cells)
0	2.78 ± 0.25	3. 91± 1.51	<0.5
33	4.76 ± 0.37	12.72 ± 1.38	0.56 ± 0.22
100	18.25 ± 1.38	21.48 ± 1.45	0.84 ± 0.39
400	52.28 ± 3.62	35.8 ± 1.32	3.13 ± 0.18

MDA MB 231 cells were grown in a growth medium (tGM), washed with PBS and placed in Experimental Medium (EM) as described in Methods for 24 h to starve for growth factors. Then cells were treated with the indicated range of oleate concentrations (bound to 0.5% BSA) for 24 h in fresh EM. At the end of the treatment period, TG content and lipolysis rate (see Methods) were measured by two complementary methods. The results show that glycerol released from lipolysis greatly exceeds the amount of FFA released (instead of being 1/3 the amount), suggesting that FFA are retained inside the cells and most likely immediately re-esterified to TG. The data represent the means and SE for two independent experiments performed in duplicate (n=4).

The data presented in Table I show the rate of lipolysis measured by the two complementary methods and reveals that the values are not stoichiometrically related as might be expected. For the cells treated with 400 μM oleate, we found only 3.13 nmol/h/10^6 cells of FFA released, instead of 3 x 35.8=107.4 nmol/h/10^6 cells calculated from the results obtained using glycerol release (1 molecule of TG is composed of 1 glycerol and 3 FFA molecules). The rate of FFA oxidation in MDA-MB-231 cells (approximately 0.05 nmol/h/10^6 cells) cannot explain the discrepancy between the amount of glycerol and FFA released. The results therefore indicate that >95% of FFA are indeed recycled back to TG in these cells rather than being oxidized. The net effect is that these human breast cancer cells when exposed to exogenous oleate, both synthesize and maintain TG in lipid droplets very efficiently (Figure 15A), in spite of a very high rate of lipolysis (Figure 15B). These results suggest that both arms of the TG/FFA cycling pathway, esterification and lipolysis are very active and that the entire pathway is markedly upregulated by oleate in MDA-MB-231 tumor cells.

3.2.2 *Short-term oleate treatment promotes long-term serum-free cell survival in MDA-MB-231 human breast tumor cells*

To test the hypothesis that the presence of elevated levels of intracellular TG induced by exposure to exogenous oleate might be involved in the protection against apoptosis induced by serum withdrawal, we studied the long-term survival of MDA-MB-231 cells exposed briefly to oleate. After a 24 h treatment with various concentrations of exogenous oleate in EM, the cells were maintained in EM for an additional eight days (with daily changes of media) and the live adherent cells were counted daily. Figure 16A shows that serum-starved cells without oleate treatment died, however oleate-treated cells survived for many days. Short-term (24 h) treatment with 400 μM oleate afforded complete protection against apoptosis induced by serum withdrawal for at least 10 days, while lower concentrations of oleate provided partial protection in a dose dependent manner.

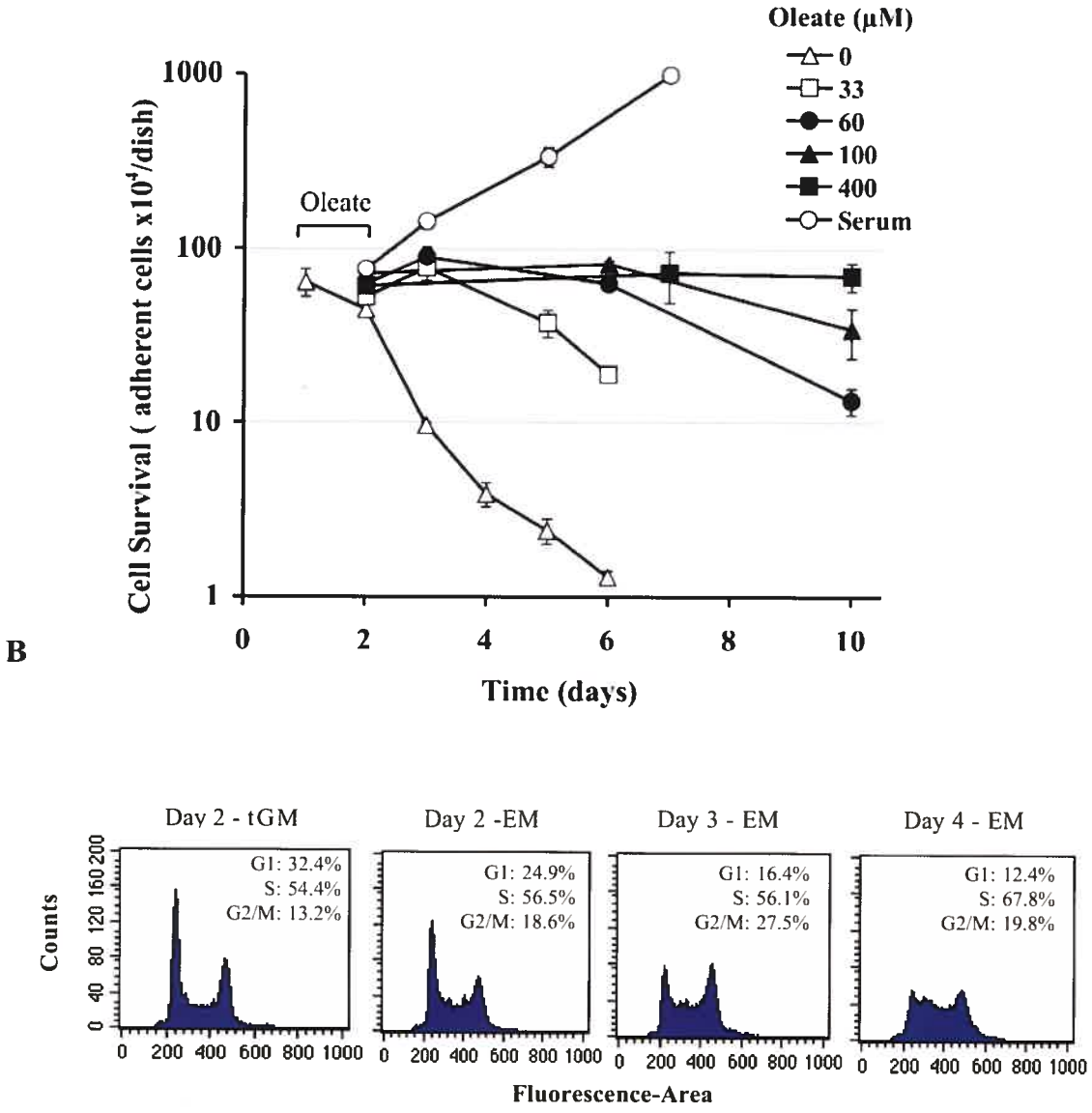


Figure 16. Effect of short-term oleate treatment on long-term serum-free survival and cell cycle distribution of MDA-MB-231 cells

A, Cells were grown in growth medium (tGM), washed with PBS (Day 0), and placed in experimental medium (EM) for 24 h (Day 1). Cells were treated with a range of oleate concentrations (0-400 μM) in EM for 24 h

(Day 2) transferred to fresh EM, and cultured for up to 8 days in EM. Viable cells were counted daily (see Methods) and the results were expressed as the mean number of live adherent cells/flask. The data represent the mean and SD from 3 separate flasks in a representative experiment, which was repeated two times. ***B***, FACS analysis for DNA content was performed on the indicated days using propidium iodide. Results for cells treated with 100 μM oleate are shown with untreated control cultures for comparison. The percentages of cells in the different phases of the cell cycle are shown inside the panels.

The percentage of live adherent MDA-MB-231 cells on Day 10 after the 24 h treatment with 400 μM oleate was 108 % relative to the cell number on Day 1 (Figure 16A). This suggests that cells protected from apoptosis by treatment with oleate were proliferating at a very low rate, if at all. Using FACS analysis, we determined the cell cycle distribution of serum starved and oleate treated MDA-MB-231 cells from Day 1 until Day 4 post serum withdrawal and compared these with the cell cycle distribution of cells grown in tGM (Figure 16B). A gradual decrease in the percentage of cells in G1 phase of the cell cycle was observed with a concomitant increase in the percentage of cells in S and G2/M phases. Together, these results (Figure 16A and Figure 16B) suggest that the cells rescued from apoptosis by oleate did not proliferate due to difficulty completing cell division.

Figure 17 shows that treatments as short as 3 h with 100 μM oleate protected human breast cancer cells from apoptosis induced by withdrawal of serum. This short pulse of oleate prevented activation of caspase-3 for up to 24 h. Remarkably, even a one-hour pulse was partially protective against apoptosis.

The results indicate that a short pulse of a physiological concentration of exogenous oleate protects MDA-MB-231 human breast cancer cells from apoptosis induced by serum withdrawal. The survival effect is oleate dose dependent and long lasting. Surviving cells slow down their proliferation and tend to accumulate in the S and/or G2/M phases of the cell cycle.

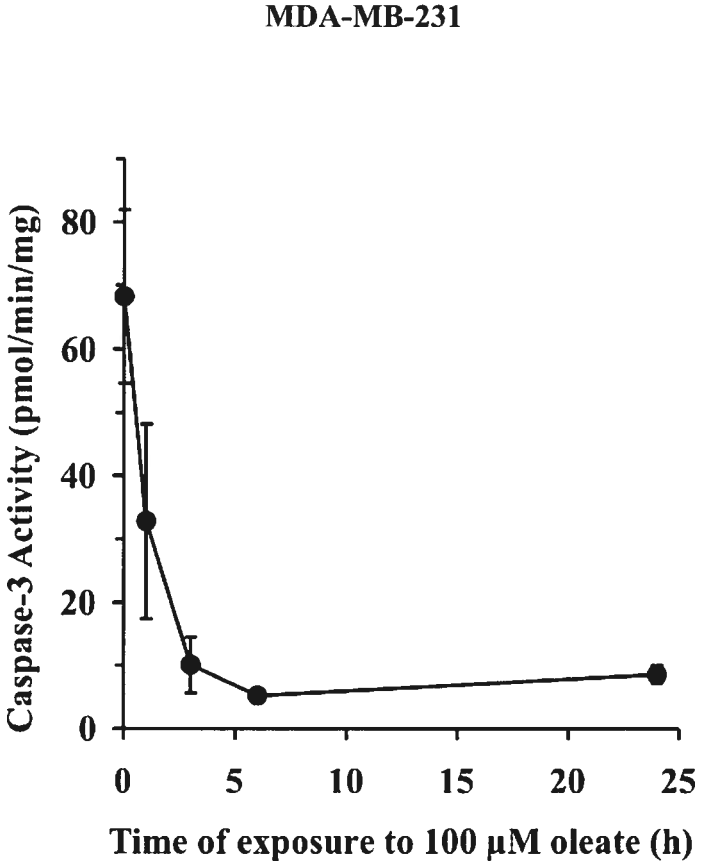


Figure 17. Effect of a short pulse of 100 µM oleate on apoptosis induced by serum withdrawal in MDA-MB-231 cells

Cells were grown in growth medium (tGM), placed in EM for 24 h, and washed with PBS. Cells were treated with 100 µM oleate for a range of time periods (1, 3, 6 and 24 h). They were then washed with PBS and incubated with fresh EM until the 24 h time point. Caspase-3 activity was assessed on total cells at the 24 h time point for all cultures. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

3.2.3 *Short-term oleate treatment causes a long-lasting change in cellular lipid metabolism in MDA-MB-231 cells*

Figure 18 shows that short-term (24 h) treatment of serum-starved MDA-MB-231 cells with 100 µM oleate leads to long-term stable (at least 7 days) elevated intracellular TG content (Panel A) and elevated lipolysis rate (Panel B). Thus, lipolysis remained high, between 20 and 17 nmol/h/10^6 cells, from Day 2 until Day 7, whereas within the same time period, cellular TG content was moderately lowered, from 20 to 12 nmol/10^6 cells. This suggests that during the 7 days post oleate treatment, the cellular TG pool continued to turnover at a high rate because of very active TG/FFA cycling.

3.2.4 *MDA-MB-231 cells rescued from apoptosis by oleate and cultured in serum-free conditions maintain a high rate of glucose oxidation*

Continuous TG/FFA cycling requires a constant supply of glycerol-3-phosphate, which can be produced by glycolysis. It was therefore important to assess the metabolism of glucose in MDA-MB-231 cells rescued from apoptosis by oleate. MDA-MB-231 cells have a high rate of glucose metabolism compared to MCF-10A and, contrary to these non-transformed cells, they maintain a high glucose oxidation rate after serum and GF withdrawal (Figure 14 and 19A). The measurements of glucose oxidation at Day 2, 48 h after serum withdrawal were performed on live adherent cells only (not on total cells), because at this time, the cells were beginning to enter apoptosis. At Day 3 post serum withdrawal, MDA-MB-231 cells rescued by short-term treatment with oleate still maintained a high glucose oxidation rate, while the majority of non-treated cells (> 90%) had detached from the dishes and were dead as determined by Trypan Blue staining. Thus, elevated glucose metabolism in MDA-MB-231 cells most likely provides glycerol-3-phosphate needed for fatty acids esterification and remains the main energy source in MDA-MB-231 cells rescued from apoptosis by short-term treatment with oleate. It is therefore unlikely that FFA released from droplets by lipolysis are needed as an alternative energy source (via fatty acid oxidation in mitochondria) to promote survival of these cells in

the absence of serum. Consistent with this hypothesis, MDA-MB-231 cells were found to have a very low rate of FFA oxidation (Figure 10A).

3.2.5 *MDA-MB-231 cells rescued from apoptosis by oleate and cultured in serum-free conditions synthesize de novo fatty acids*

We have shown that the cellular TG content of MDA-MB-231 cells increased and the rate of lipolysis was upregulated by oleate in a dose dependent manner and both effects persisted for days after oleate removal. This implies that during this time TG/FFA cycling was actively going on. We have also shown data suggesting that fatty acids are being recycled to maintain continuous synthesis of TG. However, the recycling of fatty acids is not completely efficient. Some of FFA released during lipolysis can be secreted outside the cell (1-2% as shown in Table I). Some fatty acids can be channeled to phospholipid synthesis. It was shown that MDA-MB-231 cells incorporate equal amounts of [^{14}C]-labeled oleate into TG and phospholipids during 1 h incubation [167]. Finally, some fatty acids are oxidized in mitochondria and peroxisomes. In spite of the above pathways that served to reduce available FFA for recycling, and the absence of oleate in EM to supply the TG pool in these cells, oleate treatment promoted the survival of MDA-MB-231 cells for many days. Thus we hypothesize that MDA-MB-231 cells synthesize FFA de novo from glucose. We have shown that MDA-MB-231 cells rescued from death by treatment with oleate metabolize glucose at a high rate; therefore it is likely that they produce sufficient amounts of pyruvate required for anaplerosis and de novo fatty acid synthesis. We suggest that newly synthesized fatty acids could supply the pool of endogenous FFA contributing to maintenance of the stable TG levels in these cells. Figure 19 B shows that indeed MDA-MB-231 cells rescued from death by short–term treatment with oleate, synthesize fatty acids *de novo* from acetate, and produce TG containing these newly synthesized FFA.

