EFFECT OF BIOACTIVE POLYUNSATURATED FATTY ACIDS ON DE NOVO LIPID SYNTHESIS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by
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January 2012

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Cornell University 2012

Conjugated linoleic acid (CLA) and the long chain ω-3 polyunsaturated fatty acids (LC ω-3 PUFAs) represent unique examples of bioactive nutrients that show a wide range of functions in biological systems. De novo lipogenesis (DNL) is a critical metabolic process that is implicated in wide range of physiological and pathological processes. The objective of this dissertation was to investigate the molecular mechanisms of different bioactive fatty acids (FAs) on some aspects of lipid metabolism. The initial investigation was to examine the role of thyroid hormone responsive spot 14 (S14) in the regulation of lipid synthesis in adipose tissue of growing mice and the possible role of S14 in the CLA-induced reduction in fat accretion. Wild type (Wt) and *S14* null mice were supplemented with CLA for 14 days. Knocking out the *S14* gene significantly reduced the epididymal fat depot, but there was no corresponding reduction in the mRNA abundance for lipogenic genes. CLA treatment significantly reduced the total dissected fat tissue and downregulated the mRNA abundance of *SREBF-1c*, *ACACA* and *FASN* regardless of the genotype. This suggests that the S14 protein is not involved in the transcriptional regulation of lipogenic enzymes and expression of the *S14* gene is not essential for the CLA delipidative mechanism. A second focus of this thesis was to examine the effects of LC ω-3 PUFAs on lipid synthesis in lactating mice. Mice were given a diet containing 10% fish oil (FO) from d 6 to 13 of lactation. Results showed no differences among treatments in the proportion of de novo synthesized milk FAs or the lipogenic capacity of mammary explants. In addition, mRNA abundance of key enzymes in the FA synthesis pathway were not different among treatments. In contrast, FO treatment resulted in an impressive decrease in hepatic lipid accumulation and downregulation of lipogenic

gene expression. Overall, these results demonstrate that dietary FO supplementation to lactating mice had no effect on mammary lipogenic variables, but effects on hepatic lipogenesis were obvious. The final investigation was to investigate the mechanism of CLA induced milk fat depression (MFD) in lactating ewes and to determine their suitability to serve as convenient model for investigations of mammary lipid metabolism in lactating cows. Lactating dairy ewes received a CLA supplement containing *trans*-10, *cis*-12 CLA for 10 wks. CLA supplementation resulted in a decrease in milk fat secretion with de novo synthesized FAs being most markedly affected. Consistent with this, a coordinated downregulation in the mRNA expression of key enzymes involved in DNL occurred. Thus, lactating ewes are responding to the *trans*-10, *cis*-12 CLA in a manner similar to cows and should represent a good model to continue mechanistic studies of the regulation of lipid metabolism in mammary gland.

Biographical Sketch

Mahmoud Hussein grew up in Damanhour City, north of Egypt. He was graduated from the College of Veterinary Medicine at Alexandria University in 1997. Mahmoud showed interest in academia and later in 1999 he was appointed as teaching and research assistant in the Department of Nutrition, College of Veterinary Medicine, Menufiya University. Mahmoud received his master degree in 2004 from Menufiya university where he investigated the effect of dietary supplementation of different linoleic acid to alpha linolenic acid ratios on performance and histopathology of broiler chickens under the guidance of Dr. Esam Yousuf. Later, Mahmoud was granted a scholarship from the Department of Education and Scientific Research of Egypt to pursue his PhD studies in USA. At Cornell University, Mahmoud worked under the direction of Dr. Dale Bauman with the primary objective of understanding the basics of nutritional genomics. After completion of his PhD degree, Mahmoud will work with Dr. Dale Bauman and Dr. Yves Boisclair as postdoctoral associate before joining the Department of Nutrition faculty at the College of Veterinary Medicine, Menufiya University. Mahmoud is married to Walaa Alshafie and has Omar and Menna.

To my parents, to my beloved wife, to my kids Omar and Menna, and
to those who have taught me about science and life

Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Prof. Dale Bauman for the continuous support and guidance during my study at Cornell. His encouragement and immense knowledge have guided and helped me all the way through my research and writing of this thesis. Dr. Bauman was an amazing model of how real scientist should be. Hard working, dedication and caring with ever lasting smiles and always open doors were a great compilation of Dr. Bauman's nature that inspires me. His understanding of many moments in my family life is unforgettable. Working with Dr. Bauman was a great honor and I could not have imagined having a better advisor and mentor for my Ph.D study. His impact on my life as scientist will not be forgotten and I will be always indebted for him.

My deepest thanks are also due to the members of my thesis committee: Prof. Yves Boisclair, Dr. Qiamong Long and Dr. Ling Qi, for their help, insightful comments, and hard questions. Sincere thanks goes to Dr. Boisclair for the unlimited access to his lab, interaction with his group and his valuable research comments.

This work would not be possible without the support of my country, Egypt, through the Department of High Education and Scientific Research. The financial help covered most of my study and living expenses for me and my family. For sure, this investment will pay off in the future through our research contribution and knowledge transfer to build bright and promising future for our beloved country. This will not be forgotten and someday our country will be proud of us and we will be proud of her.

This research project would not have been achieved without the support of many people. I thank my fellow lab mate Dr. Kevin Harvatine for his effort in teaching, orienting and helping me before and after his graduation. Dr. Harvatine provided me with exceptional training in molecular biology and mouse experimental procedures. Without his kind help and valuable comments, this work would not be accomplished. Also, my sincere thanks to my group members, Dr. Cindy Tyburczy, Dr. Anne O'Donnel, Dr. Monica Foote and Susanne Peloton for their valuable help, nice conversations and advising during my research.

Special thanks also to Dr. Sarah Giesy for the stimulating discussion, sharing literature and invaluable assistance. Also, I wish to express my gratitude to Dr. Patricia Johnson and her students who gave us exceptional access to the Real-Time PCR machine.

Overall my life in Cornell was amazing with the help of many people in the campus who taught me many things about life and science. Dr. Susan Suarez, department of biomedical science, she and Dr. Bauman were our family models for dedication, hard working, sincerity and for their kindness, caring and understanding. Words are not enough to speak for these people.

Lastly, I offer my regards and thanks to all of those who supported me and my family in any respect during the completion of my research projects. Dr. Ahmed Hassan, Dr. Mustafa Alsherif, Dr. Mahmoud Zeid and their families, Takashi and Mayuka Yasui, Cheryl Barbasch and Anita Watkins were our big family and made our life in Ithaca an unforgettable nice experience. We will not forget them.

Last, but not least, I wish to express my love and gratitude to my beloved parents, my lovely wife and my awesome son and daughter for their understanding and endless love, through the duration of my studies at Cornell.

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Glossary

18S	18S ribosomal RNA.
AA	Arachidonic acid.
ACAC	Acetyl-coenzyme A carboxylase.
ACACA	Acetyl-coenzyme A carboxylase alpha.
ACACB	Acetyl-coenzyme A carboxylase beta.
ACLY	ATP citrate lyase.
ACOX1	Acyl-Coenzyme A oxidase 1, palmitoyl.
ACSS1	Acyl-CoA synthetase short-chain family member 1.
ACTB	Actin, beta.
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase.
ALA	α linolenic acid.
AMPK	AMP-activated protein kinase.
ATF6	Activating transcription factor 6.
B2M	Beta-2-micro-globulin.
BW	Body weight.
C/EBP	CCAAT/enhancer binding protein.
CD36	Fatty acid translocase.

ChRE Carbohydrate response element.

ChREBP Carbohydrate response element binding protein.

CLA Conjugated linoleic acid.

COX Cyclooxygenases.

CPT1a Carnitine palmitoyltransferase 1a.

DFAS De novo FA synthesis.

DGAT Diacylglycerol O-acyltransferase.

DGAT1 Diacylglycerol O-acyltransferase 1.

DHA Docosahexaenoic acid; C22:6 $\omega-3$.

DLS De novo lipid synthesis.

DNL De novo lipogenesis.

DSFA De novo synthesized fatty acid.

eIF2α Eukaryotic initiation factor 2 α.

EPA Eicosapentaenoic acid; C20:5 $\omega-3$.

EPF Epididymal fat.

ER Endoplasmic reticulum.

FA Fatty acid.

FABP1 Fatty acid binding protein 1.

FABP3 Fatty acid binding protein 3.

FASN Fatty acid synthase.

FO Fish oil.

G6PD Glucose-6-phosphate dehydrogenase.

GLUT-4 Glucose transporter type 4.

GPAM Glycerol-3-phosphate acyltransferase, mitochondrial.

GPAT Glycerol-3-phosphate acyltransferase.

GRP78 Glucose-regulated protein.

HFC High fat control.

HNF-4 Hepatocyte nuclear factor 4.

HSL Hormone sensitive lipase.

IDH1 NADP- isocitrate dehydrogenase.

IL Interleukin.

INSIG1 Insulin induced gene 1.

INSIG2 Insulin induced gene 2.

IRE1 Inositol-requiring 1.

ISGC International Sheep Genomics Consortium.

LA linoleic acid.

LBM Lean body mass.

LC ω-3 PUFA long chain ω-3 polyunsaturated fatty acids.

LFC Low fat control.

LOX Lipoxygenases.

L-PK L-type pyruvate kinase.

LPL Lipoprotein lipase.

LT Leukotrienes.

LXR Liver X receptor.

MAPK Mitogen activated protein kinases.

MBTPS1 Membrane-bound transcription factor peptidase, site 1.

MCFAs Medium chain fatty acids.

MEF Mesentric fat.

MFD Milk fat depression.

MIG12 S14 realted protein or MIG12.

MLX MAX-like protein X.

NCBI National Center for Biotechnology Information.

NEFA Non-esterified fatty acids.

NFκB Nuclear factor kappa B.

nSREBF Nuclear SREBF.

null *S14* partial knockout mice.

PERK PKR-like ER kinase.

PG Prostaglandins.

PGD Phosphogluconate dehydrogenase.

PPAR Peroxisome proliferator-activated receptor.

PPARa Peroxisome proliferator-activated receptor alpha.

PUFAs Polyunsaturated fatty acids.

qRT-PCR Quantitative real-time PCR.

RPF Retroperitoneal fat.

RPL13A Ribosomal protein L13a.

RPS13 Ribosomal protein S13.

RPS15 Ribosomal protein S15.

RPS20 Ribosomal protein S20.

RXR Retinoid X receptor.

S14 Thyroid hormone responsive spot 14.

SCAP SREBF chaperone.

SCD Stearoyl-coenzyme A desaturases.

SCD1 Stearoyl-coenzyme A desaturase 1.

SCF Subcutaneous fat.

SREBF Sterol regulatory element binding transcription factor.

SREBF1 Sterol regulatory element binding transcription factor 1.

SREBF1-c Sterol regulatory element binding transcription factor 1-c.

T3 Triiodothyronine.

TG Triglycerides.

TH Thyroid hormone.

TNFα Tumor necrosis factor.

TX Thromboxanes.

UCP Uncoupling proteins.

UPR Unfolded protein response.

WAT White adipose tissue.

XBP1 X-box binding protein 1.

Chapter 1

Introduction

In affluent societies the science of nutrition is at a new frontier. It is progressing from the concept of 'adequate nutrition' for survival to that of 'optimal nutrition' for improving health and prevention of chronic diseases [1]. Until recently, nutrition research concentrated on nutrient deficiencies and impairment of health. However, due to a growing realization that the effects of nutrition on health and disease cannot be understood without a profound understanding of how nutrients act at the molecular level, nutrition research has undergone an important shift in focus from epidemiology and physiology to molecular biology and genetics. The advent of genomics has created unprecedented opportunities for increasing our understanding of how nutrients modulate gene and protein expression and ultimately influence cellular and organismal metabolism.

From a nutrigenomics perspective, nutrients are considered to be signaling molecules that, through appropriate cellular sensing mechanisms, result in translation of these dietary signals into changes in gene, protein, and metabolite expression [2]. The information that allows nutrients to activate specific signaling pathways is contained within their molecular structure and minor changes in structure, for example, saturated vs unsaturated fatty acids (FAs) or the position and geometry of the double bonds in a FA can have a profound influence on which sensor pathways are activated. Nutrients can be regarded as functional if they have been proved to affect one or more target functions in the body, beyond the traditional

nutritional effects, in a way that is relevant to an improved state of health and well-being and a reduction in the risk of chronic diseases [3].

Fat has traditionally been regarded to be important as a calorie-dense nutrient and as a source for essential FAs. In recent years, specific FAs have been increasingly recognized as important biological regulators. In this regard, CLA and the LC ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have attracted considerable attention because of their reported anticarcinogenic, antiatherogenic, antiobesity, antiadipogenic, and antiinflammatory biological activities [4,5]. All of these potentially positive target functions highlight the importance of these bioactive polyunsaturated fatty acids (PUFA) at a time when the increasing prevalence of obesity and the metabolic syndrome affects not only adults but also a worrisome proportion of adolescents and children. CLA refers to all the positional and geometric isomers of linoleic acid. CLA isomers are formed when reactions shift the location of one or both of the double bonds of linoleic acid in such a manner that the two double bonds are no longer separated by an interceding carbon. On the other hand, FA that have a longer chain length, higher number of double bonds, and an n-3 double bond arrangement represent LC ω-3 PUFAs with distinct and unique biological properties that separate them and their metabolic products from other FAs. Many of the effects of these bioactive PUFAs in both cell biology and human health and disease relate to their ability to regulate gene expression and subsequent downstream events.

The DNL from simple substrates, glucose and acetate in monogastrics and ruminant animals respectively, requires collaboration and harmony of multiple metabolic pathways, including glycolysis and pyruvate oxidation or activation of acetate to generate acetyl-CoA for FA synthesis, NADPH generation to supply the reductive power, packaging of FAs into a glycerophosphate backbone, and finally, packaging to export triglycerides in liver (lipoproteins) or mammary tissue (milk fat globules). This diverse array of metabolic pathways and their key enzymes are highly active in the liver, adipose and lactating mammary gland and interestingly in many human tumors.

In addition to genetic, hormonal and transcriptional control, DNL is nutritionally regulated and highly responsive to dietary FA composition. For example, dietary supplementation of the *trans*-10, *cis*-12 CLA was able to induce milk fat depression (MFD) in ruminant and non-ruminant animal models [6]. Moreover, the dietary LC ω-3 PUFAs has been shown to downregulate hepatic glycolysis and de novo lipid synthesis pathways [7].

The proposed mechanisms by which bioactive FAs effect lipid metabolism involves regulation of the abundance and activity of metabolic nuclear receptors and lipogenic enzymes. While the mechanistic details are not completely elucidated, it is clear that there are species and tissue differences and even differences among specific FA isomers.

The overall objective of this dissertation was to investigate the molecular mechanisms of different bioactive FAs on some aspects of lipid metabolism in different lipogenic tissues using ruminant and non-ruminant animal models. One investigation examined the role of thyroid hormone responsive spot 14 (S14) protein in mediating the antiobesity effect of CLA using a mouse model. The S14 gene encodes for a small nuclear protein and is predominantly expressed in lipid synthesizing tissues; its exact function is not clear, but several lines of evidence in rodent models suggest that it might be required for normal lipogenesis [8]. Similarly, S14 gene expression was highly correlated with downregulation of mammary lipogenesis in lactating dairy cow and body fat deposition in growing mice receiving *trans*-10, *cis*-12 CLA treatment [9]. Therefore, we hypothesize that S14 gene might be essential for normal lipogenesis in white adipose tissue and, moreover that S14 may be implicated in the mechanism by which CLA is able to reduce body fat accretion.

A second area investigated in this dissertation involved the ability of LC ω-3 PUFAs to affect lipid metabolism. Studies involving oral administration of fish oil or purified ethyl esters of EPA and DHA in both experimental animals and humans have observed a decrease in serum and hepatic levels of triglycerides, cholesterol and phospholipid [10], and a reduction in body fat deposition [11]. However, the effect of LC ω-3 PUFAs on the biology of mammary gland, the major lipid synthesizing organ during lactation, has not been investi-

gated extensively and to the best of our knowledge the effects on mammary lipogenesis per se have not been reported. Therefore, a second thesis objective was to examine the effects of fish oil supplementation on mammary lipogenesis and the expression of lipogenic genes and transcription factors in mammary and hepatic tissues of lactating mice.

The final investigation of the current dissertation was an examination of the molecular mechanisms mediating the CLA-induced downregulation of mammary lipid synthesis. There have been several published studies characterizing the cellular changes in CLA-induced MFD in dairy cow [6]. Other studies have shown that CLA also induces MFD in lactating ewes [12], but, the molecular basis for MFD, whether induced by diet or CLA supplementation, has not been investigated. Thus, the final thesis objective was to use lactating ewes as a model and investigate the molecular changes during CLA-induced MFD.

Chapter 2

Literature Review

2.1 Lipogenesis

De novo lipogenesis (DNL) involves the metabolic pathways leading to synthesis of fatty acids (FAs), which are ultimately esterified with glycerol-3-phosphate to form triglycerides (TG) [13]. FAs are essential components of all biological membranes and represent an important form of energy storage in both animals and plants [14]. As a consequence, the enzymatic pathways of DNL are present in all living organisms [15] and most tissues synthesize FAs at low rates that are not under hormonal or dietary regulation [16]. In contrast, the FA synthesis in liver, adipose tissue and lactating mammary gland, the major sites for DNL [17,18], can occur at rates 10 - 1,000 times those in other tissues and is regulated by diet and hormones [16]. The relative contribution of the lipogenic tissues to total FA synthesis depends on the species and the physiological status of the animal itself [16,17]. For example, FA synthesis occurs primarily in the liver of humans and birds. Rodents can synthesize FAs at high rates in both liver and adipose tissue, whereas in pigs and ruminants, adipose tissue is the primary site [16,19]. On the other hand, in lactating mammals the mammary glands are the major site of DNL with the FA being used for milk fat production. For example, whereas the liver and adipose tissue contribute about equally to total FA synthesis in the virgin mouse, by mid-lactation the contribution of the mammary gland accounted for approximately 75%

of the total rate of FA synthesis which mostly secreted into milk [20].