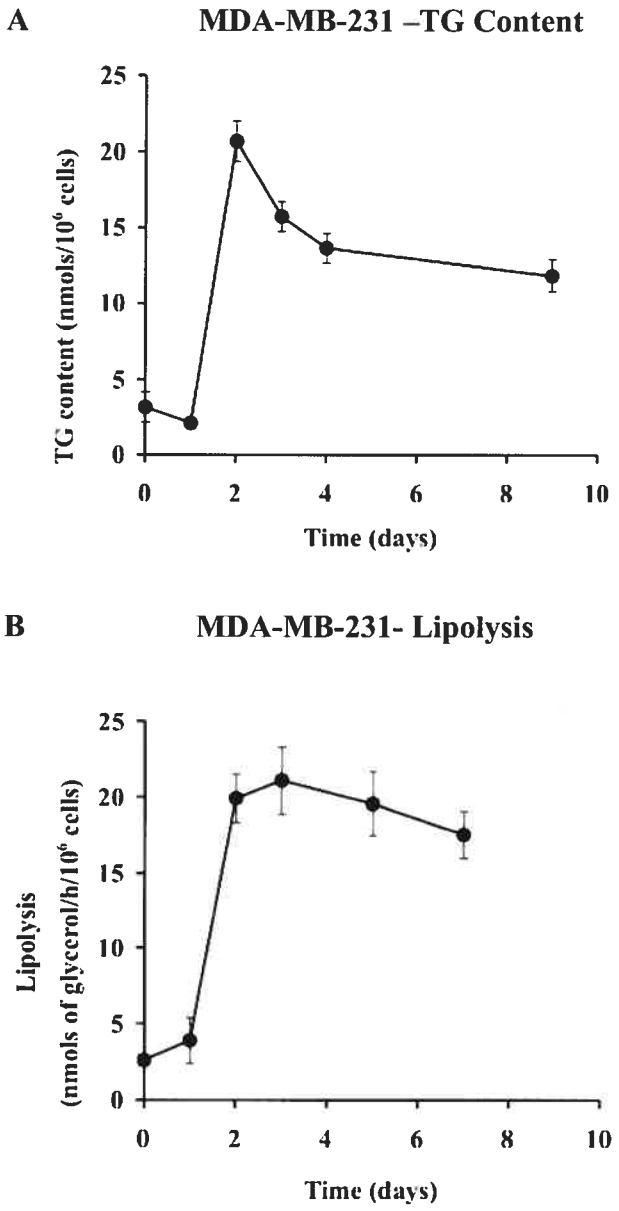


Figure 18. Long-term effect of 24 h treatment with 100 µM oleate on TG content and lipolysis rate in MDA-MB-231 cells

A, ***B,*** Cells were grown in growth medium (tGM), placed in EM for 24 h, washed with PBS, and treated with 100 µM oleate for 24 h in EM. Subsequently cells were cultured for up to 7 additional days in EM. Total cellular TG content (***A***) and lipolysis rate (***B***) in adherent cells was determined at the indicated time points. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

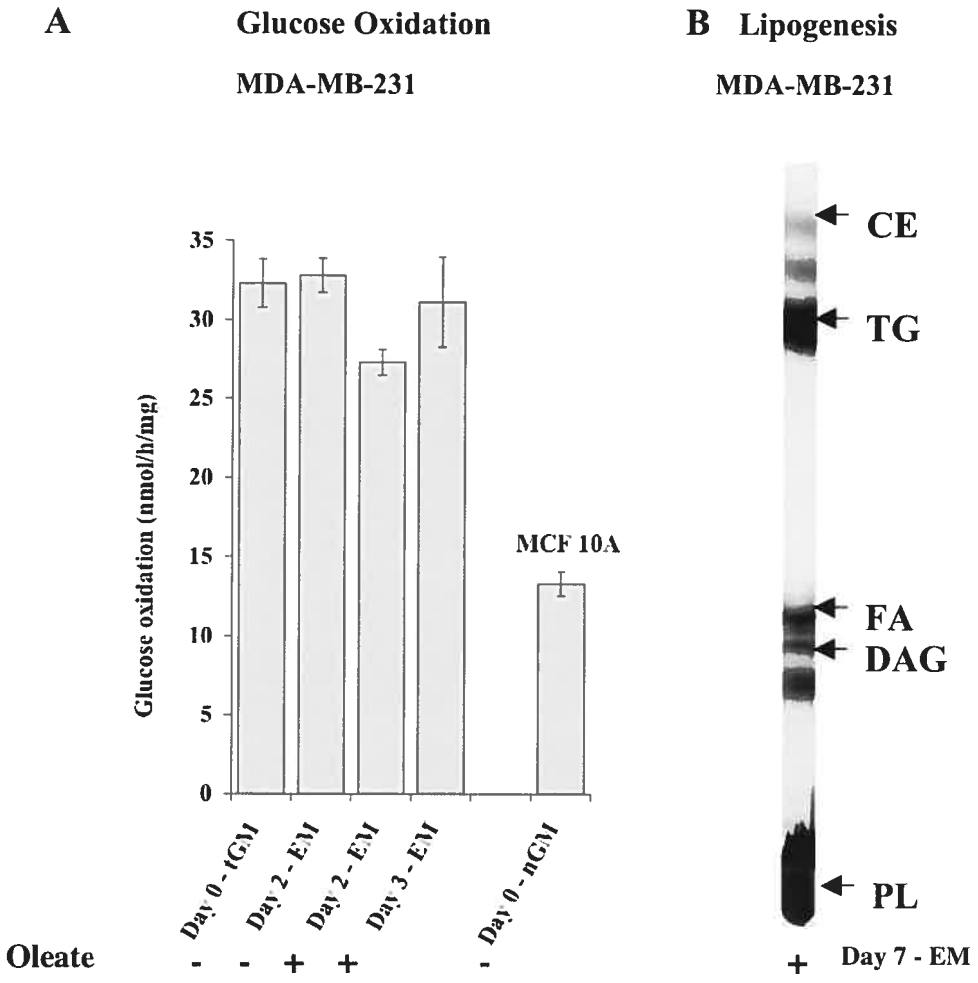


Figure 19. Long-term effect of 24 h treatment with 100 μM oleate on glucose oxidation rate and lipogenesis (fat synthesis) in MDA-MB-231 cells

A, ***B***, Cells were grown in growth media, placed in EM for 24 h, washed with PBS, and treated with 100 μM oleate and 0 μM oleate (0.5% BSA) for another 24 h. Subsequently, cells were cultured for up to 7 additional days in EM. *A*, Glucose oxidation was measured on the indicated days (see Methods) in MDA-MB-231 cells. Oxidation rate in MCF-10A cells is also shown for comparison. The data represent the mean and SE for two

independent experiments performed in duplicate (n=4). ***B***, Lipogenesis was measured in MDA-MB-213 cells on Day 7 as described in Methods. The autoradiogram shown represents a TLC plate on which total lipid extracts were separated. The markers used to identify the different class of lipids were non-radioactive; they were visualized by staining with iodine vapors. Abbreviations: CE, cholesterol ester; DAG, diacylglycerol; FA, fatty acids; PL, phospholipids; TG, triacylglycerol.

3.2.6 *High capacity to store TG is correlated with enhanced serum-free survival in various human breast cancer cell lines*

The effect of oleate treatment on cell survival was tested in three other human breast cancer cell lines; together with the non-transformed human breast epithelial cell strain MCF-10A (Figure 20). The design of the experiment was similar to that shown in Figure 16A and cells were counted on Day 6. The results are expressed as a percentage of the cell number at Day 1 (before the addition of oleate). It should be noted that each of the different cell lines had different growth characteristics. MCF-10A was the only cells line in our panel that needed supplementary GF for survival and proliferation *in vitro*. As shown in Figure 9 and 12, MCF-10A cells were very sensitive to serum withdrawal and were not protected by oleate treatment when maintained in EM. T-47D and MDA-MB-468 human breast tumor cells responded similarly to oleate treatment as did MDA-MB-231 cells, although the three cell lines differed in their sensitivity to serum withdrawal, with MDA-MB-231 being the most sensitive. Oleate treatment had no effect on cell survival in the absence of GF of the fourth cell line tested, MCF-7. The three cell lines that responded to oleate treatment accumulated high levels of cellular TG when exposed to 100 μM oleate (Figure 21A), whereas the cell line that did not respond to oleate (MCF-7) had low total TG content, as did MCF-10A cells. These results further confirm that a high capacity to store TG is associated with enhanced serum-free cell survival after treatment with oleate in three of four human breast cancer cell lines tested.

FFA esterification to form complex lipids, in particular TG, represents one of two main metabolic pathways for fatty acids within the cell, the other being fatty acid oxidation. It was of interest to examine the relative rate of fatty acid oxidation in our panel of cell lines. We expected to see an inverse correlation between oxidation and esterification. Figure 21B shows that MDA-MB-231 and T-47D cell lines, which have high capacity to store TG, had a low intrinsic fatty acid oxidation rate, while MDA-MB-468, MCF-7 and MCF-10A cell lines showed high fatty acid oxidation rates. Surprisingly however, MDA-MB-468 cells (but not MCF-7) also had a high capacity to produce TG when exposed to exogenous oleate. Thus, the rate of fatty acid oxidation of the different human breast cancer cell lines tested was variable (high and low) and did not correlate with the ability to store cellular TG or the long-term serum-free survival after short-term treatment with oleate.

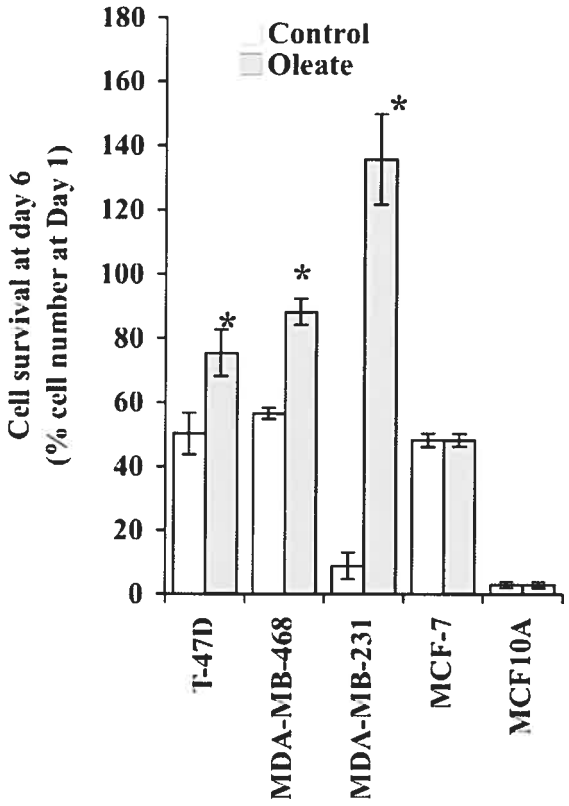


Figure 20. Effect of short-term oleate treatment on long-term serum-free survival of a panel of human breast cell lines and control MCF-10A cells

Cells were grown in growth medium (tGM), washed with PBS, and then placed in EM for 24 h (Day 1). The cells were treated with a 100 μM oleate for another 24 h in EM (Day 2), and then cultured for 6 days in EM. Viable cells were determined on Day 6 as described in Methods and the long-term serum-free cell survival was calculated as the mean percentage of the cell number at Day 1 (grey bars). Untreated controls received 0.5% BSA (white bars) and were also calculated the same way. The data shown are the means and SE for 2-4 independent experiments performed with triplicate flasks per experiment per cell type (n=6 for T-47D, n=9 for MDA-MB-231, n=12 for MDA-MB-468, n=8 for MCF-7 and n=6 for MCF-10A). *, $p < 0.01$ *versus* respective control.

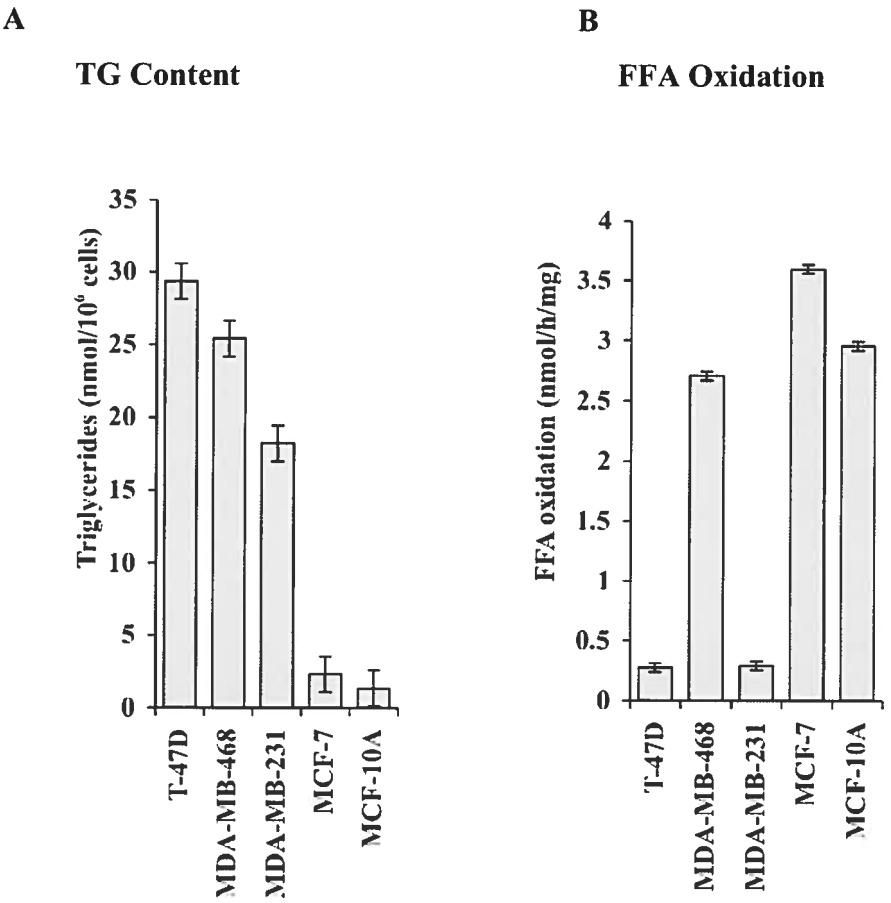


Figure 21. Effect of short-term oleate treatment on cellular TG content and FFA oxidation of a panel of human breast cell lines and control MCF-10A cells

A, Cells were grown in growth media (tGM), washed with PBS, placed in EM for 24 h (Day 1), treated with a 100 μM oleate in EM for another 24 h (Day 2), and immediately assessed for TG content. ***B***, Fatty acid oxidation rate was determined on Day 1 as described in Methods. The data in Panel ***A*** and ***B*** represent the mean and SE for two independent experiments performed in duplicate (n=4).

CHAPTER IV
DISCUSSION AND CONCLUSIONS

4.1 Upregulation of TG/FFA cycling by oleate may be involved in the maintenance of long-term serum-free survival of human breast cancer cells

We have shown that the common nutrient fatty acid oleate prevents apoptosis and promotes long-term cell survival in the absence of growth factors in three out of four human breast tumor cell lines (MDA-MB-231, MDA-MB-468, and T47D). The cells were protected for many days from apoptosis and death induced by serum withdrawal, by short-term (3-24 h) treatment with physiological concentration of oleate (100 μM). This long-term survival effect occurred in the absence of exogenous oleate and was associated with the accumulation of TG. The period of survival was oleate dose dependent as shown in MDA-MB-231 cells, even though oleate was no longer present in the media. The cells proliferated very slowly if at all in serum-free conditions, suggesting that treatment with oleate influences mainly cell survival with little effect on cell proliferation. After short-term treatment with oleate, the cellular TG pool enlarged and the rate of lipolysis (measured as glycerol released into the culture medium) increased in a dose dependent manner. Subsequently, both TG levels and the rate of lipolysis remained high for a long period of time (8-10 days).

MDA-MB-231 cells have very efficient TG/FFA cycling. Thus, they recycle FFA to maintain elevated TG content in spite of very active ongoing lipolysis. This suggests that they also have a very high rate of FFA esterification. Despite the absence of serum, glucose metabolism remains high in MDA-MB-231 cells rescued from apoptosis by short-term treatment with oleate. Thus, glucose serves as the main energy source and provides the glycerol-3-phosphate needed for FFA esterification. It is attractive to propose that the ability to efficiently esterify oleate and to maintain elevated TG content, due to upregulated TG/FFA cycling, plays a role in the antiapoptotic effect induced by treatment with oleate. Moreover, it may also be involved in the maintenance of long-term survival of human breast cancer cells in the absence of serum.

Cellular TG/FFA cycling occurs in many tissues. It is a multi-step, complex cycle involving many enzymes. It has been considered a "futile cycle" since its main

outcome is the consumption of energy (the production of 1 molecule of TG consumes 6 molecules of ATP). However, it allows the preservation of intracellular pool of fatty acid, when they are not oxidized for energy production and has been linked to thermogenesis in brown adipose tissue [130]. The present study suggests a novel role for TG/FFA cycling, which is enhancement of human breast cancer cell survival.

4.2 Oleate cannot modify survival of GF-dependent non-transformed cells

The relationship between the regulation of cellular metabolic pathways and the signaling pathways for cell survival and proliferation is not well understood and is only beginning to be discovered. Survival of non-transformed MCF-10A cells depends on the presence of EGF, hydrocortisone and insulin in the culture medium. We have shown that glucose metabolism decreases considerably in these cells in serum and GF-free conditions. Downregulation of the metabolism of glucose in MCF-10A cells upon withdrawal of GF is consistent with the notion that GF regulates cell survival by controlling cell access to nutrients in addition to inhibiting apoptosis [75,184]. Thus, MCF-10A cells could suffer from atrophy when they are withdrawn from GF, even in conditions of nutrient abundance. MDA-MB-231 breast cancer cells, on the other hand, maintain a high rate of glucose oxidation even in serum-free conditions. Thus, we would like to propose that oleate can promote survival of certain cancer cells like MDA-MB-231 that acquire upregulated and GF independent metabolism of glucose. In contrast, it cannot modify survival of GF dependent cells like MCF-10A. This is consistent with the hypothesis described above, which suggests that oleate induced protection against cell death involves upregulation of TG/FFA cycling. TG/FFA cycling requires a source of glycerol-3-phosphate for continuous FFA esterification. This requirement can be met in cells like MDA-MB-231 at serum-free conditions, but not in MCF-10A cells, which downregulate the metabolism of glucose upon withdrawal of serum and GF.

4.3 Model of Alternative NAD+ regeneration system linking TG/FFA cycling to the metabolism of glucose

We have shown that the TG pool in MDA-MB-231 cells is relatively constant for a long period of time after short-term treatment with oleate. Our data shows that TG/FFA cycling is upregulated and *de novo* fatty acid synthesis could supply the FFA pool that is required for cycling. We have shown that the rate of lipolysis is also constant, and depends on cellular TG content. Figure 22 presents a schematic model, which describes how we think the metabolic pathways for FFA, TG, glucose, and NAD are linked into a larger metabolic circuit that can be upregulated by the initial supply of exogenous oleate. FFA are esterified to form TG. The increase in the TG content is proportional to exogenous oleate dose. The TG pool is not inert but is constantly turning over due to high rates of both lipolysis and esterification involved in TG/FFA cycling. The rate of cycling increases as a function of the original exogenous oleate concentration. Cycling needs G3P for esterifcation to produce TG, the production of which is coupled to NAD^+ regeneration. Therefore, the high rate of TG/FFA cycling sets the high rate of NAD^+ regeneration, which in turn maintains the high rate of glycolysis. This allows the production of sufficient amounts of pyruvate required for anaplerosis and de novo fatty acids synthesis. De novo synthesized FFA support TG production and completes the circuit.

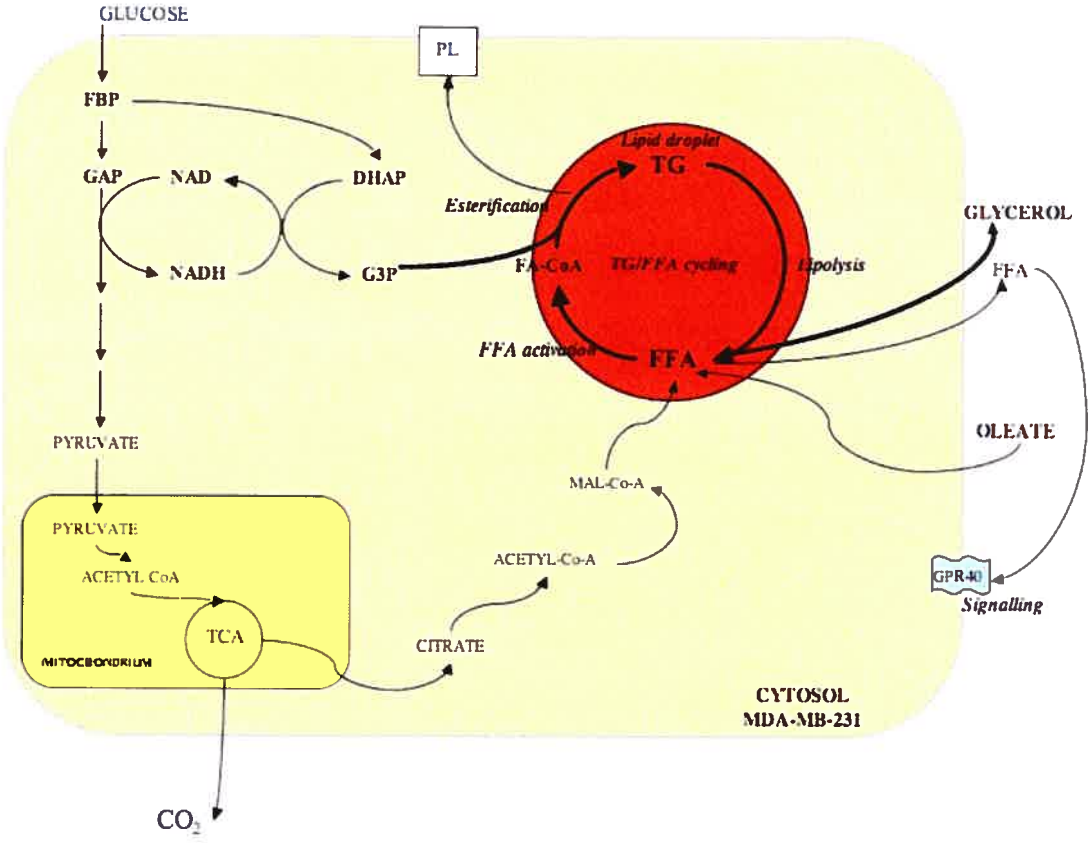


Figure 22. Model of the Alternative NAD Regeneration System, which sets a stable rate for cytoplasmic NAD^+ regeneration

Exogenous FFA (oleate) enter the cell and are activated to form FA-CoA, which is esterified to glycerol-3-phosphate (G3P) to form TG. Cellular TG content increases proportionally to the exogenous oleate dose. The TG pool is not inert but is constantly turning over due to high rates of both lipolysis and esterification involved in TG/FFA cycling. The rate of TG/FFA cycling increases as a function of the original exogenous FFA (oleate) concentration. TG/FFA cycling needs G3P, the production of which is coupled to NAD regeneration. Therefore, the high rate of TG/FFA cycling sets the stable rate of cellular NAD regeneration, which in turn allows the

maintenance of a high rate of glycolysis. This allows the production of the sufficient amounts of pyruvate required for anaplerosis and de novo FFA synthesis, which can supply the pool of FFA and contribute to continuing TG/FFA cycling. FFA released from the cell can bind to GPR40, the FFA receptor, to initiate intracellular signaling cascade.

Abbreviations: FBP, fructose 1,6-bi phosphate; GAP, glyceraldehydes-3-phosphate; DHAP, dihydroxyacetone phosphate; G3, glycerol-3-phosphate; TCA, tricarboxylic acid cycle; MAL-CoA, malonyl CoA; FFA, fatty acids; FA-CoA, Fatty acyl CoA; TG, triglycerides.

4.4. TG/FFA cycling might preserve the pool of FFA to ensure long-term signaling via GPR40

As it was mentioned above, MDA-MB-231 breast cancer cells appear to have acquired autonomy in controlling their access to glucose, but their apoptotic machinery can still respond to a lack of serum growth factors. The best-studied system responsible for cell survival and extracellular nutrient uptake is the PI3K/AKT pathway [reviwed in 73]. Our previous work [173], as well as that of others [185], demonstrated that this pathway could be stimulated via the G protein coupled FFA receptors GPR40 and GPR120. It is possible that the antiapoptotic effect described here, which involves upregulation of TG/FFA cycling, is also in part GPR40 mediated. Thus, TG/FFA cycling which preserves the pool of FFA inside the cells for long periods of time would ensure the continuous availability of small amounts of FFA released into the medium upon lipolysis, for binding to the GPR40 receptor and activation of the survival signaling cascade.

4.5. Relevance of the oleate-induced long-term survival effect to cancer

The human breast cancer cell lines used in this study are derived from metastases, so they represent aggressive, advanced breast tumors [186]. The ability of oleate to modify their survival *in vitro* appears to be independent of their ER status. However, it seems to be positively correlated with their invasiveness. Thus, two cell lines, which responded to oleate, were estrogen receptor (ER) negative (MDA-MB-231, MDA-MB-468), while another one was ER positive (T-47D). Furthermore, MCF-7 cells that did not respond to oleate at all are the least invasive of the cancer cell lines used in this study, while MDA-MB-231 cells, which were the most responsive to oleate, are known to be the most invasive [187]. Additional experiments are required to determine whether the response to oleate might provide a good prognostic marker of breast cancer cell invasiveness.

MDA-MB-231 cells rescued from death by treatment with oleate were viable for long periods of time but blocked in cell division. In this respect, they resemble disseminated tumor cells (DTC), which have been detected in the bone marrow of patients with breast tumors [188]. Most DTC are not actively proliferating and remain in a state of "dormancy" for many years [reviwed in 43]. The Persistence of DTC is associated with a poor clinical outcome [189]. It would be tempting to speculate that oleate could contribute to the survival of DTC via upregulating TG/FFA cycling. Furthermore, we suggest that upregulation of TG/FFA cycling, which may confer resistance to apoptosis, may also contribute to the drug resistance common in DTC [190]. More evidence, especially studies *in vivo* with primary tumors and metastasis, are needed to verify if the TG/FFA cycle could be indeed a marker of tumor invasiveness or could contribute to DTC survival and/or drug resistance.

4.6. Search for markers which could identify cancer phenotype sensitive to treatment with oleate

Cells with a high capacity for FFA esterification can be identified phenotypically under the light microscope following exposure to 100 μM exogenous oleate for 24 h and staining with Oil Red O to visualize lipid droplets (lipid bodies) in the cytoplasm. All three cell lines protected from apoptosis induced by GF withdrawal by short-term (24 h) treatment with 100 μM oleate contained many lipid droplets in the cytoplasm (data shown only for MDA-MB-231 cells) and showed elevated TG content. Thus, it is tempting to speculate that the presence of lipid droplets may be a marker for resistance to apoptosis in tumor cells. However, MCF-7 breast cancer cells, as well as MCF-10A non-transformed cells, which were not protected from apoptosis by the treatment with oleate, produced large droplets when the FFA concentration in the medium was increased to 400 μM oleate (data shown for MCF-10A cells only). When extrapolating this to the in vivo situation, we predict that obese, insulin resistant cancer patients, who have constantly elevated levels of FFA in the blood, may contain lipid droplets in many cell types in the body, including tumor cells, but this may not indicate that these cells are capable to upregulate their TG/FFA cycle and become resistance to apoptosis. Thus, the presence of lipid droplets *per se* may not be a good marker for resistance to apoptosis in breast tumor cells. A low rate of FFA oxidation, which is often correlated with the high capacity for esterification may not be a good marker either. Three out of four breast cancer cell lines tested showed an inverse correlation between their capacity for oleate esterification and their rate of FFA oxidation (MDA-MB-231, T47-D and MCF-7). However, the two main metabolic pathways for FFA metabolism, oxidation and esterification, may not be mutually exclusive in all human breast tumor cells since MDA-MB-468 had both a relatively high ability for oleate esterification and a high FFA oxidation rate. According to our data possible markers for the antiapoptotic phenotype described in this thesis could be a combination of a high glycolytic rate and a high rate of lipolysis. This however, needs further investigation using more defined cell models such as non-transformed

breast epithelial cells that stably express myristoylated Akt, to test if upregulation of aerobic glycolysis brought about by Akt activation could lead to stable long-term upregulation of lipolysis and long-term GF-free survival after short-term treatment with oleate [70, reviewed in 191].

4.7 Conclusions

The data described here suggest that the metabolism of fatty acids plays an active role in the control of breast cancer cell survival. We have found that the common nutrient fatty acid oleate prevents apoptosis and promotes long-term cell survival in the absence of serum in three out of four human breast tumor cell lines tested. The long-term serum-free survival of these cell lines is associated with a high capacity to store TG and is oleate dose dependent. We propose that the ability to efficiently esterify oleate and to maintain elevated TG content due to upregulated TG/FFA cycling may play a role in the antiapoptotic effect induced by treatment with exogenous oleate and may be involved in the maintenance of long-term GF-free survival of human breast tumor cells. This is the first report proposing a role of the TG/FFA cycle in cancer cell survival. We also propose that treatment with oleate may protect tumors, which have acquired GF independent and upregulated metabolism of glucose from apoptosis. The *in vitro* results presented here provide support for epidemiological studies which show a positive correlation between elevated oleate content in breast tissue and increased breast cancer risk [192,193]. They are also consistent with recent reports showing that dietary fat reduction significantly reduces (up to 42%) the risk of tumor recurrence in postmenopausal women [63]. Further studies are required to elucidate how the complex TG/FFA cycle is regulated in breast tumor cells, particularly how the TG/FFA cycle is linked to GF signaling and how it may enhance cell survival in GF independent cancer cells. As well, further studies are needed to elucidate whether upregulated TG/FFA cycling promotes tumor cell survival by NAD+ regeneration and/or FFA signaling, possibly via GPR40 or another FFA receptor. It would also be important to

determine whether our *in vitro* results are relevant to human breast cancer cell metabolism *in vivo*, for both primary tumors and their metastases.

The important points to retain from this work can be summarized as follows: 1) Metabolic and signaling pathways for cell survival and proliferation are intertwined and the GF status of the cells may deeply influence their response to nutrients. 2) The metabolism of fuel substrates, glucose and FFA, is coordinately regulated in individual cells, thus suggesting that FFA metabolism should be studied in the context of the metabolism of glucose. 3) Cancer cells, which become GF independent may be vulnerable to the nutritional environment, may not be able to deal with the lack of some basic nutrients like glucose (they are glucose dependent) or they may be susceptible to overabundance of nutrients. In other words, they may be incapable to adjust their rate of metabolism to their energetic requirements, particularly under conditions like cell cycle arrest, when these requirements markedly decline. 4) The metabolism of FFA is different in cancer cells versus normal, non-transformed cells, and it may vary between individual cancers. However, it may also vary between different stages of cancer development, and thus may have a prognostic potential. Further research in the area of glucose and FFA metabolism may help to design novel strategies for cancer staging and classification independent form existing ones and may help to design cancer prevention strategies based on diet and therapies that directly or indirectly modify tumor cell metabolism to reduce tumor cell survival.

REFERENCES

1. Watson JD, Crick FH: Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* 1953, 171:737-738.
2. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, et al.: Initial sequencing and analysis of the human genome. *Nature* 2001, 409:860-921.
3. Stein LD: Human genome: end of the beginning. *Nature* 2004, 431:915-916.
4. Nowell PC: The clonal evolution of tumor cell populations. *Science* 1976, 194:23-28.
5. Nowell PC: Tumor progression: a brief historical perspective. *Semin Cancer Biol* 2002, 12:261-266.
6. Hanahan D, Weinberg RA: The hallmarks of cancer. *Cell* 2000, 100:57-70.
7. Sonnenschein C, Soto AM: Somatic mutation theory of carcinogenesis: why it should be dropped and replaced. *Mol Carcinog* 2000, 29:205-211.
8. Soto AM, Sonnenschein C: The somatic mutation theory of cancer: growing problems with the paradigm? *Bioessays* 2004, 26:1097-1107.
9. Triolo VA: Nineteenth Century Foundations Of Cancer Research Advances In Tumor Pathology, Nomenclature, And Theories Of Oncogenesis. *Cancer Res* 1965, 25:75-106.
10. Gilbert SF: Opening Darwin's black box: teaching evolution through developmental genetics. *Nat Rev Genet* 2003, 4:735-741.
11. Kuperwasser C, Chavarria T, Wu M, Magrane G, Gray JW, Carey L, Richardson A, Weinberg RA: Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc Natl Acad Sci U S A* 2004, 101:4966-4971.
12. Bhowmick NA, Moses HL: Tumor-stroma interactions. *Curr Opin Genet Dev* 2005, 15:97-101.
13. Roskelley CD, Bissell MJ: The dominance of the microenvironment in breast and ovarian cancer. *Semin Cancer Biol* 2002, 12:97-104.
14. Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C: The stroma as a crucial target in rat mammary gland carcinogenesis. *J Cell Sci* 2004, 117:1495-1502.

15. Weaver VM, Gilbert P: Watch thy neighbor: cancer is a communal affair. *J Cell Sci* 2004, 117:1287-1290.

16. Sell S: Stem cell origin of cancer and differentiation therapy. *Crit Rev Oncol Hematol* 2004, 51:1-28.

17. Al-Hajj M, Clarke MF: Self-renewal and solid tumor stem cells. *Oncogene* 2004, 23:7274-7282.

18. Genetics of breast and ovarian cancer. *National Cancer Institute, US NIH, www.cancer.gov* 2006.

19. Canadian Cancer Statistics 2006. *Canadian Cancer Society, NCIC, www.cancer.ca* 2006.

20. Couch JF, Weber BL: *Breast Cancer in The genetic basis of human cancer.* Edited by Vogelstei B, Kinzler KW: McGraw-Hill Companies, inc.; 1998.

21. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005, 365:1687-1717.

22. Lux MP, Fasching PA, Beckmann MW: Hereditary breast and ovarian cancer: review and future perspectives. *J Mol Med* 2006, 84:16-28.

23. Hall JM, Lee MK, Newman B, Morrow JE, Anderson LA, Huey B, King MC: Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 1990, 250:1684-1689.

24. Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, Collins N, Nguyen K, Seal S, Tran T, Averill D, et al.: Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science* 1994, 265:2088-2090.

25. Tonin P, Serova O, Simard J, Lenoir G, Feunteun J, Morgan K, Lynch H, Narod S: The gene for hereditary breast-ovarian cancer, BRCA1, maps distal to EDH17B2 in chromosome region 17q12-q21. *Hum Mol Genet* 1994, 3:1679-1682.

26. Sinilnikova OM, Mazoyer S, Bonnardel C, Lynch HT, Narod SA, Lenoir GM: BRCA1 and BRCA2 mutations in breast and ovarian cancer syndrome: reflection on the Creighton University historical series of high risk families. *Fam Cancer* 2006, 5:15-20.

27. Boulton SJ: Cellular functions of the BRCA tumour-suppressor proteins. *Biochem Soc Trans* 2006, 34:633-645.

28. Wooster R, Weber BL: Breast and ovarian cancer. *N Engl J Med* 2003, 348:2339-2347.

29. de Jong MM, Nolte IM, te Meerman GJ, van der Graaf WT, Oosterwijk JC, Kleibeuker JH, Schaapveld M, de Vries EG: Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. *J Med Genet* 2002, 39:225-242.

30. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 1997, 138:863-870.

31. Clarke RB, Howell A, Potten CS, Anderson E: Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 1997, 57:4987-4991.

32. Clarke RB: Human breast cell proliferation and its relationship to steroid receptor expression. *Climacteric* 2004, 7:129-137.

33. Cheng G, Weihua Z, Warner M, Gustafsson JA: Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. *Proc Natl Acad Sci U S A* 2004, 101:3739-3746.

34. Mallepell S, Krust A, Chambon P, Brisken C: Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. *Proc Natl Acad Sci U S A* 2006, 103:2196-2201.

35. Kelloff GJ, Lippman SM, Dannenberg AJ, Sigman CC, Pearce HL, Reid BJ, Szabo E, Jordan VC, Spitz MR, Mills GB, et al.: Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer--a plan to move forward. *Clin Cancer Res* 2006, 12:3661-3697.

36. Lewis JS, Jordan VC: Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. *Mutat Res* 2005, 591:247-263.

37. Jordan VC: Chemoprevention in the 21st century: is a balance best or should women have no estrogen at all? *J Clin Oncol* 2005, 23:1598-1600.

38. Girault I, Bieche I, Lidereau R: Role of estrogen receptor alpha transcriptional coregulators in tamoxifen resistance in breast cancer. *Maturitas* 2006, 54:342-351.

39. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R: Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004, 96:926-935.

40. Osborne CK, Schiff R: Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. *Breast* 2003, 12:362-367.

41. Lupu R, Menendez JA: Targeting fatty acid synthase in breast and endometrial cancer: An alternative to selective estrogen receptor modulators? *Endocrinology* 2006, 147:4056-4066.