In non-ruminants, the enzymes involved in the lipogenic pathway include: (i) glucokinase and L-type pyruvate kinase (L-PK) for glycolysis; (ii) ATP citrate lyase (ACYL), acetyl-coenzyme A carboxylase (ACAC) and fatty acid synthase (FASN) for lipogenesis and long-chain elongase and stearoyl-coenzyme A desaturases (SCD) catalyzing FA elongation and desaturation steps; and (iii) glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) and diacylglycerol O-acyltransferase (DGAT) for TG synthesis [13].

FAs are synthesized by an extramitochondrial system that is responsible for the complete synthesis of palmitate from acetyl-CoA in the cytosol [21]. The synthesis of FAs de novo is achieved by the sequential condensation of two-carbon units derived initially from acetyl-CoA [14]. Glucose is the primary substrate for FA synthesis in most tissues in the body, including liver, adipose tissue, and mammary gland of non-ruminants [16]. A well recognized and major metabolic difference between the ruminant and non-ruminant is the failure of carbon from glucose to contribute to FA synthesis within the tissues of a ruminant, including mammary gland. This phenomenon is accounted for in part by the negligible activity of ACYL and NADP-malate dehydrogenase [19, 21]. Rather, acetate and to a lesser extent β-hydroxybutyrate, serve as the precursors for FA synthesis in both adipose tissue and mammary gland of ruminants. Acetate is converted to acetyl-CoA in the cytosol and this is the principal building block of FA. β-hydroxybutyrate use is more limited as it can serve only as the first four-carbon primer in the initiation of FA synthesis [22].

The biosynthesis of FAs apart from carbon substrate also requires considerable amounts of reducing equivalents in the form of NADPH. The cytosolic enzymes which potentially could be involved in NADPH production are glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), NADP-malate dehydrogenase and NADP- isocitrate dehydrogenase (IDH1) [19,22]. It takes two NADPH for each two carbon addition from malonyl-CoA. In rats, the first two enzymes of the pentose phosphate pathway (G6PD and

PGD) contribute about one-half of the reducing power for FA synthesis with the remainder coming from the malate transhydrogenation cycle via NADP-malate dehydrogenase. In ruminants, NADP-malate dehydrogenase is negligible and its contribution of NADPH is replaced by cytosolic IDH1 [19, 22].

ACAC is a complex enzyme system which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in FA synthesis. There are two ACAC forms, acetyl-coenzyme A carboxylase alpha (ACACA) and acetyl-coenzyme A carboxylase beta (ACACB), encoded by two different genes. ACACA is expressed in all cell types but is found at its highest levels in the lipogenic tissues [23]. The activity of ACACA is regulated in a complex fashion. In the short term ACACA is regulated through allosteric mechanisms with cellular metabolites possessing positive (citrate) or negative effect (malonyl-CoA and long-chain acyl-CoA), and reversible activation/deactivation via phosphorylation on a number of specific serine residues [24]. On the long term, ACACA is regulated through complex transcriptional regulation involving the presence of multiple promoters and splice variants that are expressed in a tissue and signal specific manner [23].

The entire pathway of palmitate synthesis from malonyl-CoA in mammals is catalyzed by a single, homodimeric, multifunctional protein, FASN [25]. The synthesis of palmitate is carried out by a very complex overall reaction that involves sequential condensation of seven two-carbon units derived from malonyl-CoA to the primer acetyl moiety derived from acetyl-CoA [26]. Contrary to what is observed in rodent mammary gland, FASN in ruminant mammary tissue synthesizes short-and medium chain fatty acids (MCFAs) without the implication of a thioesterase II [27]. In addition to being able to load acetyl-CoA, malonyl-CoA, and butyryl-CoA, ruminant FASN contains a loading acyltransferase whose substrate specificity extends to up to C12, with the result that it is able to load and also release these short-and MCFAs [28]. This mechanism is specific to the lactating mammary gland, whereas the product of FASN in other lipogenic tissues is predominantly C16:0 for both ruminants and non-ruminants [19, 22, 29].

SCD catalyzes a rate-limiting step in the synthesis of unsaturated FAs through Δ-9 desaturation, introducing a *cis* double bond. The principal product of SCD1 is oleic acid, which is formed by desaturation of stearic acid. Although the kinetics differ, SCD1 is able to act on a spectrum of fatty acyl-CoA substrates, including FAs ranging from C14 to C19. For example, palmitoleoyl-CoA and oleyl-CoA are synthesized from palmitoyl-CoA and stearoyl-CoA, respectively, by the action of SCD1 [30]. In addition, in bovine mammary gland, SCD1 is responsible for the synthesis of the major portion of two CLA isomers found in milk fat, *cis*-9, *trans*-11 CLA and *trans*-7, *cis*-9- CLA [31, 32]. Four SCD isoforms, SCD1 through SCD4, have been identified in mouse. In contrast, only 2 SCD isoforms, SCD1 and SCD5, have been identified in human and cow.

The first step in TG biosynthesis is the esterification of glycerol-3-phosphate in the sn-1 position; this reaction is catalyzed by GPAT. Two isoforms of GPAT have been identified in mammals and these differ in their subcellular location (mitochondrial vs. endoplasmic reticulum) [23]. In rodents, both isoforms have a role in the TG synthesis in the liver and adipose tissue [33]. The second step of TG synthesis is committed by AGPAT. AGPAT has a greater affinity for saturated fatty acyl-CoA in the order C16 > C14 > C12 > C10 > C8, which is in accordance with the observed high proportion of medium- and long-chain saturated FAs at the sn-2 position in milk, with palmitate as the major FA (representing 43% of the total palmitate found in triacylglycerol) [23]. The third enzyme, DGAT, is located on the endoplasmic reticulum membrane. DGAT is the only protein that is specific to triacylglycerol synthesis and therefore may play an important regulatory role [33].

2.2 Thyroid Hormone Responsive Spot 14 and Lipogenesis

The thyroid hormone responsive spot 14 (*S14*) gene codes for a 17-kDa acidic protein that lacks any well-recognized functional motifs [8]. However, several lines of evidence suggest that this protein may be involved in lipogenesis. For example, *S14* mRNA has been de-

tected predominantly in lipogenic tissues [34]. In addition, levels of hepatic *S14* mRNA are extremely low in fetal and newborn rats but increase at the time of weaning [35] and the increase corresponds to a similar increase in lipogenesis and lipogenic enzymes [8]. Moreover, alterations in the expression of *S14* in lipogenic tissues also correspond to changes in the expression of other lipogenic enzymes in other physiological situations. On the other hand, *S14* gene expression in liver is controlled by a wide variety of hormones and dietary factors in parallel with the major lipogenic enzyme genes [36]. A particularly striking aspect of *S14* expression is its rapid and robust response to thyroid hormone (TH) [37] and carbohydrate [38], which made expression of *S14* very useful in the study of TH action [8]. The direct role of TH and carbohydrate in regulation of the expression of *S14* gene, has been confirmed by the identification of several TH [39] and a carbohydrate [40] response elements in the *S14* gene promotor region. In addition, hepatic *S14* mRNA abundance is reduced in diabetic rats and insulin administration to streptozotocin-induced diabetic rats restored levels of hepatic *S14* mRNA to normal within 4 h [41]. Moreover, in coordination with other lipogenic genes, administration of long-chain PUFAs to rats downregulated the abundance of *S14* mRNA [42].

To better understand the role of S14 in lipogenesis, hepatocytes were transfected with an *S14* antisense oligonucleotide. Interestingly, the transfected cells showed lower levels of lipogenesis, compared with controls, and this was associated with a diminished immunoreactivity of ACYL and FASN, reduced induction of malic enzyme by T3 and carbohydrate, and decreased malic enzyme mRNA abundance [43]. Furthermore, an experiment using siRNA to knock down *S14* expression in breast cancer cells showed that S14 was required for lipogenesis [44].

The results obtained from the oligonucleotide model are supported by studies with mice that had a knockout of the *S14* gene. The mammary lipogenic rate of knockout dams was decreased by 62% compared to wild-type dam and this correspond to a similar reduction in the de novo synthesis of FAs [45]. However, despite the lower production of FAs and the

decreased rate of lipogenesis, there was no reduction in the activity and mRNA abundance of key enzymes involved in lipogenesis (*FASN*, *ACACA*). These data indicate that the regulatory impact of S14 on lipogenesis does not occur by altering the transcription of the rate-limiting lipogenic enzymes [8]. Indeed, malonyl-CoA, the substrate of the FASN reaction, has been shown to be increased in the mammary gland of the *S14* knockout animal which suggests that the defect in lipogenesis occurs because of an in vivo decrease in FASN activity [8]. Thus, the S14 protein may act as an allosteric regulator of the in vivo activity of FASN [8]. Moreover, the lack of S14 in liver did not affect hepatic lipogenesis which suggests that there may be another related protein that can compensate for the lack of S14 [8]. Indeed, a paralog of *S14* has been identified and given the name S14 related protein (*MIG12*) [45]. Interestingly, *MIG12* was less abundant in mammary gland compared with liver which may explain the contrast in the lipogenic phenotype between both organs in the knockout animal [45]. Using small interfering RNA to simultaneously reduce levels of *S14* and *MIG12* in cultured primary hepatocytes, the rates of lipogenesis were decreased by approximately 65% in cells treated with insulin and high glucose. Interestingly, expression of either *S14* or *MIG12* gene products was sufficient to fully restore normal lipogenesis [36].