42. Marches R, Scheuermann R, Uhr J: Cancer dormancy: from mice to man. *Cell Cycle* 2006, 5:1772-1778.

43. Pantel K, Woelfle U: Detection and molecular characterisation of disseminated tumour cells: implications for anti-cancer therapy. *Biochim Biophys Acta* 2005, 1756:53-64.

44. Calle EE, Kaaks R: Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer* 2004, 4:579-591.

45. Pan SY, Johnson KC, Ugnat AM, Wen SW, Mao Y: Association of obesity and cancer risk in Canada. *Am J Epidemiol* 2004, 159:259-268.

46. Boyd DB: Insulin and cancer. *Integr Cancer Ther* 2003, 2:315-329.

47. Harvie M, Hooper L, Howell AH: Central obesity and breast cancer risk: a systematic review. *Obes Rev* 2003, 4:157-173.

48. Goodwin PJ, Boyd NF, Hanna W, Hartwick W, Murray D, Qizilbash A, Redwood S, Hood N, DelGiudice ME, Sidlofsky S, et al.: Elevated levels of plasma triglycerides are associated with histologically defined premenopausal breast cancer risk. *Nutr Cancer* 1997, 27:284-292.

49. Muti P, Quattrin T, Grant BJ, Krogh V, Micheli A, Schunemann HJ, Ram M, Freudenheim JL, Sieri S, Trevisan M, et al.: Fasting glucose is a risk factor for breast cancer: a prospective study. *Cancer Epidemiol Biomarkers Prev* 2002, 11:1361-1368.

50. Goodwin PJ, Boyd NF: Body size and breast cancer prognosis: a critical review of the evidence. *Breast Cancer Res Treat* 1990, 16:205-214.

51. Chlebowski RT: Obesity and early-stage breast cancer. *J Clin Oncol* 2005, 23:1345-1347.

52. Enger SM, Greif JM, Polikoff J, Press M: Body weight correlates with mortality in early-stage breast cancer. *Arch Surg* 2004, 139:954-958; discussion 958-960.

53. Montague CT, O'Rahilly S: The perils of portliness: causes and consequences of visceral adiposity. *Diabetes* 2000, 49:883-888.

54. Yu YH, Ginsberg HN: Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circ Res* 2005, 96:1042-1052.

55. Lorincz AM, Sukumar S: Molecular links between obesity and breast cancer. *Endocr Relat Cancer* 2006, 13:279-292.

56. van Landeghem AA, Poortman J, Nabuurs M, Thijssen JH: Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Res* 1985, 45:2900-2906.

57. Rose DP, Komninou D, Stephenson GD: Obesity, adipocytokines, and insulin resistance in breast cancer. *Obes Rev* 2004, 5:153-165.

58. El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M: Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 2003, 144:4154-4163.

59. McGarry JD: What if Minkowski had been ageusic? An alternative angle on diabetes. *Science* 1992, 258:766-770.

60. Unger RH: Longevity, lipotoxicity and leptin: the adipocyte defense against feasting and famine. *Biochimie* 2005, 87:57-64.

61. Prentki M, Joly E, El-Assaad W, Roduit R: Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. *Diabetes* 2002, 51 Suppl 3:S405-413.

62. Holmes MD, Chen WY, Feskanich D, Kroenke CH, Colditz GA: Physical activity and survival after breast cancer diagnosis. *Jama* 2005, 293:2479-2486.

63. Chlebowski R, Blackburn G, Elashoff R, Thompson C, Goodman M, Shapiro A, Giuliano A, Karanja N, Hoy M, Nixon D: Diatery fat reduction in posmonopausal women with primary breast cancer: Phase III Women's Intervention Nutrition Study. 2005.

64. Jonat W, Hilpert F, Maass N: The use of aromatase inhibitors in adjuvant therapy for early breast cancer. *Cancer Chemother Pharmacol* 2005, 56 Suppl 1:32-38.

65. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA, et al.: Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005, 353:1673-1684.

66. Conlon I, Raff M: Size control in animal development. *Cell* 1999, 96:235-244.

67. Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB: Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol* 2001, 21:5899-5912.

68. Edinger AL, Thompson CB: Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell* 2002, 13:2276-2288.

69. Edinger AL, Cinalli RM, Thompson CB: Rab7 prevents growth factor-independent survival by inhibiting cell-autonomous nutrient transporter expression. *Dev Cell* 2003, 5:571-582.

70. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, et al.: Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* 2004, 64:3892-3899.

71. Buzzai M, Bauer DE, Jones RG, Deberardinis RJ, Hatzivassiliou G, Elstrom RL, Thompson CB: The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. *Oncogene* 2005, 24:4165-4173.

72. Hatzivassiliou G, Andreadis C, Thompson C: Akt-directed metabolic alterations in cancer. *Drug Discovery Today: Disease Mechanisms* 2005, 2:255-262.

73. Thompson JE, Thompson CB: Putting the rap on Akt. *J Clin Oncol* 2004, 22:4217-4226.

74. Brazil DP, Yang ZZ, Hemmings BA: Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* 2004, 29:233-242.

75. Hammerman PS, Fox CJ, Thompson CB: Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. *Trends Biochem Sci* 2004, 29:586-592.

76. McCormick F: Cancer: survival pathways meet their end. *Nature* 2004, 428:267-269.

77. Semenza GL: Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003, 3:721-732.

78. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N: Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* 2001, 15:1406-1418.

79. Deprez J, Vertommen D, Alessi DR, Hue L, Rider MH: Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *J Biol Chem* 1997, 272:17269-17275.

80. Yang YA, Morin PJ, Han WF, Chen T, Bornman DM, Gabrielson EW, Pizer ES: Regulation of fatty acid synthase expression in breast cancer by sterol regulatory element binding protein-1c. *Exp Cell Res* 2003, 282:132-137.

81. Berwick DC, Hers I, Heesom KJ, Moule SK, Tavare JM: The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. *J Biol Chem* 2002, 277:33895-33900.

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

83. Lindsley JE, Rutter J: Nutrient sensing and metabolic decisions. *Comp Biochem Physiol B Biochem Mol Biol* 2004, 139:543-559.

84. Hardie DG: The AMP-activated protein kinase pathway--new players upstream and downstream. *J Cell Sci* 2004, 117:5479-5487.

85. Imai S, Armstrong CM, Kaeberlein M, Guarente L: Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 2000, 403:795-800.

86. Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D: Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 2004, 430:686-689.

87. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA: hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001, 107:149-159.

88. Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA: Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 2004, 305:390-392.

89. Wullschleger S, Loewith R, Hall MN: TOR signaling in growth and metabolism. *Cell* 2006, 124:471-484.

90. Inoki K, Li Y, Zhu T, Wu J, Guan KL: TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002, 4:648-657.

91. Azoulay-Zohar H, Israelson A, Abu-Hamad S, Shoshan-Barmatz V: In self-defence: hexokinase promotes voltage-dependent anion channel closure and prevents mitochondria-mediated apoptotic cell death. *Biochem J* 2004, 377:347-355.

92. Robey RB, Hay N: Mitochondrial hexokinases: guardians of the mitochondria. *Cell Cycle* 2005, 4:654-658.

93. Kim JW, Dang CV: Multifaceted roles of glycolytic enzymes. *Trends Biochem Sci* 2005, 30:142-150.

94. Sirover MA: New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1999, 1432:159-184.

95. O'Keefe P, Gallet B: Update of nuclear medicine--PET imaging. *Mo Med* 2005, 102:127-130.

96. Warburg O: On respiratory impairment in cancer cells. *Science* 1956, 124:269-270.

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

98. Semenza G: Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol* 2002, 64:993-998.

99. Semenza GL: HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* 2002, 8:S62-67.

100. Kaelin WG, Jr.: von Hippel–Lindau-associated malignancies: Mechanisms and therapeutic opportunities. *Drug Discovery Today: Disease Mechanisms* 2005, 2:225-231.

101. Baysal B: Krebs cycle enzymes as tumor suppressors. *Drug Discovery Today: Disease Mechanisms* 2005, 2.

102. Janssen HL, Haustermans KM, Sprong D, Blommestijn G, Hofland I, Hoebers FJ, Blijweert E, Raleigh JA, Semenza GL, Varia MA, et al.: HIF-1A, pimonidazole, and iododeoxyuridine to estimate hypoxia and perfusion in human head-and-neck tumors. *Int J Radiat Oncol Biol Phys* 2002, 54:1537-1549.

103. Kim JW, Gardner LB, Dang CV: Oncogenic alterations of metabolism and the Warburg effect. *Drug Discovery Today: Disease Mechanism* 2005, 2:233-238.

104. Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV: Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. *Mol Cell Biol* 2004, 24:5923-5936.

105. Arsham AM, Plas DR, Thompson CB, Simon MC: Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. *Cancer Res* 2004, 64:3500-3507.

106. Garber K: Energy boost: the Warburg effect returns in a new theory of cancer. *J Natl Cancer Inst* 2004, 96:1805-1806.

107. Faraj M, Lu HL, Cianflone K: Diabetes, lipids, and adipocyte secretagogues. *Biochem Cell Biol* 2004, 82:170-190.

108. Phan CT, Tso P: Intestinal lipid absorption and transport. *Front Biosci* 2001, 6:D299-319.

109. Evans K, Burdge GC, Wootton SA, Clark ML, Frayn KN: Regulation of dietary fatty acid entrapment in subcutaneous adipose tissue and skeletal muscle. *Diabetes* 2002, 51:2684-2690.

110. Yeaman SJ: Hormone-sensitive lipase--new roles for an old enzyme. *Biochem J* 2004, 379:11-22.

111. Zechner R, Strauss JG, Haemmerle G, Lass A, Zimmermann R: Lipolysis: pathway under construction. *Curr Opin Lipidol* 2005, 16:333-340.

112. Kitamura Y, Accili D: New insights into the integrated physiology of insulin action. *Rev Endocr Metab Disord* 2004, 5:143-149.

113. Hamilton JA: Fast flip-flop of cholesterol and fatty acids in membranes: implications for membrane transport proteins. *Curr Opin Lipidol* 2003, 14:263-271.

114. Glatz JF, Luiken JJ, Bonen A: Involvement of membrane-associated proteins in the acute regulation of cellular fatty acid uptake. *J Mol Neurosci* 2001, 16:123-132; discussion 151-127.

115. Voet D, Voet J, Pratt C: *Fundamentals of Biochemistry (2 nd Upgrade Edition)*. New York: John Wiley and Sons, inc.; 2001.

116. Coleman RA, Lee DP: Enzymes of triacylglycerol synthesis and their regulation. *Prog Lipid Res* 2004, 43:134-176.

117. Bartlett K, Eaton S: Mitochondrial beta-oxidation. *Eur J Biochem* 2004, 271:462-469.

118. Frayn KN: The glucose-fatty acid cycle: a physiological perspective. *Biochem Soc Trans* 2003, 31:1115-1119.

119. Dulloo AG, Gubler M, Montani JP, Seydoux J, Solinas G: Substrate cycling between de novo lipogenesis and lipid oxidation: a thermogenic mechanism against skeletal muscle lipotoxicity and glucolipotoxicity. *Int J Obes Relat Metab Disord* 2004, 28 Suppl 4:S29-37.

120. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963, 1:785-789.

121. McGarry JD, Mannaerts GP, Foster DW: A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest* 1977, 60:265-270.

122. Ruderman NB, Saha AK, Kraegen EW: Minireview: malonyl CoA, AMP-activated protein kinase, and adiposity. *Endocrinology* 2003, 144:5166-5171.

123. Kahn BB, Alquier T, Carling D, Hardie DG: AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 2005, 1:15-25.

124. Hardie DG: New roles for the LKB1-->AMPK pathway. *Curr Opin Cell Biol* 2005, 17:167-173.

125. Ruderman N, Prentki M: AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome. *Nat Rev Drug Discov* 2004, 3:340-351.

126. Devlin T: *Textbook of Biochemistry with Clinical Correlations* edn Fourth edition. New York/ Chichester/ Weinheim/ Brisbane/ Singapore/ Toronto: John Wiley and Sons, Inc.; 1997.

127. Vaughan M: The production and release of glycerol by adipose tissue incubated in vitro. *J Biol Chem* 1962, 237:3354-3358.

128. Reshef L, Olswang Y, Cassuto H, Blum B, Croniger CM, Kalhan SC, Tilghman SM, Hanson RW: Glyceroneogenesis and the triglyceride/fatty acid cycle. *J Biol Chem* 2003, 278:30413-30416.

129. Coleman RA, Lewin TM, Van Horn CG, Gonzalez-Baro MR: Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? *J Nutr* 2002, 132:2123-2126.

130. Bertin R: Glycerokinase activity and lipolysis regulation in brown adipose tissue of cold acclimated rats. *Biochimie* 1976, 58:431-434.

131. Pol A, Martin S, Fernandez MA, Ferguson C, Carozzi A, Luetterforst R, Enrich C, Parton RG: Dynamic and regulated association of caveolin with lipid bodies: modulation of lipid body motility and function by a dominant negative mutant. *Mol Biol Cell* 2004, 15:99-110.

132. Martin S, Parton RG: Caveolin, cholesterol, and lipid bodies. *Semin Cell Dev Biol* 2005, 16:163-174.

133. Martin S, Parton RG: Lipid droplets: a unified view of a dynamic organelle. *Nat Rev Mol Cell Biol* 2006, 7:373-378.

134. Murphy DJ: The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Prog Lipid Res* 2001, 40:325-438.

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

136. Kuhajda FP, Jenner K, Wood FD, Hennigar RA, Jacobs LB, Dick JD, Pasternack GR: Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc Natl Acad Sci U S A* 1994, 91:6379-6383.

137. Swinnen JV, Ulrix W, Heyns W, Verhoeven G: Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. *Proc Natl Acad Sci U S A* 1997, 94:12975-12980.

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

139. Yahagi N, Shimano H, Hasegawa K, Ohashi K, Matsuzaka T, Najima Y, Sekiya M, Tomita S, Okazaki H, Tamura Y, et al.: Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma. *Eur J Cancer* 2005, 41:1316-1322.

140. Li JN, Mahmoud MA, Han WF, Ripple M, Pizer ES: Sterol regulatory element-binding protein-1 participates in the regulation of fatty acid synthase expression in colorectal neoplasia. *Exp Cell Res* 2000, 261:159-165.

141. Horton JD, Goldstein JL, Brown MS: SREBPs: transcriptional mediators of lipid homeostasis. *Cold Spring Harb Symp Quant Biol* 2002, 67:491-498.

142. Anderson RG: Joe Goldstein and Mike Brown: from cholesterol homeostasis to new paradigms in membrane biology. *Trends Cell Biol* 2003, 13:534-539.

143. Chirala SS, Chang H, Matzuk M, Abu-Elheiga L, Mao J, Mahon K, Finegold M, Wakil SJ: Fatty acid synthesis is essential in embryonic development: fatty

acid synthase null mutants and most of the heterozygotes die in utero. *Proc Natl Acad Sci U S A* 2003, 100:6358-6363.

144. Pizer ES, Kurman RJ, Pasternack GR, Kuhajda FP: Expression of fatty acid synthase is closely linked to proliferation and stromal decidualization in cycling endometrium. *Int J Gynecol Pathol* 1997, 16:45-51.

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

146. Wang HQ, Altomare DA, Skele KL, Poulikakos PI, Kuhajda FP, Di Cristofano A, Testa JR: Positive feedback regulation between AKT activation and fatty acid synthase expression in ovarian carcinoma cells. *Oncogene* 2005, 24:3574-3582.

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

148. Kumar-Sinha C, Ignatoski KW, Lippman ME, Ethier SP, Chinnaiyan AM: Transcriptome analysis of HER2 reveals a molecular connection to fatty acid synthesis. *Cancer Res* 2003, 63:132-139.

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

150. Menendez JA, Oza BP, Atlas E, Verma VA, Mehmi I, Lupu R: Inhibition of tumor-associated fatty acid synthase activity antagonizes estradiol- and tamoxifen-induced agonist transactivation of estrogen receptor (ER) in human endometrial adenocarcinoma cells. *Oncogene* 2004, 23:4945-4958.

151. Katz-Brull R, Lavin PT, Lenkinski RE: Clinical utility of proton magnetic resonance spectroscopy in characterizing breast lesions. *J Natl Cancer Inst* 2002, 94:1197-1203.

152. Glunde K, Jie C, Bhujwalla ZM: Molecular causes of the aberrant choline phospholipid metabolism in breast cancer. *Cancer Res* 2004, 64:4270-4276.

153. Wright MM, Howe AG, Zaremberg V: Cell membranes and apoptosis: role of cardiolipin, phosphatidylcholine, and anticancer lipid analogues. *Biochem Cell Biol* 2004, 82:18-26.

154. Finney RE, Nudelman E, White T, Bursten S, Klein P, Leer LL, Wang N, Waggoner D, Singer JW, Lewis RA: Pharmacological inhibition of phosphatidylcholine biosynthesis is associated with induction of phosphatidylinositol accumulation and cytolysis of neoplastic cell lines. *Cancer Res* 2000, 60:5204-5213.

155. Terry PD, Rohan TE, Wolk A: Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. *Am J Clin Nutr* 2003, 77:532-543.

156. Kota BP, Huang TH, Roufogalis BD: An overview on biological mechanisms of PPARs. *Pharmacol Res* 2005, 51:85-94.

157. Nakamura MT, Cheon Y, Li Y, Nara TY: Mechanisms of regulation of gene expression by fatty acids. *Lipids* 2004, 39:1077-1083.

158. Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A: Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr* 2004, 79:935-945.

159. Sampath H, Ntambi JM: Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu Rev Nutr* 2005, 25:317-340.

160. Saadatian-Elahi M, Norat T, Goudable J, Riboli E: Biomarkers of dietary fatty acid intake and the risk of breast cancer: a meta-analysis. *Int J Cancer* 2004, 111:584-591.

161. Gottlicher M, Widmark E, Li Q, Gustafsson JA: Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* 1992, 89:4653-4657.

162. Hardy S, Langelier Y, Prentki M: Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Res* 2000, 60:6353-6358.

163. Paumen MB, Ishida Y, Muramatsu M, Yamamoto M, Honjo T: Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J Biol Chem* 1997, 272:3324-3329.

164. Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 1998, 95:2498-2502.

165. Hickson-Bick DL, Buja ML, McMillin JB: Palmitate-mediated alterations in the fatty acid metabolism of rat neonatal cardiac myocytes. *J Mol Cell Cardiol* 2000, 32:511-519.

166. Blazquez C, Galve-Roperh I, Guzman M: De novo-synthesized ceramide signals apoptosis in astrocytes via extracellular signal-regulated kinase. *Faseb J* 2000, 14:2315-2322.

167. Hardy S, El-Assaad W, Przybytkowski E, Joly E, Prentki M, Langelier Y: Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. *J Biol Chem* 2003, 278:31861-31870.

168. Brown AJ, Jupe S, Briscoe CP: A family of fatty acid binding receptors. *DNA Cell Biol* 2005, 24:54-61.

169. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G: Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 2005, 11:90-94.

170. Kostenis E: A glance at G-protein-coupled receptors for lipid mediators: a growing receptor family with remarkably diverse ligands. *Pharmacol Ther* 2004, 102:243-257.

171. Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K, Offermanns S: PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nat Med* 2003, 9:352-355.

172. Hebert SC: Physiology: orphan detectors of metabolism. *Nature* 2004, 429:143-145.

173. Hardy S, St-Onge GG, Joly E, Langelier Y, Prentki M: Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. *J Biol Chem* 2005, 280:13285-13291.

174. de Vries JE, Vork MM, Roemen TH, de Jong YF, Cleutjens JP, van der Vusse GJ, van Bilsen M: Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes. *J Lipid Res* 1997, 38:1384-1394.

175. Rosenthal MD: Accumulation of neutral lipids by human skin fibroblasts: differential effects of saturated and unsaturated fatty acids. *Lipids* 1981, 16:173-182.

176. Buttke TM: Inhibition of lymphocyte proliferation by free fatty acids. I. Differential effects on mouse B and T lymphocytes. *Immunology* 1984, 53:235-242.

177. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV, Jr., Ory DS, Schaffer JE: Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A* 2003, 100:3077-3082.

178. Welters HJ, Tadayyon M, Scarpello JH, Smith SA, Morgan NG: Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett* 2004, 560:103-108.

179. Averill-Bates DA, Przybytkowski E: The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. *Arch Biochem Biophys* 1994, 312:52-58.

180. Heimberg H, De Vos A, Vandercammen A, Van Schaftingen E, Pipeleers D, Schuit F: Heterogeneity in glucose sensitivity among pancreatic beta-cells is correlated to differences in glucose phosphorylation rather than glucose transport. *Embo J* 1993, 12:2873-2879.

181. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959, 37:911-917.

182. Van Veldhoven PP, Swinnen JV, Esquenet M, Verhoeven G: Lipase-based quantitation of triacylglycerols in cellular lipid extracts: requirement for presence of detergent and prior separation by thin-layer chromatography. *Lipids* 1997, 32:1297-1300.

183. Trimboli AJ, Waite BM, Atsumi G, Fonteh AN, Namen AM, Clay CE, Kute TE, High KP, Willingham MC, Chilton FH: Influence of coenzyme A-independent transacylase and cyclooxygenase inhibitors on the proliferation of breast cancer cells. *Cancer Res* 1999, 59:6171-6177.

184. Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, Thompson CB: Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 2005, 120:237-248.

185. Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G: Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. *J Biol Chem* 2005, 280:19507-19515.

186. Burdall SE, Hanby AM, Lansdown MR, Speirs V: Breast cancer cell lines: friend or foe? *Breast Cancer Res* 2003, 5:89-95.

187. Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ: Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. *J Natl Cancer Inst* 2002, 94:1494-1503.

188. Janni W, Rack B, Schindlbeck C, Strobl B, Rjosk D, Braun S, Sommer H, Pantel K, Gerber B, Friese K: The persistence of isolated tumor cells in bone marrow from patients with breast carcinoma predicts an increased risk for recurrence. *Cancer* 2005, 103:884-891.

189. Wiedswang G, Borgen E, Karesen R, Qvist H, Janbu J, Kvalheim G, Nesland JM, Naume B: Isolated tumor cells in bone marrow three years after diagnosis in disease-free breast cancer patients predict unfavorable clinical outcome. *Clin Cancer Res* 2004, 10:5342-5348.

190. Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, Janicke F, Pantel K: Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. *Clin Cancer Res* 2005, 11:3678-3685.

191. Plas DR, Thompson CB: Akt-dependent transformation: there is more to growth than just surviving. *Oncogene* 2005, 24:7435-7442.

192. Petrek JA, Hudgins LC, Ho M, Bajorunas DR, Hirsch J: Fatty acid composition of adipose tissue, an indication of dietary fatty acids, and breast cancer prognosis. *J Clin Oncol* 1997, 15:1377-1384.

193. Perez Alenza D, Rutteman GR, Pena L, Beynen AC, Cuesta P: Relation between habitual diet and canine mammary tumors in a case-control study. *J Vet intern Med* 1998, 12:132-139.

APPENDIX I

Manuscript submitted to Molecular Cancer Research

Upregulation of Cellular Triacylglycerol/Free Fatty Acid Cycling by Oleate Is Associated with Long-Term Growth Factor Independent Survival of Human Breast Cancer Cells

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Running title: TG/FFA cycling and breast cancer cell survival

Key words: breast cancer, cell survival, fatty acid, triacylglycerol, lipolysis

Abstract

We previously showed that exogenous oleate protects MDA-MB-231 human breast cancer cells against palmitate-induced apoptosis in part by increasing esterification of this free fatty acid (FFA) into triacylglycerol (TG). The aim of this study was to better understand the mechanism whereby oleate protects human breast cancer cells against apoptosis induced by serum withdrawal. The metabolism of FFA, TG and glucose, in parallel with long-term cell survival in the absence of serum, was investigated in MDA-MB-231 cells treated with exogenous oleate. The results were extended to a panel of human breast cancer cell lines (MDA-MB-468, T-47D, MCF-7) and the non-transformed cell strain MCF-10A. Short-term (3-24 h) exposure of MDA-MB-231 cells to exogenous oleate resulted in dose dependent long-term (10 days) serum-free survival and correlated with accumulation of TG in lipid droplets. The cellular TG content increased and the rate of lipolysis was upregulated by oleate in a dose dependent manner and both effects persisted after oleate removal. Rapid TG lipolysis and FFA reesterification, supported by high rates of glycolysis that provides the glycerol backbone for TG synthesis, are consistent with the presence of very active TG/FFA cycling in human breast cancer cells. Only the cancer cell lines capable of accumulating TG showed long-term serum-free survival after treatment with oleate. The results suggest that upregulation of TG/FFA cycling induced by short-term treatment with oleate may be involved in maintenance of human breast cancer cell survival and may provide one of the links between lipid metabolism and cancer.

Introduction

Epidemiological evidence indicates that obesity increases the risk of developing several diseases, including breast cancer (rev. in 1, 2, 3). Excess adiposity is considered harmful because it is associated with increased plasma triacylglycerols (TG) and free fatty acids (FFA) (4) that contribute to ectopic deposition of lipids in nonadipocytes. This abnormal fat deposition is implicated in the development of insulin resistance and lipotoxic tissue damage (5, rev. in 6, 7, 8). The long chain saturated FA palmitate (C16:0) and the monounsaturated FA oleate (C18:1) are the most common FA in our diet, blood, and other tissues. Lipotoxicity *in vitro* is induced by palmitate but not by oleate (5, 9, 10). In addition, oleate protects against apoptosis *in vitro* and prevents insulin resistance *in vivo* (11).

We previously showed that oleate supplementation leads to TG accumulation in MDA-MB-231 cells and protects against lipotoxicity by channeling saturated FFA to the intracellular lipid storage pool and by sustaining the synthesis of cardiolipin for mitochondrial membrane integrity (9). In addition, cells with increased capacity for unsaturated FA synthesis (cells overexpressing stearoyl-CoA desaturase), which have increased endogenous levels of monounsaturated fatty acids, are resistant to palmitate-induced lipotoxicity, whereas cells with impaired TG synthetic activity are sensitive to lipotoxicity induced by both saturated and monounsaturated FFA (12). Therefore, accumulation of TG promoted by exogenous and/or endogenous oleate may have an adaptive function and protect against lipotoxicity.

Monounsaturated FFA may also be protective against apoptosis induced by factors other than palmitate. For example, the monounsaturated FFA palmitoleate is

protective against apoptosis induced by serum withdrawal and cytokines in rat β-cells (13) and oleate prevents apoptosis induced by serum withdrawal in MDA-MB-231 cells (10). Protection by oleate against apoptosis induced by serum withdrawal might involve signaling via the cell-surface G protein coupled receptor GPR40 and activation of the phosphatidylinositol 3-kinase/Akt survival pathway (14).

FFA and glucose are both fuel substrates and their metabolic pathways are interdependent and often reciprocally regulated (rev. in 15). Warburg (16, 17) showed over 70 years ago that cancer cells have increased rates of glucose metabolism. However, it was shown only recently that oncogenic mutations are directly responsible for some of these changes (18, 19) and that glucose metabolism and survival pathways are intertwined (rev. in 20, 21). Tumor cells have both elevated glycolytic and synthetic processes in order to meet the need for continuous growth (rev. in 22, 23). Little is known, however, how these changes in glucose metabolism affect the metabolism of FFA or TG. The question becomes especially important in the context of obese individuals and obese cancer patients where both fuels may be available in excess.

The aim of the present study was to better understand the biochemical basis of the antiapoptotic action of oleate in human breast cancer cells. Specifically, we addressed the question of how the metabolism of oleate and TG accumulation could be involved in protection of human breast cancer cells against apoptosis induced by serum withdrawal. The metabolism of oleate, TG and glucose were examined after short-term oleate treatment (24 h) and related to long-term (8-10 days) cell survival in the absence of serum growth factors or FFA. We show that human breast cancer

cells treated with oleate respond by upregulating their level of TG/FFA cycling and that this response correlates with oleate induced long-term serum-free survival in those cell lines which also have an elevated capacity for fat storage.

Results

Short-Term Oleate Treatment Promotes Long-Term Serum-Free Cell Survival

Long-term survival of MDA-MB-231 cells exposed briefly to oleate was measured. After 24 h exposure to various concentrations of exogenous oleate, the cells were maintained in culture without serum or oleate for an additional eight days (with daily changes of media) and the live adherent cells were counted daily. Figure 1A shows that serum-starved cells without oleate treatment died whereas oleate-treated cells survived for many days. Short-term (24 h) treatment with 400 μM oleate afforded complete protection against apoptosis induced by serum withdrawal for at least 10 days, while lower concentrations of oleate provided partial protection in a dose dependent manner.

The percentage of live adherent MDA-MB-231 cells at Day 10 post 24 h treatment with 400 μM oleate was 108.4 % relative to the cell number at Day 1 (Fig. 1A). This suggests that cells protected against apoptosis by treatment with oleate were proliferating at a very low rate, if at all. Using FACS analysis, we examined the cell cycle distribution of serum-starved and oleate-treated MDA-MB-231 cells from Day 1 until Day 4 post serum withdrawal and compared these with the 5% FBS control at Day 2 (Fig. 1B). A gradual decrease in the percentage of cells in G1 phase of the cell cycle was observed with a concomitant increase in the percentage of cells in S and G2/M phases. Together these results (Fig. 1A and B) suggest that cells protected against apoptosis by oleate did not proliferate due to difficulty completing their cell cycle.

Figure 1C shows that treatments as short as 3 h with 100 μM oleate protected human breast cancer cells against apoptosis induced by serum withdrawal. This short pulse of oleate prevented activation of caspase-3 for up to 24 h. Remarkably, even a one-hour pulse was partially protective against apoptosis.

The results indicate that a short pulse of a physiological concentration of exogenous oleate protects human breast cancer cells against apoptosis induced by serum withdrawal. The survival effect is oleate dose dependent and long lasting. Surviving cells slow down their proliferation and tend to accumulate in the S and/or G2/M phases of the cell cycle.

MDA-MB-231 Cells Have a High Capacity To Store TG as Lipid Droplets

Figure 2A shows that a 24 h exposure of MDA-MB-231 cells to physiological concentrations of 100 μM and 400 μM of oleate bound to BSA causes the formation of many intracellular lipid droplets. The lipid droplet material that was stained by Oil Red O increased proportionally with increasing oleate concentration. Control cells (not exposed to oleate) formed few if any lipid droplets. The ability of MDA-MB-231 cells to efficiently esterify oleate for storage as TG is illustrated in Fig. 2B. Total intracellular TG content of the cells was found to increase in a dose dependent manner in response to 24 h treatment with increasing concentrations of oleate.

MDA-MB-231 Cells Have High Rates of TG/FFA Cycling

TG stored in lipid droplets can be hydrolyzed to produce FFA and glycerol. This process (lipolysis) has been mostly studied in adipocytes, where it is carried out by the highly regulated enzyme hormone-sensitive lipase in addition to other less-known lipolytic enzymes (rev. in 24). FFA that are released from TG by lipolysis can be immediately reesterified within the cell to form TG again. Lipolysis and reesterification are two opposing processes involved in what is referred to as intracellular TG/FFA cycling (25, rev. in 26).

Lipolysis in MDA-MB-231 cells was measured using two complementary assays in which the amount of either glycerol or FFA released into the cell media from the breakdown of TG was analyzed (Fig. 2C and Table 1). Figure 2C shows that MDA-MB-231 cells have a high rate of lipolysis as measured by glycerol released relative to their total TG content. Moreover, the rate of lipolysis increased dramatically with exposure to increasing concentrations of exogenous oleate. Thus, following 24 h of incubation with the highest concentration of exogenous oleate (400 μM), cellular TG content was 52 nmol/10^6 cells and glycerol released during 1 hr was 35 nmol/10^6 cells. This suggests that MDA-MB-231 cells reesterify FFA very efficiently and have very active TG/FFA cycling.

The data presented in Table 1 shows the rate of lipolysis measured by the two methods and reveals that the values are not stoichiometrically related as might be expected. For the cells treated with 400 μM oleate, we found only 3.13 nmol/h/10^6 cells of FFA released, instead of 3 x 35.8=107.4 nmol/h/10^6 cells calculated from the results obtained using glycerol release (1 molecule of TG is composed of 1 glycerol and 3 FA). The rate of FFA oxidation in MDA-MB-231 cells (~0.05 nmol/h/10^6

cells) cannot explain the discrepancy between the amount of glycerol and FFA released. The results therefore indicate that >95% of FFA are indeed recycled back to TG in these cells rather than being oxidized. The net effect is that these cells when exposed to exogenous oleate, both synthesize and maintain TG in lipid droplets very efficiently (Fig. 2B) in spite of a very high rate of lipolysis (Fig. 2C).

Short-term Oleate Treatment, which Protects MDA-MB-231 Cells against Apoptosis, Causes a Long-lasting Change in Cellular Lipid Metabolism

Figure 3 shows that short-term (24 h) treatment of serum-starved MDA-MB-231 cells with 100 μM oleate leads to long-term stable (at least 7 days) intracellular TG content (Fig. 3A) and elevated lipolysis rate (Fig. 3B). Thus, lipolysis remained elevated between 20 and 17 nmol/h/10^6 cells from Day 2 until Day 7, whereas within the same time period, cellular TG content was moderately lowered from 20 to 12 nmol/10^6 cells. This suggests that during the 7 days post oleate treatment, the cellular TG pool continued to turnover at a high rate because of very active TG/FFA cycling.

MDA-MB-231 Cells Protected against Apoptosis by Oleate and Cultured in Serum-Free Conditions, Maintain a High Rate of Glucose Oxidation

A number of growth factors-dependent cells downregulate their metabolism of glucose when growth factors are withdrawn (27). It was therefore important to assess the metabolism of glucose in MDA-MB-231 cells under serum-free conditions. Continuous TG/FFA cycling requires a constant supply of glycerol-3-phosphate, which can be produced by glycolysis. Figure 3C shows that MDA-MB-

231 cells have a high rate of glucose metabolism, a characteristic typical of many cancer cells. In control conditions (presence of serum) MDA-MB-231 cells exhibited 160% higher glucose oxidation compared to non-transformed breast epithelial cells MCF-10A. In addition, MDA-MB-231 cells maintained a high glucose oxidation rate until Day 2 post serum withdrawal, contrary to MCF-10A cells that downregulated glucose metabolism by 65% 24 h after withdrawal of serum and growth factors. The latter measurements are probably overestimated since they were performed on live adherent cells (not on total cells) because, at Day 2 post serum withdrawal, the cells began to enter apoptosis. At Day 3 post serum withdrawal, MDA-MB-231 cells protected by short-term treatment with oleate still maintained a high glucose oxidation rate, while the majority of non-treated cells (> 90%) had detached from the dishes and were dead as determined by Trypan Blue staining (see Figure 1A).