2.3 Bioactive Fatty Acids

2.3.1 Conjugated Linoleic Acid

CLA is a generic term used to describe positional and geometric isomers of linoleic acid (*cis*-9, *cis*-12 octadecadienoic acid). In CLA isomers, the two double bonds are located adjacent to one another rather than being separated by an interceding carbon as occurs in most PUFAs. The two double bonds in CLA can vary in location (for example 8 and 10, 9 and 11, 10 and 12, or 11 and 13) and the double bonds can be any combination of *cis* or *trans* configuration [32, 46].

CLA originate from two sources - industrial or natural [32]. The CLA from industrial

sources originates from the partial hydrogenation of vegetable oils. Natural sources are CLA isomers produced by rumen bacteria as intermediates in the biohydrogenation of dietary PUFAs. For this reason, CLA is found naturally mainly in ruminant meat and dairy products. Although there are over 20 different CLA isomers found in ruminant-sourced foods, the *cis*-9, *trans*-11 CLA , also known as rumenic acid, is the most abundant isomer accounting for 75-90% of total CLA [32]. *Trans*-7, *cis*-9 CLA is the second most common isomer, representing about 10%. Other CLA isomers generally comprise only 1-2% each of total CLA in ruminant fat [32, 47, 48]. After formation in the rumen, most *cis*-9, *trans*-11 CLA is biohydrogenated by rumen microorganisms to *trans*-11-octadecenoic acid (vaccenic acid) and then to stearic acid. However, some rumenic acid does pass from the rumen and is absorbed. It was originally thought that this was the source of rumenic acid in ruminant fat, but subsequent work showed the major source was endogenous synthesis [32]. Endogenous synthesis involves vaccenic acid which is also absorbed and then may be converted by SCD1 within mammalian cells back to *cis*-9, *trans*-11 CLA by incorporating a *cis* double bond in position 9. Likewise, *trans*-7, *cis*-9 CLA also arises predominantly from endogenous synthesis via stearoyl-coenzyme A desaturase 1 (SCD1) with the substrate being *trans*-7-18:1, another intermediate produced in rumen biohydrogenation [49].

The CLA content of ruminant-sourced food is variable and depends on several factors, including season, the animal's breed, nutritional status and age of the cows [50]. However, diet is the major factor affecting ruminant fat content of CLA with the proportion of CLA generally ranging from 0.34% to 1.07% of FAs in dairy products, and from 0.12% to 0.68% of the total FAs in raw or processed beef products [32]. Rumenic acid has been shown to have anticarcinogenic and antiatherogenic effects in biomedical studies with animal models. In addition several of the CLA isomers of minor concentration in ruminant fat also have biological activity. In particular, *trans*-10, *cis*-12 CLA is a potent inhibitor of body fat accretion and milk fat synthesis as will be discussed in later sections.

In addition to natural sources, chemical synthesis of CLA has been achieved to produce

products with different CLA isomers [51]. These laboratory methods of chemical synthesis of CLA isomers typically involve a partial hydrogenation of linoleic acid [48]. Initial methods resulted in CLA preparations that had four isomers (*trans*-8, *cis*-10; *cis*-9, *trans*-11; *trans*-10, *cis*-12 and; *cis*-11, *trans*-13) and these were used in many biomedical studies with animal models. Subsequently, the method of partial hydrogenation was modified to produce a product that consisted mainly of two isomers [32, 51]. CLA content in these preparations was about 90% and this was made up of 50:50 mixture of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA with less than 5% of the total CLA being made up of trace amounts of other CLA isomers. Over the last decade, this mixture of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA has been used in most experimental studies and this is also the CLA mixture that is commercially available as a supplement for humans. However, recent evidence indicates that both isomers exhibit significant biological activities, which in some cases are similar but in other cases the two isomers have opposite biological effects. With technology advancements, enriched or purified *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA preparations have become commercially available in recent years, so that the effects of individual isomers can be examined in health-related disorders [48].

2.3.2 Omega-3 Polyunsaturated Fatty Acids

The ω-3 FAs are PUFAs where the first double bond is three carbon atoms from the methyl terminal; this results in distinct properties that separate these FAs from the more common ω-6 or ω-9 FAs. The long chain PUFAs of the ω-3 series docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) represent a unique class of food constituents that show a wide range of functions in biological systems. The effects include the ability to modulate gene expression [52], inflammatory processes [53] and cellular membrane structure and function as well as modulate signaling pathways involved in normal and pathological cell functions [54]. Recent epidemiological and clinical studies with humans and biomedical studies using animal models indicate that food sources of EPA and DHA may be useful for reducing the risk of

coronary heart disease and atherosclerosis, treating inflammatory conditions and preventing certain types of cancer [5,55].

PUFAs in general are fundamental components of phospholipids in cellular membranes. They are usually located in the sn-2 position, whereas saturated or monounsaturated FAs are usually bound in the sn-1 position of phospholipid molecules [11]. The relative abundance or ratio between the long chain ω-6 (arachidonic acid (AA)) and ω-3 (EPA and DHA) FAs in cell membranes depends on their supply in the diet and the conversion (elongation and desaturation) of their precursors, linoleic acid (LA) and α linolenic acid (ALA), respectively. LA and ALA are considered essential FAs and must be supplied in the diet as the body can not synthesize them in adequate amounts [56]. Although conversion of ALA to LC ω-3 PUFAs is important to maintain constant levels of EPA and DHA [57], emerging evidence suggests that the process is relatively inefficient; in human only about 5% of ALA is converted to EPA and less than 0.5% is converted to DHA [56]. On the other hand, the conversion of LA to AA is much more efficient [58]. Therefore, the tissue content of LC ω-3 PUFAs largely depends on the exogenous supply of EPA and DHA.

Fish are the primarily source of LC ω-3 PUFAs and their levels of EPA and DHA vary according to the fish species, season, fishing area, and age and gender of the fish [59]. EPA and DHA can also be obtained from other sources such as marine microalgae, the primary producers of EPA and DHA, and krill oil, extracted from marine invertebrates in the zooplankton [59]. Most dietary ω-3-rich fish oils and many supplements provide EPA and DHA as esterified in a TG. On the other hand, supplementation with free FAs and FA esters can provide higher levels of EPA and DHA, but the free FAs form can cause gastrointestinal complaints [59]. Thus, esterified forms of EPA and DHA have been developed and the ethyl esters are the most common supplement form of EPA and DHA due to their greater stability and the fact they avoid the potential toxic effects of methanol released from methyl esters [60]. The TG and the ethyl ester forms of EPA and DHA appear to have slight differences in absorption rate, but the total levels of EPA and DHA incorporated into plasma

lipids have been shown to be equivalent for both forms [60, 61]. Moreover, ethyl esters of EPA and DHA have also been associated with positive health outcomes in research trials and are being utilized in therapeutics [59].

The LC ω-3 PUFAs are able to markedly affect lipid metabolism in liver and to a lesser extent adipose tissue. Studies involving oral administration of fish oil or purified ethyl esters of EPA and DHA in both experimental animals and humans report a decrease in serum and hepatic levels of TG, serum concentration of cholesterol and phospholipid [10] and a reduction in body fat deposition [11, 62]. Although mechanistic details are not fully understood, the effects of LC ω-3 PUFAs in liver involve the regulation of the activity of metabolic nuclear receptors which results in repartitioning of metabolic fuel (i.e, FAs) away from TG synthesis and storage toward oxidation; the net effect is a decrease in the substrate available for very low density lipoprotein synthesis and secretion [63].

The effect of LC ω-3 PUFAs on the biology of mammary gland, the major lipid synthesizing organ during lactation, has not been investigated extensively and to the best of our knowledge the effect of LC ω-3 PUFAs on mammary lipogenesis per se has not been reported. Investigations of FO or LC ω-3 PUFAs on the milk FA composition in lactating rats indicate a trend toward an increase in the proportion of MCFAs in milk fat relative to the control diets [64–68]. Most diets are devoid of MCFAs so their presence in milk fat may be indicative of mammary de novo FA synthesis (DFAS). However, these experiments were not designed specifically to address effect on rates of de novo FA synthesis (DFAS) and an increase would be contrary to the well established downregulation effect of LC ω-3 PUFAs on the hepatic lipid synthesis. Moreover, when lactating cow were abomasally infused with FO, EPA and DHA content of milk fat increased indicating these LC ω-3 PUFAs were taken-up and utilized by the mammary gland, but milk fat yield was unaffected [69].