Thus, elevated glucose metabolism in MDA-MB-231 cells most likely provides glycerol-3-phosphate needed for FFA esterification and remains the main energy source in MDA-MB-231 cells protected against apoptosis by short-term treatment with oleate. It is therefore unlikely that FFA released from droplets by lipolysis are needed as an alternative energy source (via FFA oxidation in mitochondria) to promote survival of these cells in the absence of serum. Consistent with this, MDA-MB-231 cells were found to have a very low rate of FFA oxidation (Fig. 4C).

High Capacity to Store TG Is Correlated With Enhanced Serum-Free Cell Survival in Various Human Breast Cancer Cell Lines

The effect of oleate treatment on cell survival was tested in three other human breast cancer cell lines, together with the non-transformed human breast epithelial cell line (MCF-10A) (Fig. 4). The design of the experiment was similar to that shown in Fig. 1A and cells were counted on Day 6. The results are expressed as percentage of control culture cell number at Day 1 (before addition of oleate). It should be noted that each of the different cell lines had different growth characteristics. The MCF-10A non-transformed cells were dependent on both serum and growth factors for survival and were not protected by oleate treatment in the absence of these additives (Fig. 4A). T-47D and MDA-MB-468 cells responded similarly to oleate treatment as did MDA-MB-231 cells, although the three cell lines differed in their sensitivity to serum withdrawal, with MDA-MB-231 being the most sensitive. Oleate treatment in the absence of serum had no effect on the survival of the fourth cell line tested, MCF-7. The three cell lines that responded to oleate treatment accumulated high levels of cellular TG, as shown in Fig. 4B, whereas the cell line that did not respond to oleate (MCF-7) had low total TG content, as did MCF-10A cells. These results further confirm that high cellular TG content is associated with enhanced serum-free cell survival after treatment with oleate in three of four human breast cancer cell lines.

FFA esterification to form complex lipids, in particular TG, represents one of two main metabolic pathways for FFA within the cell, the other being FFA

oxidation. It was of interest to examine the relative rate of FFA oxidation in our panel of cell lines. We expected to see an inverse correlation between oxidation and esterification. Figure 4C shows that MDA-MB-231 and T-47D cell lines, which have high TG content, had a low intrinsic FFA oxidation rate, while MDA-MB-468, MCF 7 and MCF-10A cell lines showed high FFA oxidation rates. Surprisingly, however, MDA-MB-468 cells had both a high FFA oxidation rate and a high TG content after exposure to exogenous oleate. Thus, the rate of FFA oxidation of the different human breast cancer cell lines tested was variable (high and low) and did not correlate with the cellular TG content or the ability for long-term serum-free survival induced by treatment with oleate.

Discussion

We found that three out of four human breast cancer cell lines tested (MDA-MB-231, MDA-MB-468, and T47D) were protected for many days against apoptosis and death induced by serum withdrawal by short-term (24 h) treatment with oleate. These cells proliferated very slowly if at all, suggesting that treatment with oleate influences mainly cell survival with little effect on cell proliferation. The long-term serum-free survival in these human breast cancer cell lines was associated with elevated TG content and was oleate dose dependent. After short-term treatment with oleate, the cellular TG pool increased significantly and the rate of lipolysis (measured as glycerol released into the culture medium) was upregulated. Subsequently, both TG levels and the rate of lipolysis remained high for a long period of time (8-10 days). These results demonstrate that MDA-MB-231 cells have very efficient TG/FFA cycling, allowing maintenance of elevated TG content in spite of very active ongoing lipolysis. Glucose metabolism remained high in serum-starved MDA-MB-231 cells protected against apoptosis by short-term treatment with oleate, providing glycerol-3-phosphate needed for FFA esterification and being the main energy source in these cells. Thus, it is attractive to propose that elevated TG/FFA cycling after exposure to exogenous oleate is involved in the maintenance of long-term serum-free survival of human breast cancer cells.

Cellular TG/FFA cycling occurs in many tissues. It is a multi-step, complex cycle involving many enzymes. At least one enzyme activates FFA to form FA-CoA (28), then at least five others are involved in sequential esterification of FA-CoA to glycerol backbone (29); subsequently at least three different lipases are involved in

lipolysis (24). TG/FFA cycling is considered to be a "futile cycle" since its main outcome is the consumption of energy (production of 1 molecule of TG consumes 6 molecules of ATP). However, this process allows preservation of intracellular FA pools when they are not oxidized for energy production and has been linked to thermogenesis in brown adipose tissue (30). The present study suggests a novel role for TG/FFA cycling, which is enhancement of human breast cancer cell survival. A model illustrating this relationship is shown in Figure 5 and is explained below.

The model is based on the fact that many cancer cells, including the MDA-MB-231 cell line used in this study, show increased glycolytic rate. This allows rapidly proliferating cancer cells to use glycolytic intermediates for synthesis of nucleic acids and amino acids, and also supplies their energy needs. In addition, glycolysis-derived pyruvate carbons are transported from the mitochondrial matrix to the cytoplasm in the form of citrate, which becomes a precursor for *de novo* FFA synthesis. Thus, cancer cells are fully prepared for continuous growth and proliferation by being able to synthesize many of their substrates and complex molecules *de novo* from glucose. The weakness of the efficient cancer cell synthetic machine, however, is that the high glycolytic rate requires constant regeneration of cytosolic NAD^+. In many cell types, lactate dehydrogenase (LDH) is a key enzyme involved in this process. LDH oxidizes pyruvate to lactate with simultaneous regeneration of NAD^+ from NADH, and lactate is secreted from the cell. Our model in Figure 5 proposes that rapid TG/FFA cycling provides a second (alternative) route for NAD^+ regeneration.

The esterification phase of TG/FFA cycle requires glycerol-3-phosphate (G3P). G3P is produce by G3P dehydrogenase (GPDH) from dihydroxyacetone phosphate (DHAP) with simultaneous regeneration of NAD^+ from NADH. We propose that channeling of G3P to FFA esterification to form TG followed by rapid hydrolysis and release of glycerol from the cell, allows the system to run continuously as long as FFA for reesterification are available. A very efficient recycling of FFA in MDA-MB-231 cells preserves the intracellular pool of FA. In addition *de novo* synthesis of FA can possibly also supply the pool. The alternative NAD regeneration system described here and shown in Fig. 5 enhances tumor cell survival by setting a stable rate of cytoplasmic NAD^+ regeneration, which would otherwise be limiting and cause cancer cell apoptosis and death.

It was recently postulated that signaling for cell survival might involve controlling cell access to nutrients in addition to inhibiting apoptosis (21, 31, 32). MDA-MB-231 breast cancer cells appear to have acquired autonomy in controlling their access to glucose, but their apoptotic machinery can still respond to a lack of serum growth factors. The best-studied system responsible for cell survival and extracellular nutrient uptake is the phosphatidylinositol 3-kinase/Akt pathway (rev. in 33). Our previous work (14) as well as that of others (34) demonstrated that this pathway could be stimulated via the G protein coupled FFA receptors, GPR40 and GPR120. It is possible that the survival effect described here, which involves up regulation of TG/FFA cycling, is also in part GPR40-mediated. Thus, TG/FFA cycling by preserving the pool of FA inside the cells for long periods of time would assure continuing availability of small amounts of FFA released into the medium

upon lipolysis, for binding to the GPR40 receptor and activation of the survival signaling cascade.

The human breast cancer cell lines used in this study derived from metastases, so they represent aggressive, advanced breast tumors (35). Two of those, which responded to oleate are estrogen receptor (ER) negative (MDA-MB-231, MDA-MB-468) while one is ER positive (T-47D). Furthermore, MCF-7 cells, which did not respond to oleate at all, are the least invasive of the cancer cell lines used in this study. MDA-MB-231 cells, which were the most responsive to oleate, are known to be the most invasive (36). Therefore, the ability of oleate to modify human breast cancer cell survival *in vitro* appears to be independent of their ER status, however it may be positively correlated with their invasiveness.

MDA-MB-231 cells protected against death by treatment with oleate were viable for long periods of time but blocked in cell division. In this respect they resemble disseminated tumor cells, which have been detected in the bone marrow of patients with breast tumors. Most are not actively proliferating and remain in a state of "dormancy" for many years (rev. in 37). Persistence of disseminated tumor cells is associated with a poor clinical outcome (38). It is tempting to speculate that oleate could contribute to the survival of disseminated tumor cells via upregulating TG/FFA cycling. Furthermore, upregulation of TG/FFA cycling, which confers resistance to apoptosis may also contribute to drug resistance common for disseminated tumor cells (39). More evidence, especially studies *in vivo* with primary tumors and metastasis are needed to verify if increased rate of TG/FFA

cycling could indeed be a marker of tumor invasiveness or could contribute to disseminated tumor cells survival and/or drug resistance.

The *in vitro* results presented here provide support for epidemiological studies, which show a positive correlation between elevated oleate content in breast tissue and increased breast cancer risk (40, 41). They are also consistent with the recent evidence suggesting that obesity is associated with increased breast cancer recurrence and mortality, and that life style interventions, like dietary fat reduction, significantly improve survival in postmenopausal women (42). Further work is required to determine whether enhanced TG/FFA cycling promotes tumor cell survival by NAD+ regeneration and/or FFA signaling, possibly via GPR40 or another FFA receptor, and whether our *in vitro* results are relevant to human breast cancer cell metabolism *in vivo*.

Material and Methods

Materials

FFA sodium salts were purchased from Nu-Check, Prep (Elysian, MN). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA) and was heat-inactivated at 56°C for 30 min. FFA-free BSA (Fraction V, 96% FFA-free, was obtained from Sigma (St-Louis, MO). [1-^{14}C]palmitic acid (55 mCi/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). D-[U-^{14}C] glucose (317 mCi/mmol) was obtained from Amersham Biosciences (Baie d'Urfé, Québec, Canada). Unlabeled and labeled albumin-bound fatty acids (oleate and palmitate) were prepared as previously described (9).

Cell Lines and Culture Conditions

The tumorigenic human breast cancer cell lines, MDA-MB-231, MDA-MB-468, T-47D and MCF-7, and the non-tumorigenic cell line derived from normal human epithelial cells MCF-10A, were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in a 50/50 mixture of Dulbecco's modified Eagle's Medium and Ham's F-12 (DMEM/F12; Wisent; St-Bruno, Québec) containing 2 mM glutamine and 5% heat-inactivated FBS. For MCF-10A cells, DMEM/F12 was supplemented with insulin 10 µg/ml, hydrocortisone 0.5 µg/ml (both from Sigma, St-Louis, MO) and recombinant human epidermal growth factor 20 ng/ml (Invitrogen). All experiments were performed in minimal essential media (MEM) as described below.

Experimental Procedure for Oleate Treatment

Cells were plated in triplicate in 25 cm^2 flasks at a low density of 1 x 10^5 cells per flask in MEM (phenol red-free; Sigma-Aldrich, Oakville, ON, Canada) supplemented with 5% heat inactivated FBS. For total TG content and lipolysis measurements, cells were similarly plated in 75 cm^2 flasks at $3x10^5$ cells per flask. After two days of growth, cells were washed with PBS and the medium was replaced with fresh MEM with no additives for 18 h. After this period of serum starvation, the medium was replaced with fresh MEM (serum/growth factor free) containing albumin-bound oleate at concentrations ranging from 0 to 400 μM complemented with 0.5% BSA. For long-term survival experiments after exposure to oleate for 24 h, cells were cultured in MEM without serum, growth factor, BSA or FFA, and media were changed daily.

Caspase 3 Assay

Caspase 3 activity as an index of apoptosis was determined in cell lysates prepared from individual cultures using the protocol described in CaspACE™ Fluoremetric assay system (Promega, Madison, WI) with Ac-DEVD-AFC as the caspase-3 substrate (BIOMOL; Plymouth Meeting, PA). Cell protein concentration was determined using the BCA Protein Assay kit (Pierce; Rockford, IL) with BSA as standard.

Fatty Acid and Glucose Oxidation

Fatty acid oxidation was determined as the amount of $^{14}CO_2$ liberated from samples incubated with [1-^{14}C] palmitic acid or [1-^{14}C] oleic acid using a modified procedure described by (43). Briefly, cells were grown according to experimental procedure for oleate treatment in duplicate 25 cm^2 flasks. Then, media were discarded and replaced with 0.9 ml of fresh MEM containing 0.1% BSA and incubated at 37°C for 30 min in a CO_2 incubator. Subsequently, 100 µl of the 10X reaction mix (prepared freshly 2 h in advance and containing 10 mM carnitine, 1 mM palmitate, 4% BSA and 0.5 µCi per culture flask of labeled FA) was added to each flask. The flasks were immediately sealed with rubber serum vial stoppers. The stoppers were fitted with plastic tubes containing folded glass fiber filter paper (Whatman GF/B) saturated with 0.15 ml of 5% KOH. The sealed flasks were incubated for 1 h at 37°C. Control blank flasks contained all reagents without cells. The reaction was stopped by injecting 0.3 ml of 40% perchloric acid through the serum stopper into each flask with a syringe and the flasks were then shaken gently for 24 h at RT. Filters were then removed and placed into scintillation vials containing scintillation liquid. Radioactivity was counted 24 h later, using a liquid scintillation counter (Tri-Carb 2100TR, Perkin Elmer). Results were expressed as nmol of FFA released/h/mg of cell protein.

Glucose oxidation was measured by determining the release of $^{14}CO_2$ from [U-^{14}C] glucose. The experimental setup to capture $^{14}CO_2$ was as described above for FFA oxidation. Cells were preincubated in 0.9 ml MEM medium (with or without serum or with 0.1% BSA) for 30 min at 37°C in a CO_2 incubator (5% CO_2 and 95% air) after which 100µl aliquot of D-[U-^{14}C] glucose diluted in MEM was added to

each flask (0.5 µCi/flask). The flasks were then sealed and incubated for 1 h at 37°C. The reaction was stopped and the radioactivity captured by the filters was measured as described above. The results were expressed as nmol of glucose oxidized/h/mg of protein.

Triglyceride Assay

Cellular TG content was determined using the GPO-Trinder kit (Sigma Diagnostics, St-Louis, MO) and samples were prepared as described by (44). Two million cells harvested on ice by scraping were washed twice with cold PBS and lipids were extracted as described (45). The extracted lipids were dried under N_2, dissolved in 100 µl of 2% v/v Thesit detergent (Sigma, St-Louis, MO) in chloroform, dried again under N_2, and resuspended in 50 µl of water. The samples were then vortexed and sonicated in a water bath sonicator (Crest Tru-Sweep (50/60 Hz), Trenton, NJ) for 15 min at RT. Two hundred µl of GPO-Trinder kit reagent was added directly to each sample tube, mixed gently and incubated for 5 min at 37°C. The reaction mixture was transferred to a 1.5 ml microfuge tube and centrifuged at 12,000 x *g* at 4°C for 5 min. The supernatants were transferred to a 96 well-plate and the OD measured at 540 nm using a microplate reader (Bio-Rad 3550). Triolein (glyceryl trioleate; Sigma-Aldrich) was used as standard.

Lipid Droplets Staining

Lipid droplets were visualized using staining with Oil Red O. Stock solution was prepared by dissolving 300 mg Oil Red O (Allied Chemical, Brighton, UK) in

100 ml of 99% 2-propanol. Working solution prepared freshly by diluting the stock solution with water (3:2) was kept at RT for 10 min and filtered through a 0.22 µm filter (Corning PES filter). Cells in duplicate Petri dishes (60 mm) were washed twice with 5 ml PBS and incubated with 3 ml of Oil Red O working solution for 15 min at RT with gentle shaking. The cells were then washed once with PBS and fixed with 10% formalin (Anachemia, Lachine, QC, Canada) for 25 min. The cells were washed again with PBS, stained for 5 min with Harris Hematoxilin (Sigma-Aldrich) diluted 50% with PBS, washed with PBS, and 3 ml of 10% glycerol in PBS was added to each dish to prevent drying. Photomicrographs were taken from a representative field using an inverted microscope (Nikon Eclipse TE300) at 400X magnification and a digital camera (Nikon CoolPix 990).

Lipolysis

Cells were grown in 2 x 75 cm^2 flasks, trypsinized, pooled, resuspended in serum-free MEM medium containing 0.1% BSA and counted. The cells were then resuspended in fresh MEM medium containing 0.5% BSA at a density 2 x 10^6 cells/100 µl and incubated in 48 well-plates (100 µl/well) for 3 h at 37°C in a CO_2 incubator. Samples were then transferred to Eppendorf tubes and centrifuged at 1,500 rpm for 5 min at 4°C. The supernatants were collected and frozen at -80°C. The rate of lipolysis was measured using two different assays. Glycerol released from the cells was measured in triplicate using the Triglyceride GPO-Trinder kit (Sigma Diagnostics, St-Louis, MO). Total FFA released were determined in

duplicate using the Wako NEFA test kit (Wako Chemicals USA, Inc. Richmond, VA) and expressed as nmol glycerol or FFA released/h/10^6 cells.

FACS Analysis

DNA staining for FACS analysis was performed as described (46). Briefly, cells were harvested by trypsinization, washed with PBS plus 0.1% BSA at 4°C and the cells were fixed with 70% ethanol for 10 min at -20°C. They were then collected by centrifugation (300 x *g*, 5 min) and resuspended in staining buffer prepared by adding NP40 (0.6% v/v) and 36 µg/ml RNase to a 1 x dilution of a 20 x stock (1.17 g of sodium chloride, 2.13 g of sodium citrate, and 0.10 g of propidium iodide in 100 ml of water, pH 7.6 adjusted with acetic acid). Stained cells (10,000) were analyzed with a flow cytometer (FACS SCAN, Beckton Dickinson) and cell cycle analysis was performed using the Cell Quest Pro software.

Statistical Analysis.

Statistical significance was calculated with the Student's t-test. A P-value of <0.01 was considered significant.

Table 1. The Rates of Lipolysis in Oleate-Treated MDA-MB-231 Cells Evaluated by Measurements of the Amounts of Glycerol or FFA Released into the Medium.

Oleate (μM)	TG content (nmol/10^6 cells)	Glycerol released (nmol/h/10^6 cells)	FFA released (nmol/h/10^6 cells)
0	2.78 ± 0.25	3. 91± 1.51	<0.5
33	4.76 ± 0.37	12.72 ± 1.38	0.56 ± 0.22
100	18.25 ± 1.38	21.48 ± 1.45	0.84 ± 0.39
400	52.28 ± 3.62	35.8 ± 1.32	3.13 ± 0.18

Note: Cells were serum-starved for 24 h and then treated with oleate for another 24 h. Immediately after oleate treatment, cells were collected for lipid extraction and for determination of the rate of lipolysis as described in Methods. The data represent the means and SE for two independent experiments performed in duplicate (n=4).

Figure Legends

Figure 1: Effect of short-term oleate treatment on long-term serum-free survival and cell cycle distribution of MDA-MB-231 cells. **A**. Two days after seeding (Day 0), cells were serum-starved for 24 h (Day 1) and treated with a range of oleate concentrations (0-400 μM) for 24 h (Day 2). Cells were cultured for up to 8 days in medium without serum and oleate and, viable cells (cells excluding trypan blue), were counted daily. Cell survival is expressed as the mean number of live adherent cells/flask. The data represent the mean and SD for a representative experiment (n=3), which was repeated two times. **B**. FACS analysis for DNA content is shown for control cells grown in 5% FBS (Day 2) or for oleate-treated cells collected at various days. **C**. Cells serum-starved for 24 h were treated with 100 μM oleate for a range of times (0-24 h). Cells were then washed with PBS and incubated with medium containing no serum. Caspase-3 activity was assayed on total cells at the 24 h time point. The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

Figure 2: Relationship between short-term oleate treatment and cellular lipid droplet formation, total cell TG content, and lipolysis rate in MDA-MB-231 cells. Cells serum-starved for 24 h were treated with a range of oleate concentrations (0-400 μM) for 24 h **A**. Representative photomicrographs of Oil Red O-stained cells are shown (400X). Control cells were grown in media containing 5% FBS. **B** and **C**. At the end of oleate treatment, cells were immediately assayed for TG content (**B**) or

lipolysis rate (**C**). The data represent the mean and SE for two independent experiments performed in duplicate (n=4).

Figure 3: TG content, lipolysis rate and glucose oxidation in MDA-MB-231 cells treated with oleate and maintained in serum-free conditions. **A**, **B** and **C**. Cells serum-starved for 24 h were treated with 100 μM oleate for 24 h. Then, they were cultured for up to 7 additional days in medium without serum and oleate. Total cellular TG content (**A**), lipolysis (**B**) or glucose oxidation (**C**) of adherent cells was determined at specific days. Glucose oxidation is expressed as nmol/h/mg of protein. Glucose oxidation in MCF-10A cells is also shown for comparison (**C**). The data in all panels represent the mean and SE for two independent experiments performed in duplicate (n=4).

Figure 4: Effect of short-term oleate treatment on long-term serum-free survival, cellular TG content and FFA oxidation in a panel of human breast cell lines. **A**. Cells serum-starved for 24 h were treated with 100 μM oleate for 24 h. They were then cultured for 6 days in medium without serum or oleate. At Day 6 the number of viable cells was determined and the long-term serum-free survival was calculated as the mean percentage of control at Day 1 (before addition of oleate). The data are the means and SE for 2-4 independent experiments performed with triplicate flasks per experiment (n=6 for T-47D, n=9 for MDA-MB-231, n=12 for MDA-MB-468, n=8 for MCF 7 and n=4 for MCF-10A). *, $p < 0.01$ *versus* respective control. **B**. After 24 h of serum starvation, cells were treated with a 100 μM oleate for 24 h and assayed

for TG content **C**. Fatty acid oxidation was measured after 24 h of serum starvation. For (**B**) and (**C**), the data represent the mean and SE for two independent experiments performed in duplicate (n=4).

Figure 5: Model illustrating the possible link between TG/FFA cycling and breast cancer cell growth/survival. Exogenous FFA after entering the cell are activated to form FA-CoA (Step #1) and esterified to form TG (Step #2). Cellular TG content increases proportionally to exogenous oleate dose. The TG pool is not inert but is constantly turning over due to high rates of both lipolysis and reesterification involved in TG/FFA cycling (Step #3). The rate of TG/FFA cycling increases as a function of the original exogenous FFA (oleate) concentration. TG/FFA cycling needs G3P, production of which is coupled to NAD regeneration (Step #4). Therefore, the high rate of TG/FFA cycling sets the stable rate of cellular NAD regeneration, which in turn allows the maintenance of a high rate of glycolysis. This allows the production of sufficient amounts of pyruvate (Step #5) required for anaplerosis (Step #6) and de novo FA synthesis (Step #7), which can supply the intracellular pool of FA and contribute to continuing TG/FFA cycling.

References

1. Calle, E. E. and Thun, M. J. Obesity and cancer. Oncogene, *23:* 6365-6378, 2004.
2. Calle, E. E. and Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer, *4:* 579-591, 2004.
3. Kuhl, H. Breast cancer risk in the WHI study: the problem of obesity. Maturitas, *51:* 83-97, 2005.
4. Felber, J. P. and Golay, A. Pathways from obesity to diabetes. Int J Obes Relat Metab Disord, *26 Suppl 2:* S39-45, 2002.
5. El-Assaad, W., Buteau, J., Peyot, M. L., Nolan, C., Roduit, R., Hardy, S., Joly, E., Dbaibo, G., Rosenberg, L., and Prentki, M. Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. Endocrinology, *144:* 4154-4163, 2003.
6. McGarry, J. D. What if Minkowski had been ageusic? An alternative angle on diabetes. Science, *258:* 766-770, 1992.
7. Unger, R. H. Longevity, lipotoxicity and leptin: the adipocyte defense against feasting and famine. Biochimie, *87:* 57-64, 2005.
8. Prentki, M., Joly, E., El-Assaad, W., and Roduit, R. Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. Diabetes, *51 Suppl 3:* S405-413, 2002.
9. Hardy, S., El-Assaad, W., Przybytkowski, E., Joly, E., Prentki, M., and Langelier, Y. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. J Biol Chem, *278:* 31861-31870, 2003.
10. Hardy, S., Langelier, Y., and Prentki, M. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res, *60:* 6353-6358, 2000.
11. Soriguer, F., Esteva, I., Rojo-Martinez, G., Ruiz de Adana, M. S., Dobarganes, M. C., Garcia-Almeida, J. M., Tinahones, F., Beltran, M., Gonzalez-Romero, S., Olveira, G., and Gomez-Zumaquero, J. M. Oleic acid from cooking oils is associated with lower insulin resistance in the general population (Pizarra study). Eur J Endocrinol, *150:* 33-39, 2004.
12. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Jr., Ory, D. S., and Schaffer, J. E. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A, *100:* 3077-3082, 2003.
13. Welters, H. J., Tadayyon, M., Scarpello, J. H., Smith, S. A., and Morgan, N. G. Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. FEBS Lett, *560:* 103-108, 2004.
14. Hardy, S., St-Onge, G. G., Joly, E., Langelier, Y., and Prentki, M. Oleate promotes the proliferation of breast cancer cells via the G protein-coupled receptor GPR40. J Biol Chem, *280:* 13285-13291, 2005.
15. Dulloo, A. G., Gubler, M., Montani, J. P., Seydoux, J., and Solinas, G. Substrate cycling between de novo lipogenesis and lipid oxidation: a

thermogenic mechanism against skeletal muscle lipotoxicity and glucolipotoxicity. Int J Obes Relat Metab Disord, *28 Suppl 4:* S29-37, 2004.
16. Warburg, O. On the origin of cancer cells. Science, *123:* 309-314, 1956.
17. Warburg, O., Posener, K., and Negelein, E. Uber den stoffwechsel der tumoren. Biochem Z, *152:* 319-344, 1924.
18. Dang, C. V. and Semenza, G. L. Oncogenic alterations of metabolism. Trends Biochem Sci, *24:* 68-72, 1999.
19. Osthus, R. C., Shim, H., Kim, S., Li, Q., Reddy, R., Mukherjee, M., Xu, Y., Wonsey, D., Lee, L. A., and Dang, C. V. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem, *275:* 21797-21800, 2000.
20. Plas, D. R. and Thompson, C. B. Cell metabolism in the regulation of programmed cell death. Trends Endocrinol Metab, *13:* 75-78, 2002.
21. Hammerman, P. S., Fox, C. J., and Thompson, C. B. Beginnings of a signal-transduction pathway for bioenergetic control of cell survival. Trends Biochem Sci, *29:* 586-592, 2004.
22. Mazurek, S. and Eigenbrodt, E. The tumor metabolome. Anticancer Res, *23:* 1149-1154, 2003.
23. Stubbs, M., Bashford, C. L., and Griffiths, J. R. Understanding the tumor metabolic phenotype in the genomic era. Curr Mol Med, *3:* 49-59, 2003.
24. Zechner, R., Strauss, J. G., Haemmerle, G., Lass, A., and Zimmermann, R. Lipolysis: pathway under construction. Curr Opin Lipidol, *16:* 333-340, 2005.
25. Vaughan, M. The production and release of glycerol by adipose tissue incubated in vitro. J Biol Chem, *237:* 3354-3358, 1962.
26. Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C. M., Kalhan, S. C., Tilghman, S. M., and Hanson, R. W. Glyceroneogenesis and the triglyceride/fatty acid cycle. J Biol Chem, *278:* 30413-30416, 2003.
27. Vander Heiden, M. G., Plas, D. R., Rathmell, J. C., Fox, C. J., Harris, M. H., and Thompson, C. B. Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol, *21:* 5899-5912, 2001.
28. Coleman, R. A., Lewin, T. M., Van Horn, C. G., and Gonzalez-Baro, M. R. Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways? J Nutr, *132:* 2123-2126, 2002.
29. Coleman, R. A. and Lee, D. P. Enzymes of triacylglycerol synthesis and their regulation. Prog Lipid Res, *43:* 134-176, 2004.
30. Bertin, R. Glycerokinase activity and lipolysis regulation in brown adipose tissue of cold acclimated rats. Biochimie, *58:* 431-434, 1976.
31. Edinger, A. L., Cinalli, R. M., and Thompson, C. B. Rab7 prevents growth factor-independent survival by inhibiting cell-autonomous nutrient transporter expression. Dev Cell, *5:* 571-582, 2003.
32. Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T., and Thompson, C. B. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell, *120:* 237-248, 2005.
33. Thompson, J. E. and Thompson, C. B. Putting the rap on Akt. J Clin Oncol, *22:* 4217-4226, 2004.

34. Katsuma, S., Hatae, N., Yano, T., Ruike, Y., Kimura, M., Hirasawa, A., and Tsujimoto, G. Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. J Biol Chem, *280:* 19507-19515, 2005.
35. Burdall, S. E., Hanby, A. M., Lansdown, M. R., and Speirs, V. Breast cancer cell lines: friend or foe? Breast Cancer Res, *5:* 89-95, 2003.
36. Wang, F., Hansen, R. K., Radisky, D., Yoneda, T., Barcellos-Hoff, M. H., Petersen, O. W., Turley, E. A., and Bissell, M. J. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst, *94:* 1494-1503, 2002.
37. Pantel, K. and Woelfle, U. Detection and molecular characterisation of disseminated tumour cells: implications for anti-cancer therapy. Biochim Biophys Acta, *1756:* 53-64, 2005.
38. Wiedswang, G., Borgen, E., Karesen, R., Qvist, H., Janbu, J., Kvalheim, G., Nesland, J. M., and Naume, B. Isolated tumor cells in bone marrow three years after diagnosis in disease-free breast cancer patients predict unfavorable clinical outcome. Clin Cancer Res, *10:* 5342-5348, 2004.
39. Muller, V., Stahmann, N., Riethdorf, S., Rau, T., Zabel, T., Goetz, A., Janicke, F., and Pantel, K. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res, *11:* 3678-3685, 2005.
40. Petrek, J. A., Hudgins, L. C., Ho, M., Bajorunas, D. R., and Hirsch, J. Fatty acid composition of adipose tissue, an indication of dietary fatty acids, and breast cancer prognosis. J Clin Oncol, *15:* 1377-1384, 1997.
41. Perez Alenza, D., Rutteman, G. R., Pena, L., Beynen, A. C., and Cuesta, P. Relation between habitual diet and canine mammary tumors in a case-control study. J Vet Intern Med, *12:* 132-139, 1998.
42. Chlebowski, R., Blackburn, G., Elashoff, R., Thompson, C., Goodman, M., Shapiro, A., Giuliano, A., Karanja, N., Hoy, M., and Nixon, D. Diatery fat reduction in posmonopausal women with primary breast cancer: Phase III Women's Intervention Nutrition Study. 2005.
43. Averill-Bates, D. A. and Przybytkowski, E. The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. Arch Biochem Biophys, *312:* 52-58, 1994.
44. Van Veldhoven, P. P., Swinnen, J. V., Esquenet, M., and Verhoeven, G. Lipase-based quantitation of triacylglycerols in cellular lipid extracts: requirement for presence of detergent and prior separation by thin-layer chromatography. Lipids, *32:* 1297-1300, 1997.
45. Bligh, E. G. and Dyer, W. J. A rapid method of total lipid extraction and purification. Can J Biochem Physiol, *37:* 911-917, 1959.
46. Trimboli, A. J., Waite, B. M., Atsumi, G., Fonteh, A. N., Namen, A. M., Clay, C. E., Kute, T. E., High, K. P., Willingham, M. C., and Chilton, F. H. Influence of coenzyme A-independent transacylase and cyclooxygenase inhibitors on the proliferation of breast cancer cells. Cancer Res, *59:* 6171-6177, 1999.

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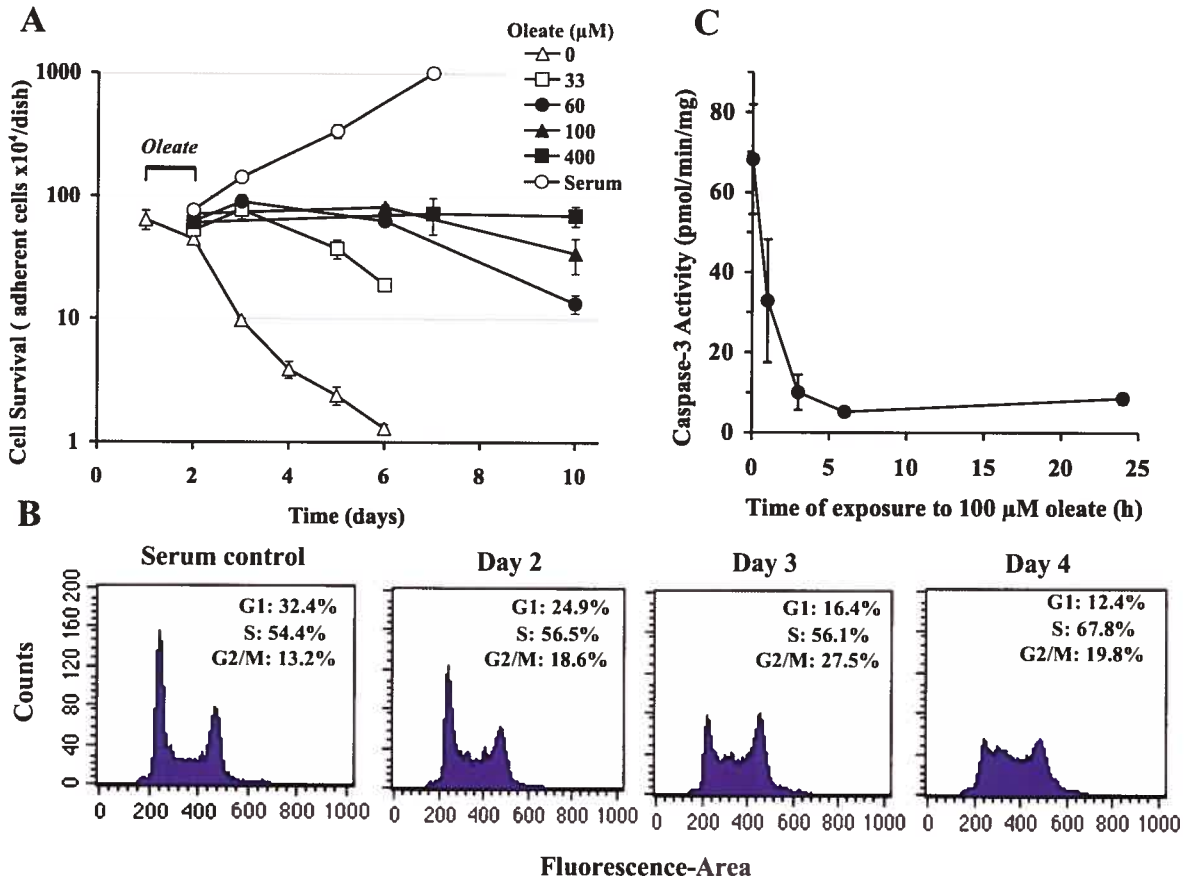
A
Oleate (μM)
0
33
60
100
400
Serum
Oleate
Cell Survival (adherent cells x10⁴/dish)
1000
100
10
1
0
2
4
6
8
10
Time (days)
C
Caspase-3 Activity (pmol/min/mg)
80
60
40
20
0
0
5
10
15
20
25
Time of exposure to 100 μM oleate (h)
B
Serum control
Day 2
Day 3
Day 4
G1: 32.4%
S: 54.4%
G2/M: 13.2%
G1: 24.9%
S: 56.5%
G2/M: 18.6%
G1: 16.4%
S: 56.1%
G2/M: 27.5%
G1: 12.4%
S: 67.8%
G2/M: 19.8%
Counts
0 40 80 120 160 200
0 200 400 600 800 1000
Fluorescence-Area