In addition to their ability to modulate the activity and/or abundance of nuclear receptors, many effects of LC-PUFAs depend on the formation of their active metabolites, eicosanoids and other lipid mediators, which have diverse physiological effects [11]. Follow-

ing cell activation by inflammatory stimuli, PUFAs in membrane phospholipids of various cell types are released by phospholipase A2 and converted to eicosanoids by cyclooxygenases (COX) and the lipoxygenases (LOX) [58]. Metabolism of the ω-6 AA by the COX pathway produces prostaglandins (PG) and thromboxanes (TX), while leukotrienes (LT) are the end products of the LOX pathway [58]. Many of these AA-derived eicosanoids are highly pro-inflammatory. On the other hand, the ω-3 FA EPA is metabolized by mammalian cells through the same COX and LOX pathways to form a different series of PG, and LT which are much less pro-inflammatory and can even have opposing effects as compared to their counterparts derived from AA [53]. Thus, inhibition of the formation of AA-derived pro-inflammatory mediators by competing with AA for the COX and LOX enzymes is thought to be a major mechanism underlying the antiinflammatory effect of ω-3 FA [70]. Moreover, recent studies have discovered resolvin and protectins, EPA and DHA derivatives with potent antiinflammatory properties [71].

In addition to the competition for the COX and LOX enzymes, ω-6 and ω-3 FAs also compete for the enzymes involved in the elongation and desaturation of these FAs as well as their incorporation into phospholipids. When one type of FA predominates in cell membrane phospholipids, it is the major substrate for these enzymes. For example, a flush of ω-3 ALA or EPA into cells would dramatically reduce the ω-6 LA and AA content of cellular phospholipids [58]. Thus, dietary supply and the balance between these FAs influences the composition of cell membrane phospholipids, and this modulates the production of different eicosanoids thereby affecting the physiological process [53] . For this reason, maintaining a balance of ω-6 and ω-3 PUFAs is important for optimal biochemical balance in the body [58].

Moreover, a portion of the metabolic effects on LC $\omega-3$ PUFAs in the liver, and possibly in other tissues, is mediated by the stimulation of AMP-activated protein kinase (AMPK), a metabolic sensor controlling intracellular metabolic fluxes including the partitioning between lipid oxidation and lipogenesis [72]. Phosphorylation of ACAC by AMPK leads to an inhibition of enzyme activity, resulting in a decrease in malonyl-CoA content which is a

key lipogenic intermediate that also inhibits mitochondrial carnitine palmitoyltransferase-1 (CPT-1). Thus, AMPK inhibits lipogenesis while stimulating β-oxidation.

2.4 Transcriptional Control of Lipogenesis by PUFAs

Metabolic regulation is one of the most remarkable features of living organisms. Of thousands of catalyzed reactions that can take place in a cell, there is probably not one that escapes some form of regulation [73]. It is commonly accepted that metabolic regulation in complex organisms relies on three main types of control. The first corresponds to the classic allosteric control of the activity of a key enzyme along a metabolic pathway triggered by the binding of an activator or inhibitor, which often is the substrate or product of the enzyme itself. The second mechanism involves various translational modifications such as proteolytic cleavage, phosphorylation, glycosylation and acetylation, which may shift the equilibrium between an inactive and active forms of the enzyme within seconds and/or affect protein stability. The third mechanism is transcriptional regulation, which affects the level of expression of key enzymes and is effective on a longer time scale. It is clear that the regulation of metabolic process involve a coordination of these various mechanisms [74].

This thesis focuses on transcriptional regulation and understanding the transcriptional control of metabolism relies on three complementary pieces of information: 1) events upstream of transcriptional activity, which define the signals involved and their route to the nucleus; 2) the molecular mechanisms by which transcription factors operate; and 3) events downstream of transcriptional activity, which depend on the groups of genes that are targeted and how additional signals are generated to reach the dynamic equilibrium of homeostasis [74]. The transcription regulation of a set of genes is mediated through the action on nuclear receptors; activation induces a conformational change in the receptor that promotes an exchange of regulatory factors interacting with the nuclear receptor and hence activate the transcription of the target genes [74]. Many transcription factors have been identified to

regulate the expression of enzymes involved in the lipogenic pathway which include sterol regulatory element binding transcription factor (SREBF), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), hepatocyte nuclear factor 4 (HNF-4), and retinoid X receptor (RXR).

2.4.1 Transcription Factors Regulating Lipogenesis

2.4.1.1 SREBF

SREBFs are a family of transcription factors which function as global regulators of lipid synthesis (see reviews [75,76]). Two genes, *SREBF1* and *SREBF2*, encode three proteins of SREBF family. The *SREBF2* gene encodes a single protein that has approx. 50% homology with SREBF1. As a result of alternative splicing and the use of alternative promoters, *SREBF1* gene encodes two proteins, SREBF1-a and SREBF1-c, that differ only in the length of the amino terminal transactivation domain [75]. SREBFs are synthesized and located on the endoplasmic reticulum endoplasmic reticulum (ER) as membrane-bound precursors that require cleavage by a two-step proteolytic process in order to release their amino-terminal domain (nuclear SREBF) [76]. The SREBF cleavage-activating protein (SCAP) functions as a cholesterol sensor. When the cellular cholesterol levels are depleted, SCAP binds to and escorts SREBFs to the golgi apparatus, where the site 1 and site 2 proteases cleave the SREBF. The cleavage form of SREBF is referred to as nuclear SREBF or mature SREBF; it enters the nucleus and in conjunction with other DNA binding proteins, binds to a sterol regulatory element in the promotor region of target genes with subsequent recruitment of coactivator proteins to initiate transcription. Upon restoration of cellular cholesterol, insulin-induced gene (INSIG), another key regulator of ER membrane proteins, traps and retains the SREBF–SCAP complex at the ER thereby inhibiting SREBF trafficking to the golgi for cleavage to form nuclear SREBF. SREBF1-c is also subject to the SCAP–INSIG cleavage regulation system described above for all SREBFs, but it is not strictly under sterol regulation [76]. SREBF1-c, SREBF1-a and SREBF2 belong to the same family, but there are distinct differences regarding

tissue distribution and their target genes [76, 77]. *SREBF2* mRNA is expressed at a similar level in most tissues but the relative levels of *SREBF1-a* and *SREBF1-c* differ significantly. Whereas in culture cells, expression of genes encoding *SREBF1-a* > *SREBF1-c*, the ratio of *SREBF1-c* : *SREBF1-a* varies significantly in animal tissues being 9, 3, or 0.1 in liver, adipose tissue or spleen, respectively [78]. However, recent studies demonstrated that the relative expression of *SREBF*-1a, -1c, and -2 in vivo is complex and can be affected by the nutritional and hormonal status of the animal [75]. Furthermore, the proteolytic cleavage of the precursor SREBF1 and SREBF2 can be regulated independently [75]. On the other hand, while SREBF target genes identified to date include many that are involved in the control of cholesterol synthesis, FA synthesis, TG synthesis and glucose metabolism [75, 77] , SREBF2 and SREBF1-a show a tendency toward activation of genes involved in cholesterol synthesis more than those involved in the pathways of FA synthesis . Overexpression of mature SREBF2 or SREBF1-a transcriptionally activates genes involved in both cholesterol and FA synthesis, although, the ratio of cholesterol : FA synthesis is greater in the SREBF2 than in the SREBF1-a overexpressing animals [75]. In contrast, SREBF1-c acts more specifically on genes involved in FA synthesis. It is highly expressed in the liver and the white adipose tissue and is sensitive to multiple regulatory signals. Furthermore, overexpression of SREBF1-c and formation of the nuclear form strongly increases the TG content of the liver, with no parallel accumulation of cholesterol [74].

The transcriptional effect of insulin, long thought to be the main inducer of glycolytic and lipogenic gene transcription, is mediated by SREBF1-c. Insulin stimulation of the transcription of SREBF1-c and the formation of the nuclear SREBF1-c, together with a signal derived from glucose, result in enhanced transcription of genes involved in both lipogenesis and glucose metabolism such as the genes encoding ACAC, FASN, Elovl-6, and SCD. Therefore, most of the lipogenic effects of insulin are dependent on the induced expression of *SREBF1-c* and the subsequent increased expression of genes for key enzymes in the FA synthesis pathway.

Although the post-transcriptional maturation of SREBFs is an important regulatory event, the transcriptional regulation of *SREBF1-c* expression parallels the activity of the transcription factor [74]. *SREBF1-c* expression is also stimulated by the LXR, via two LXR binding sites present in the *SREBF1-c* promoter [74]. In addition, *SREBF1-c* expression is induced by itself.

2.4.1.2 PPAR

PPARs are key transcriptional factors that play a central role in regulating the oxidation and storage of dietary lipids, essentially by serving as sensors for FAs and their metabolic intermediates [79]. PPARs control expression of various genes that are crucial for lipid and glucose metabolism. To date, three major types of PPAR, have been identified, namely α, γ, and β/δ [79]. Each type is encoded by a separate gene and they vary in ligand specificity and tissue distribution; hence these isoforms serve different biological functions [79]. Natural FAs and the fibrate class of hypolipidaemic drugs are known activators of PPARs [79].

The gene transcription mechanism is identical in all PPAR subtypes [79]. PPARs form heterodimers with (RXR α) [80] and bind to the specific DNA sequence designated PPRE (peroxisome proliferator response elements) present in the promoter region of PPAR-regulated genes. Binding of exogenous (drugs) or endogenous (FAs, PG, etc.) ligands [79] is essential for the PPAR heterodimer to initiate a complicated transcription process, which includes the dissociation of corepressor protein complex and association or recruitment of coactivator protein complexes [81]. This results in the increase in transcription activities of various genes involved in diverse biological processes. In addition, PPARs can be activated by phosphorylation and the PPAR:RXR heterodimer can be activated by RXR ligands [82]. These different activation mechanisms, which can act concomitantly, illustrate the capacity of fine-tuning that allows for the orchestration by PPAR actions [74].

PPARα serves as the master regulator of FA oxidation in liver [81] in which it controls a comprehensive set of genes that regulate most aspects of lipid catabolism [74]. It also

stimulates the cellular uptake of FAs by increasing the expression of the FA transport protein and FA translocase [83]. PPARα is expressed in numerous tissues in rodents and humans including liver, kidney, heart, skeletal muscle and brown fat [84, 85]. On the other hand, PPARγ is an important transcription factor involved in adipogenesis, glucose homeostasis and lipid metabolism; it is considered to be the master architect of adipocyte differentiation and is required for adipose tissue formation in vivo [86]. PPARγ has two isoforms, PPARγ1, and PPARγ2, encoded from the same gene by selective usage of two distinct promoters and alternate splicing [87]. PPARγ1 is found in a broad range of tissues, whereas PPARγ2 is restricted to adipose tissue [79]. PPARγ target genes include adipocyte FA binding protein, LPL, and LXRα. In adipose tissue, activation of these genes by PPARγ promotes fat storage and reduces serum lipid levels [86]. The third type, PPARβ/δ is expressed in most tissues and is also responsible for widespread effects on energy oxidation mainly in extrahepatic tissues [88].

2.4.1.3 ChREBP

Carbohydrate response element binding protein (ChREBP) is a transcription factor that mediates the glucose effect on both glycolytic and lipogenic gene expression [89]. ChREBP is predominantly expressed in liver, kidney, white adipose tissue and brown adipose tissue [89]. Under basal conditions ChREBP is localized in the cytosol, and its nuclear translocation is rapidly induced under high glucose concentrations. Nuclear translocation and DNA binding of ChREBP is controlled by dephosphorylation of several serine and threonine residues [89]. Glucose- or carbohydrate-response elements (ChoREs) that mediate the transcriptional response of glucose have been identified in the promoters of most lipogenic genes including *FASN* and *ACAC* [90, 91]. ChREBP does not act alone, but instead functions in a heterodimeric complex with the transcription factor MAX-like protein X (MLX). Together, these two transcription factors bind to and activate transcription of glycolytic and lipogenic genes containing a ChRE.

ChREBP is considered to be a key determinant of lipid synthesis in liver [92] where it acts as a central modulator of FA concentrations in liver by transcriptionally controlling the key enzymes in the lipogenic program (ACAC, FASN, SCD-1) and TG synthesis (GPAT) [89]. The liver-specific inhibition of ChREBP markedly affects the expression of *ACACA*, *FASN* and *SCD1* [89] and *ChREBP* gene knockout mice exhibit glucose and insulin intolerance and have impaired glycolytic and lipogenic pathways in liver [93].

2.4.1.4 LXR

LXR regulates the expression of genes involved in bile acid synthesis, reverse cholesterol transport, clearance of blood lipids, lipogenesis, and glucose uptake [94]. There are two LXR receptors, LXRα and LXRβ. Whereas LXRβ has a ubiquitous tissue distribution, LXRα predominates in the liver, adipose tissue, and macrophages [86]. The two forms appear to respond to the same natural and synthetic ligands, and the natural LXR ligands include physiological concentrations of sterol metabolites. LXR/RXR heterodimers are constitutively nuclear and bound to LXR response elements in the promotors of regulated genes. More than a dozen target genes for LXR have been identified, and many of them are integral parts of the cholesterol and FA metabolic pathways [86]. LXRα-null mice show reduced expression of the major lipogenic regulators in the liver including *SREBF1-c*, *FASN*, and *SCD1* [95]. Giving synthetic LXR ligands to mice triggers the lipogenic program and results in elevated TG levels in the plasma and liver [96].

2.4.1.5 XBP1

A recent report has suggested that X-box binding protein 1 (XBP1) is a novel transcription factor governing hepatic lipogenesis. XBP1 deficiency resulted in profound compromise of de novo hepatic lipid synthesis, leading to concomitant decreases in serum TG, cholesterol, and free FAs without causing hepatic steatosis. XBP1 was induced upon feeding high-carbohydrate diet and directly activated the transcription of key lipogenic genes involved

in hepatic FA synthesis [97]. XBP1 is a key regulator of the mammalian unfolded protein response (UPR) or ER stress response [98]. Accumulating evidence suggests that UPR may be implicated in the regulation of cellular lipogenesis playing an important role in both FA synthesis and cholesterol metabolism [99].

UPR is activated under situations where accumulation of abnormally folded proteins or unassembled subunits occurs in the ER [100, 101]. UPR functions to restore the ER homeostasis by reducing the amount of proteins translocated into the ER lumen, increasing retrotranslocation and degradation of ER-localized proteins, and inducing transcription of components of the ER machinery involved in folding, quality control, redox and lipid biogenesis [100, 101]. Activation of the UPR has cytoprotective effects and allows for cell survival under moderate ER stress conditions. However, prolonged or severe ER stress can lead to proapoptotic signals [102].

The UPR involves three integrated pathways that are activated through the ER transmembrane proteins; inositol-requiring 1 (IRE1) α, PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [100,102]. In a well-functioning and "stress-free" ER, these three transmembrane proteins are bound by a chaperone, glucose-regulated protein (GRP78), in their intraluminal domains and rendered inactive [99]. Under ER stress conditions, dissociation of GRP78 from the ER luminal domains of IRE1α, PERK and/or ATF6 allows them to be activated. [101].

Upon ER stress, the proximal sensor and endoribonuclease IRE1α induces unconventional splicing of *XBP1* mRNA to generate a mature mRNA encoding an active transcription factor, XBP1s [97,100,101]. XBP1s, alone or in conjunction with ATF6α, launches a transcriptional program to produce chaperones and proteins involved in ER biogenesis, ER-associated protein degradation, and phospholipid synthesis [99] which leads to expansion of the ER membrane, a structural hallmark of the UPR [100].

All three UPR pathways have been reported to be involved in the regulation of lipid metabolism [101]. In addition to XBP1, via IRE1α pathway, the UPR transducer ATF6 was

also involved in phospholipid biosynthesis and ER expansion as well as hepatic lipid homeostasis associated with acute ER stress. ATF6α knockout mice displayed no obvious phenotype under normal conditions but showed profound hepatic steatosis under acute ER stress induced by tunicamycin challenge [101]. The UPR branch mediated through PERK/eukaryotic initiation factor 2 α (eIF2α) has also been implicated in regulating lipogenesis. In the high fat-fed mice, PERK-mediated eIF2α phosphorylation was crucial for the expression of lipogenic genes and the development of hepatic steatosis [101]. In addition, UPR has been shown to promote the proteolytic cleavage of SREBF (1c and 2) transcription factors leading to up-regulation of FA and cholesterol synthesis, respectively [100, 101]. In fact, SREBFs may be critical mediators in the integration of lipid metabolism with the UPR [99]. Conditions such as hyperhomocysteinemia and high alcohol consumption can lead to hepatic steatosis and have been linked to ER stress-induced SREBF1-c activation [102]. Consistent with this observation, overexpression of GRP78, the master negative regulator of the UPR, in the liver of obese (ob/ob) mice can inhibit SREBF1-c cleavage and the expression of SREBF1-c and SREBF2 target genes [101].

In a related vein, PERK-deficient mammary epithelial cells in mice have an altered FA content of milk due to the lack of expression of genes involved in FA synthesis, such as *FASN*, *ACYL*, and *SCD1* [99]. SREBF1 expression was significantly downregulated in the PERK-deficient mammary gland cells; therefore, PERK-mediated UPR pathway likely regulates SREBF1-related mammary de novo lipid synthesis [101]. Additionally, a recent study demonstrated that the IRE1α/XBP1 UPR pathway is indispensable for adipogenesis [101]. XBP1-deficient mouse embryonic fibroblasts and 3T3-L1 cells with XBP1 or IRE1α knockdown exhibit profound defects in adipogenesis. All together, these reports confirmed crucial roles of the UPR pathways in lipogenesis and the pathogenesis of lipid-associated metabolic disease [101].