Figure 1

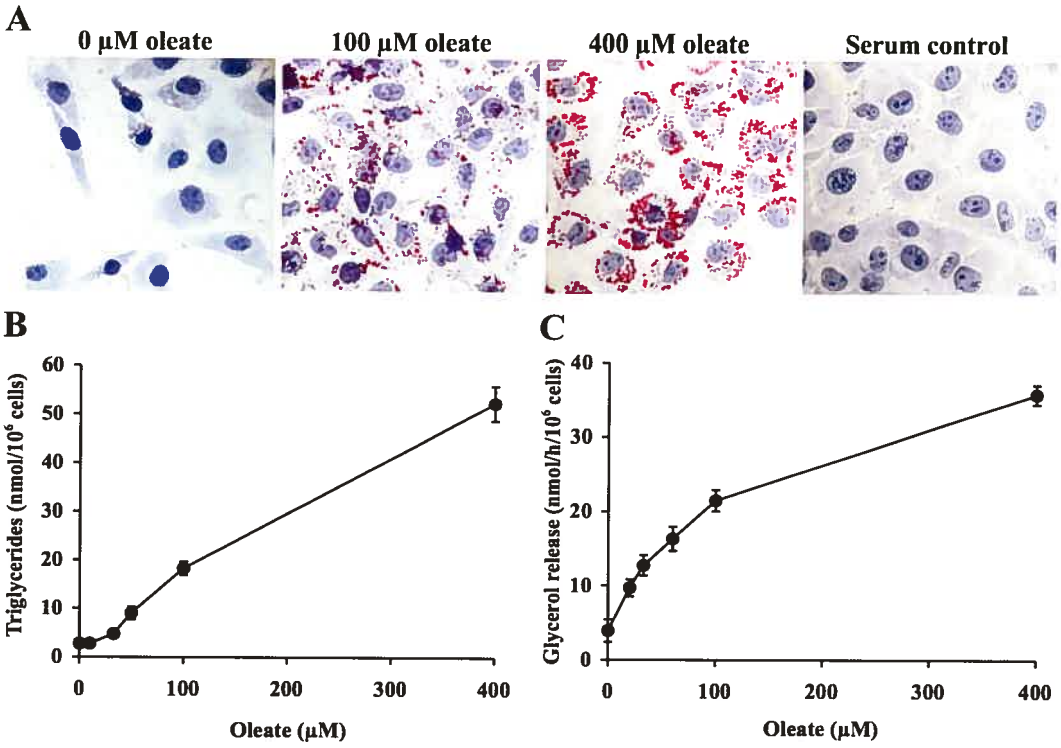
A
0 µM oleate
100 µM oleate
400 µM oleate
Serum control
B
Triglycerides (nmol/10^6 cells)
60
50
40
30
20
10
0
0
100
200
300
400
Oleate (µM)
C
Glycerol release (nmol/h/10^6 cells)
40
30
20
10
0
0
100
200
300
400
Oleate (µM)

Figure 2

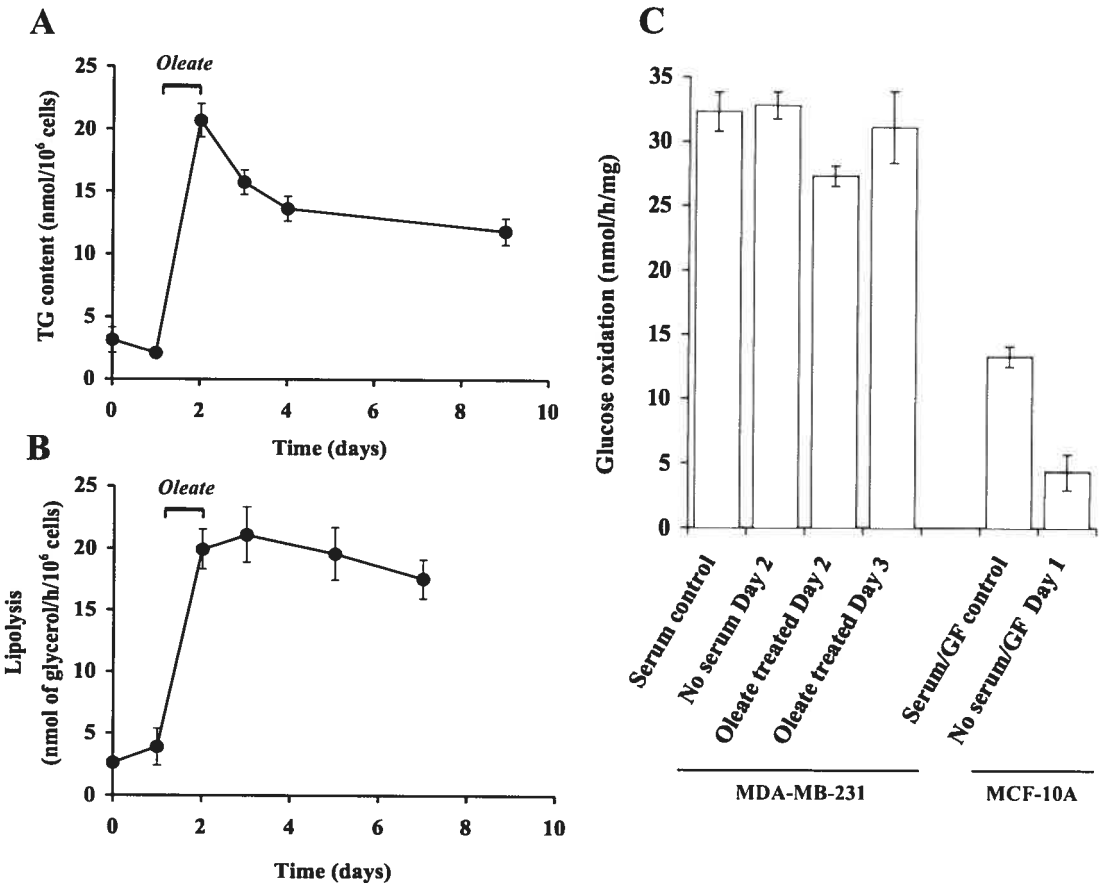
A
Oleate
TG content (nmol/10^6 cells)
25
20
15
10
5
0
0
2
4
6
8
10
Time (days)
B
Oleate
Lipolysis
(nmol of glycerol/h/10^6 cells)
25
20
15
10
5
0
0
2
4
6
8
10
Time (days)
C
Glucose oxidation (nmol/h/mg)
35
30
25
20
15
10
5
0
Serum control
No serum Day 2
Oleate treated Day 2
Oleate treated Day 3
Serum/GF control
No serum/GF Day 1
MDA-MB-231
MCF-10A

Figure 3

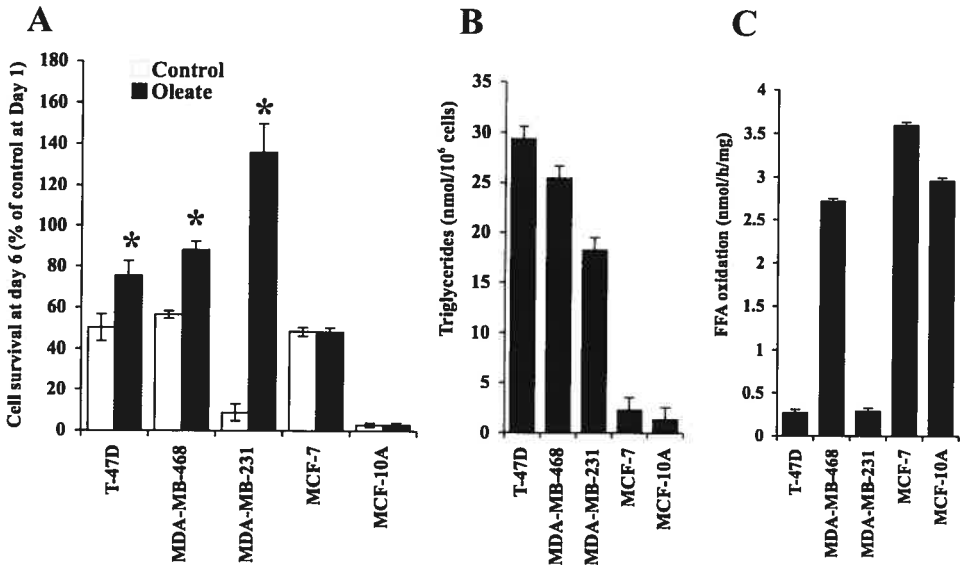
A
B
C
Control
Oleate
Cell survival at day 6 (% of control at Day 1)
Triglycerides (nmol/10^6 cells)
FFA oxidation (nmol/h/mg)
T-47D
MDA-MB-468
MDA-MB-231
MCF-7
MCF-10A

Figure 4

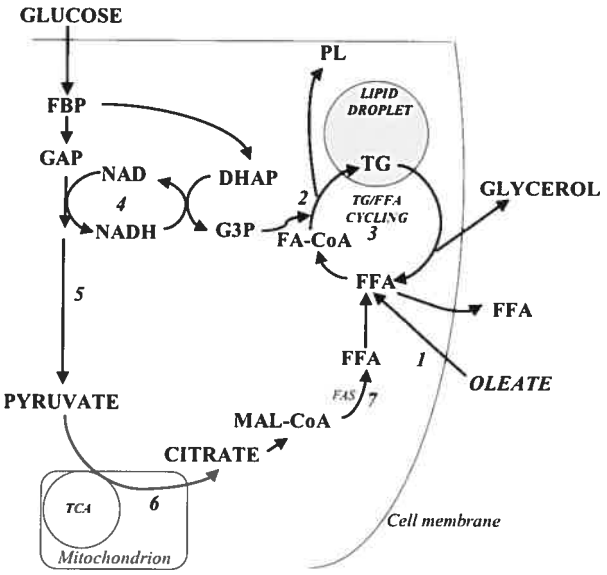
GLUCOSE
PL
LIPID DROPLET
FBP
GAP
NAD
DHAP
TG
GLYCEROL
4
2
TG/FFA CYCLING
NADH
G3P
FA-CoA
3
FFA
5
FFA
FFA
1
OLEATE
PYRUVATE
FAS
7
MAL-CoA
CITRATE
TCA
6
Cell membrane
Mitochondrion

Figure 5

APPENDIX II
CURRICULUM VITAE

EWA PRZYBTKOWSKI

(Molecular Biology, Université de Montréal, 2006)

Education:

2006 **Ph.D.** Molecular Biology, Programmes de biologie moléculaire, Université de Montréal, Quebec, Canada.

1979 **M.Sc.** Molecular Biology, University of Warsaw, Warsaw, Poland.

Graduate Student Scholarships and Awards (1999-2006):

2005-2006 Bourse d'excellence, Fondation Canderel/Institute du cancer de Montréal

2004-2005 Bourse, Faculté des etudes supérieures, Université de Montréal, (Molecular Biology)

2003-2004 Bourse d'excellence, R. Bourassa, l'Assemble National du Québec

2003 Prix d'excellence, Congres du CHUM

2001-2002 Bourse d'excellence, Fondation Marc Bourgie, Institute du Cancer de Montréal

1999-2001 Bourse, Université de Montréal (Molecular Biology).

1999 Bourse, Centre de Recherche du CHUM.

Research Work Experience: (1979-2006)

2006 **Posdoctoral Fellow,** Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada.

1998-2001 **Research Assistant**, Centre de recherche du Centre hospitalier de l'Université de Montréal. Montréal, Québec, Canada.

1996-1998 **Research Assistant,** Laboratory of Intermediary Metabolism. Institut de recherche cliniques de Montréal. Montréal, Québec, Canada.

1989-1995 **Research Assistant**, Département de Chimie/Biochimie, Université du Québec à Montréal, Montréal, Québec, Canada.

1984-1986 **Research Assistant**, McGill Cancer Center, McGill University, Montréal, Québec, Canada.

1983 **Research Assistant**, Bioresearch Department, The Ontario Cancer Institute at Princess Margaret Hospital, Toronto, Ontario, Canada.

1979-1982 **Research Assistant**, Department of Biochemistry, University of Arizona, Tucson, Arizona, USA.

Publications in Peer Reviewed Journals:

18 publications in the field of cancer biology, biochemistry, and molecular biology

First author publications (4):

1. **Przybytkowski E**, Joly E, Nolan C, Hardy S, Francoeur A-M, Langelier Y, Prentki M. Upregulation of cellular triacylglycerol/free fatty acid cycling by oleate is associated with long-term growth factor independent survival of human breast cancer cell. Submitted to Molecular Cancer Research (**2006**).
2. **Przybytkowski E**, Girouard S, Allard B, Lamarre L, Basik M. Widespread bimodal intrachromosomal genomic instability in sporadic breast cancers associated with 13q allelic imbalance. **Cancer Res.** 63:4588-93 (**2003**).
3. **Przybytkowski E,** Averill-Bates DA. Correlation between glutathione and stimulation of the pentose phosphate cycle in Chinese hamster ovary cells exposed to hydrogen peroxide. Arch. Biochem. Biophys., 325, 91-98 (1996).

4. **Przybytkowski E**, Bates J, Bates D, Mackillop W. Thermal adaptation of CHO cells at 40°C: The influence of growth conditions and the role of heat shock proteins. Radiat. Res. 107: 317-331 (1986).

Other publications (13):

5. Nolan C, Leahy J, Delghingaro-Augusto V, Moibi J, Soni K, Peyot M-L, Fortier M, Guay C, Lamontagne J, **Przybytkowski E**, Joly E, Masiello P, Wang S, Michell G, Prentki M. Beta cell compensation for insulin resistance in Zuker fatty rats: increased lipolysis and fatty acid signalling. Diabetologia 49:2120-2130 (2006).

6. Roduit R, Nolan C, Alarcon C, Moore P, Barbeau A, Delghingaro-Augusto V, **Przybykowski E**, Morin J, Masse F, Massie B, Ruderman N, Rhodes C, Poitout V, Prentki M. A role for the malonyl-CoA/long-chain acyl-CoA pathway of lipid signaling in the regulation of insulin secretion in response to both fuel and nonfuel stimuli. Diabetes 53:1007-19 (2004).

7. Hardy S, El-Assaad W, **Przybytkowski E,** Joly E, Prentki M, Langelier Y. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. J Biol Chem. 278: 31861-70 (2003).

8. Park H, Kaushik VK, Constant S, Prentki M, **Przybytkowski E**, Ruderman NB, Saha AK. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. J Biol Chem. 277:32571-7 (2002).

9. Lord-Fontaine, S., Agostinelli, E., **Przybytkowski, E.**, Averill-Bates, D.A. Amine oxidase, spermine and hyperthermia induce cytotoxicity in P-glycoprotein overexpressing multidrug resistant Chinese hamster ovary cells. Biochem. Cell Biol. 79 (2):165-175 (2001).

10. Philippoussis F, **Przybytkowski E**, Fortin M, Arguin CH, Pande SV, Steff A-M, Hugo P. Derivatives of monoglycerides as apoptotic agents in T-cells. Cell Death and Differentiation 8:1103-1112 (2001).

11. Agostinelli E. **Przybytkowski E**, Averill-Bates DA. Glucose, glutathione, and cellular response to spermine oxidation products. Free Radicals in Biology and Medicine 20:649-656 (1996).

12. Averill-Bates, D.A., Agostinelli, E., **Przybytkowski, E.**, and Mondovi', B. Aldehyde dehydrogenase and cytotoxicity of purified bovine serum amine oxidase and spermine in Chinese hamster ovary cells. Biochem. Cell Biol. 72:36-42 (1994).

13. Averill-Bates D,. **Przybytkowski E**. The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. Arch. Biochem. Biophys. 312:52-58 (1994).

14. Agostinelli E, **Przybytkowski E**, Mondovi' B, Averill-Bates DA. Heat enhancement of cytotoxicity induced by oxidation products of spermine in Chinese hamster ovary cells. Biochem. Pharmacology 48:1181-1186 (1994).

15. Averill-Bates DA, Agostinelli E, **Przybytkowski E**, Mateescu MA, Mondovi' B. Cytotoxicity and kinetic analysis of purified bovine serum amine oxidase in the presence of spermine in CHO cells. Arch. Biochem. Biophys. 300:75-79 (1993).

16. Agostinelli E, Bates DA, **Przybytkowski E**, Mateescu MA, Mondovi' B. Polyamine cytotoxicity induced by serum amine oxidase in Chinese hamster ovarian (CHO) cells. Ital. J. Biochem. 40:43A-45A (1991).

17. Bates J, **Przybytkowski E**, Bates D, Mackillop W. A model-free way of representing hyperthermia cell survival data. Radiat. Res. 107:307-316 (1986).

18. Manos MM, Munroe SD, **Przybytkowska E,** McReynolds L. Transfer of mutant viral thymidine kinase gene results in temperature-sensitive mouse cells. Molecular Cell Biology 3:490 (1983).