2.4.2 PUFA Regulation of Transcription Factors

2.4.2.1 SREBF

FAs have been shown to regulate the nuclear abundance of SREBF; however, there is no evidence for FA binding directly to SREBF1 or SREBF2 [94]. A high carbohydrate/low fat diet has been shown to induce the expression of several lipogenic and glycolytic enzymes, including ACAC, FASN, SCD1, GPAT, ACYL, pyruvate kinase, malic enzyme, S14 and G6PD. Such changes in gene expression are likely the result, at least in part, from the insulin-dependent activation of SREBF1-c. In contrast, addition of PUFAs to rodent diets results in a decline in the mRNAs encoding these same genes [75]. Recent studies with mice indicate that dietary PUFAs repress the hepatic cleavage of SREBF1 to form nuclear SREBF1, but SREBF2 cleavage is unaffected [75]. Consequently, dietary PUFAs result in a decline in the level of mature, nuclear SREBF1 and the mRNAs for SREBF1 target genes [75]. Moreover, PUFAs suppression of SREBF1 nuclear abundance accounts for much of the well characterized suppression of de novo lipogenesis by dietary PUFAs [103]. This decline in the expression of SREBF1 target genes was not observed when PUFAs were fed to transgenic mice that constitutively express mature SREBF1-a [104]. The results of these studies indicate that PUFAs likely prevent the cleavage/maturation of SREBF1. This phenomenon is seen in livers of animals fed PUFAs-containing diets, as well as in primary hepatocytes and some, but not all, SREBF1-c expressing cells lines treated with PUFAs [94]. In addition, PUFAs selectively suppress hepatic levels of SREBF1 mRNAs (1a and 1c), but not SREBF2; this reduction has been attributed to an inhibition of the transcription of the SREBF1 gene and an enhanced turnover of the mRNA encoding SREBF1 [94].

The effect of PUFAs on nuclear abundance of SREBF can be explained, in part, by their effect on the cellular cholesterol homeostasis. Increasing cellular levels of cholesterol, as well as factors that affect intracellular cholesterol distribution, influence nuclear levels of SREBF [105]. In this regard, treatment of cells with unsaturated FAs leads to a PUFAs en-

richment in membrane phospholipids and sphingolipids, and this promotes redistribution of cholesterol from the plasma membrane to the endoplasmic reticulum. In addition, sphingomyelinase activity is stimulated, leading to the release of ceramide, an important signaling molecule [105]. These events have been shown to suppress proteolytic processing of precursor SREBF and this correspond to a decline in nuclear SREBF levels and SREBF-mediated gene expression [94].

2.4.2.2 PPAR

PPAR is the only well-accepted FA-regulated nuclear receptor. All PPAR subtypes (α, γ, and β/δ) bind saturated and unsaturated FAs ranging in length from 16–20 carbons. FA binding initiates a sequence of events that leads to activation of multiple genes involved in FA transport, binding and metabolism [103]. Certain FAs, however, are more effective than others at activating PPAR . Structural studies have established that EPA is both a ligand and a robust activator of PPAR while DHA and docosapentaenoic acid (22:5 ω-3) are weak PPARα activators [103]. Challenging cells with EPA, significantly changes intracellular non-esterified fatty acids (NEFA) composition and promotes a robust response in PPARα target genes [106]. The changes in intracellular 20:5 ω-3 are recognized by PPAR and mechanisms are initiated to prevent excessive accumulation of this highly unsaturated FAs; this supports the concept that PPARs are monitors of intracellular NEFA composition and respond accordingly to induce metabolic pathways that minimize damage brought on by excessive intracellular NEFA [103].

2.4.2.3 ChREBP

ChREBP has been shown as central for the coordinated inhibition of glycolytic and lipogenic genes by PUFAs [107]. In both primary cultures of hepatocytes and in liver in vivo, PUFAs such as LA, EPA, and DHA suppresses ChREBP activity by increasing its mRNA decay and by altering ChREBP protein translocation from the cytosol to the nucleus. The PUFAs-

mediated alteration in ChREBP translocation is the result of a decrease in glucose metabolism through an inhibition of the activities of glucose kinase and G6PDH, the rate-limiting enzyme of the pentose phosphate pathway [107]. Unlike PPARα and SREBF1, PUFAs regulation of ChREBP and MLX nuclear abundance is less responsive to the type of PUFAs; the nuclear abundance of ChREBP and MLX depends to a greater extent on the amount of PUFAs in cells [108].

2.4.2.4 LXR

Unsaturated FAs antagonize oxysterol binding by LXRα and inhibit LXR action in human embryonic kidney cells [109]. The hierarchy for this effect is 20:4 ω-6 >18:2 ω-6 >18:1 ω-9; saturated FAs have no effect [94]. Moreover, it was reported that FAs interfered with LXRα/RXRβ binding to the LXR responsive element [110]. Of the two LXR subtypes, only LXRα is sensitive to FA antagonism [111].

2.5 CLA and Obesity

Due to the substantial rise in the prevalence of obesity over the past 30 years, interest in CLA as a weight loss treatment has increased [112]. The first report that suggested that CLA may have potential antiobesity effect was published in 1997, when mice were fed a diet supplemented with 0.5% CLA had a 60% decrease in body fat after 4–5 weeks [113]. Recent research on CLA shows that body fat accumulation in both humans and animals appears to be isomer specific, dose responsive and independent of dietary fat content, as well as being influenced by study duration [47]. Accumulating evidence suggests that the *trans*-10, *cis*-12 CLA isomer appears to be responsible for the reduction of fat pad weight as a result of its ability to suppress body fat accretion [112]. Supplementation with a CLA mixture (i.e., equal concentrations of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 isomers) or the *trans*-10, *cis*-12 isomer alone decreases body fat mass in many animal studies and some human studies [114].

This discovery makes this isomer a potential candidate for the treatment and management of obesity. Several mechanisms have been proposed to explain the CLA-induced reduction in obesity and fat deposition and these will be discussed.

2.5.1 CLA Regulation of Energy Metabolism

The mechanism by which CLA reduces adiposity in lab animals, and perhaps in humans, may revolve around pathways that regulate energy balance. Accordingly, potential mechanisms include decreasing energy intake or increasing energy expenditure [112]. A number of studies have shown that CLA supplementation reduces food/energy intake [114]. The reduction in food intake can be explained by either the supplement adversely affecting the palatability of the diet or effects of the absorbed CLA on aspects involved in regulating the animal's appetite. In support of the later, CLA supplementation has been shown to to affect the appetite-regulating genes in the hypothalamus. Indeed, *trans*-10, *cis*-12 CLA decreased the gene expression ratio of proopiomelanocortin to neuropeptide Y and this was associated with a 24% reduction in food intake in mice [115]. In addition, injection of mixed isomers of CLA into the hypothalamus of rats resulted in a reduction in the expression of neuropeptide Y and agouti-related neuropeptides; increases in these two neuropeptide are associated with a robust increase food intake [116]. However, other reports have shown a large decrease in body fat mass although there was no alteration in energy intake [114]. This clearly indicates that other mechanisms may be involved in the CLA effects and the reduction in energy intake is not the only proposed mechanism.

CLA has also been proposed to reduce adiposity by elevating energy expenditure via increasing basal metabolic rate (BMR), thermogenesis or lipid oxidation in animals [112]. A significant increase in energy expenditure has been observed in mice supplemented with as low as 0.25% CLA, and the increase in energy expenditure was sufficient to account for the decreased fat deposition in CLA treated mice [114]. Enhanced thermogenesis may be associated with an upregulation of uncoupling proteins (UCP) which facilitate proton transport

over the inner mitochondrial membrane, thereby causing a loss of energy as heat rather than traping it as ATP [112]. UCP2 is the most highly expressed UCP in a variety of tissues including white adipose tissue (WAT) [112]. In this regard, CLA supplementation in rodents has been shown to induce UCP2 expression in WAT [117]. CLA also increased the expression of carnitine palmitoyltransferase 1 (CPT1) in WAT which is involved in mictochondrial FA uptake and catalyze the rate limiting step of FA oxidation [118].

Other studies have demonstrated increased lean body mass (LBM) in CLA supplemented mice and this was associated with higher level of energy expenditure when expressed on a body mass basis [119, 120]. However, the proposed increase in LBM is generally based on an increase in percent body protein which is confounded by the reduction in body fat occurring in CLA treated mice; when the actual body protein mass is calculated, contrary to author conclusions no increase in total body protein is observed (eg. calculations of data from [113, 121, 122]. Nevertheless, the proposed mechanism by which CLA increases LBM is based on evidence from rodent studies [112]. CLA is thought to increase bone mineral density by upregulating osteogenic gene expression and by downregulating osteoclast bone resorbing activity [120]. Alternatively, CLA may suppress the adipogenesis of pluripotent mesenchymal stem cells in bone marrow and instead enhance their commitment to become osteoblasts (bone-forming cells). In contrast, *cis*-9, *trans*-11 CLA increased adipocyte differentiation and decreased osteoblast differentiation [112]. Consistent with these data, CLA (isomer mixture) supplementation of rats treated with corticosteroids, which decrease muscle and bone mass, prevented reductions in LBM, bone mineral density, and bone mineral content [123]. Collectively, these findings provide evidence that one mechanism by which CLA reduces adiposity may be an increased energy expenditure via increased mitochondrial uncoupling and FA oxidation in WAT, or via increased muscle or bone mass [112].

2.5.2 CLA Regulation of Preadipocyte Differentiation

The differentiation of preadipocyte into mature adipocyte is mediated by a series of programmed changes is gene expression [114]. This is mainly controlled by a cascade of transcription factors particularly CCAAT/enhancer binding protein (C/EBP) and PPAR families. C/EBPα and PPARγ are considered to be the master regulators of adipocyte differentiation [112] functioning to coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype [114]. *Trans*-10, *cis*-12 CLA treatment has been reported to reduce adipogenesis and lipogenesis by attenuating PPARγ, C/EBPα, SREBF1-c, LXRα and adipocyte-specific FA binding protein expression [112].

2.5.3 CLA Regulation of Lipid Synthesis

The CLA-induced reduction in fat deposition corresponds to decreased lipid accumulation by adipocytes [114]. Numerous proteins involved in lipogenesis are decreased with CLA treatment. For example, LPL, ACAC, FASN and SCD are all decreased in the adipose tissue of mice when the diet is supplemented with mixed CLA isomers or *trans*-10, *cis*-12 CLA [112]. The expression of lipogenic genes is regulated by SREBF1-c whose mRNA abundance has showed a tendency to decrease with CLA feeding. PPARγ, another important transcription factor in adipogenesis, was also downregulated in mice after supplementation with a CLA mixture [114]. PPARγ is a major activator of many lipogenic genes, including glycerol-3-phosphate dehydrogenase, LPL and lipin, as well as genes encoding lipid-droplet-associated proteins such as perilipin and adipocyte-differentiation-related protein [124]. LPL, a key enzyme in lipid metabolism in the adipocyte, hydrolyzes the circulating lipoprotein TG thereby releasing FAs that can then be taken-up and re-esterified by the adipocytes [114]. In 3T3-L1 adipocytes, it has been shown that LPL activity decreased in a dose-dependent pattern by CLA treatment. Moreover, the inhibition of LPL was significantly correlated with the CLA-induced reduction in lipogenesis [112]. The active CLA isomer appears to be *trans*-10, *cis*-12 because it was shown to decrease TG content of human adipocytes in culture by

decreasing glucose and FA uptake whereas *cis*-9, *trans*-11 CLA had no effect [114]. Additional support for this hypothesis arises in the observed shifts in glucose transporter type 4 (GLUT-4) protein concentration, a rate-limiting step for glucose uptake in skeletal tissue and white adipose tissue. A dietary supplement containing 1.0% CLA markedly downregulated GLUT-4 mRNA levels in white and brown adipose tissues, but upregulated GLUT-4 mRNA levels in skeletal muscle in mice [114]. The CLA-induced reductions of GLUT-4 mRNA level and protein concentration in adipose tissue are consistent with a role for CLA inhibition of the conversion of glucose into fat [114]. On the other hand, rates of lipolysis, the process by which stored TG are mobilized through the action of hormone sensitive lipase (HSL), were increased by CLA treatment in WAT thereby providing free FA for uptake in metabolically active tissues (i.e., liver and muscle). In this regard, lipolysis was increased in 3T3-L1 adipocytes or newly differentiated human adipocytes when cell culture were acutely treated with mixed CLA isomers or *trans*-10, *cis*-12 CLA [112]. Moreover, La Rosa et al. [118] observed increased mRNA abundance of HSL in mice following 3 days of *trans*-10, *cis*-12 CLA.

2.5.4 CLA Induced Inflammatory Response

The primary function of WAT is energy storage, but this tissue also has the ability to produce a number of proinflammatory cytokines [112]. These adipokines (i.e., cytokines produced by adipose tissue) can cause insulin resistance, thereby suppressing lipid synthesis and increasing lipolysis in adipocytes [112]. Induction of these inflammatory genes is dependent on various cellular kinases, including mitogen activated protein kinases (MAPK), and is driven by transcription factors such as nuclear factor kappa B (NFκB), which have been reported to directly antagonize PPARγ [112]. Tumor necrosis factor (TNFα), in particular, exerts potent antiadipogenic effects [125], and interleukin (IL)-1β and interferon γ have been observed to induce delipidation of human adipocytes [126]. Treatment with *trans*-10, *cis*-12 CLA has also been shown to increase the expression or secretion of IL-6 and IL-8 from murine [127] and human [128] adipocyte cultures, as well as TNFα and IL-1β, thereby suppressing PPARγ ac-

tivity and insulin sensitivity [129]. In human subjects, *trans*-10, *cis*-12 CLA supplementation also increases the levels of inflammatory prostaglandins [130] which has been reported to inhibit adipogenesis via phosphorylation of PPARγ by MAPKs [131] and via induction of the normoxic activation of hypoxia-inducible factor-1 [112].

2.6 CLA and Milk Fat Depression

The low-fat milk syndrome, more commonly referred to as milk fat depression (MFD), is a naturally occurring situation in dairy production when cows are fed highly fermentable diets or dietary supplements of plant or fish oils [132]. First described over a century ago, diet-induced MFD can result in a reduction in milk fat yield of up to 50%, and the decrease involves FAs of all chain lengths. According to the biohydrogenation theory, the basis for diet-induced MFD relates to an inhibition of mammary lipid synthesis by specific FAs that are intermediates in the biohydrogenation of dietary PUFAs and are produced only under certain conditions of rumen fermentation [133]. *Trans*-10, *cis*-12 CLA was the first of these unique intermediates to be identified as a potent inhibitor of milk fat synthesis, and for many situations of diet-induced MFD, the increase in milk fat content of *trans*-10, *cis*-12 CLA is correlated with the magnitude of the reduction in milk fat yield [133, 134].

CLA are produced as intermediates in the biohydrogenation of linoleic acid by rumen bacteria. Providing relatively pure CLA isomers to dairy cows by abomasal infusion, to avoid alterations by rumen bacteria, Baumgard et al. [135] clearly demonstrated that *trnas*-10, *cis*-12 CLA resulted in a reduction in milk fat in dairy cows, and milk fat was rescued when treatment ceased. On the other hand, in certain situations of diet-induced MFD, the *trans*-10, *cis*-12 CLA content and magnitude of the reduction in milk fat yield do not align with the dose-response curve generated from abomasal infusion of relatively pure *trans*-10, *cis*-12 CLA. This suggests that in these situations this single CLA isomer does not completely explain the extent of the decrease in milk fat. Thus, additional inhibitory biohydrogenation

intermediates have been proposed [133,136], and 2 additional CLA isomers that inhibit milk fat synthesis (*trans*-9, *cis*-11 and *trans*-10, *cis*-12) have recently been identified [6]. However, careful accounting of the rumen production of CLA isomers under different situations of diet-induced MFD suggests that the isomers identified to date are still not adequate to fully explain the observed decrease in milk fat yield [137].

Most research investigating CLA effects during lactation has utilized dairy cows, and results have consistently demonstrated that the inhibitory effects are specific for milk fat; yields of milk and other milk components are generally unaffected [132]. The reduction in milk fat secretion reaches a nadir by 4 to 5 d of supplementation and returns to previous levels in a similar temporal pattern when CLA treatment is terminated [6]. Most studies have lasted a few days, but long-term studies (20 wk) indicate that the reduction in milk fat persists throughout the treatment period [138, 139]. Treatment has also encompassed all phases of the lactation cycle with no adverse effects on animal health and well-being [138–140]. de Veth et al. [141] combined results from 7 studies and indicated a curvilinear relation between the increasing dose of *trans*-10, *cis*-12 CLA and the reduction in milk fat production.

The ability of CLA to regulate milk fat synthesis has also been observed in other mammals including mice, rats, pigs, sheep, goats, and humans [see review by Bauman et al. [6]]. Most of these investigations have used dietary supplements containing a mixture of CLA isomers. However, a study with rats demonstrated that milk fat content and nursing pup growth were reduced when *trans*-10, *cis*-12 CLA was provided as a dietary supplement, whereas *cis*-9, *trans*-11 CLA had no effect [142]. For all species, CLA supplements containing *trans*-10, *cis*-12 have consistently resulted in a reduction in milk fat content, milk fat yield, and/or growth rate of the nursing neonate.

Phenotypic characterization of CLA-induced MFD provides key insight into the functional mechanism of CLA. Fat is the only milk component inhibited with *trans*-10, *cis*-12 CLA treatment, and the reduction involves FAs of all chain lengths. Thus, mammary effects are

highly specific for lipid synthesis and include biochemical pathways associated with both de novo synthesis and the use of preformed FAs [6].

Evaluating the SREBF-regulatory system in bovine mammary epithelial cells (MAC-T cell line) showed decreased abundance of the nuclear SREBF1 protein during *trans*-10, *cis*-12 CLA inhibition of FA synthesis [143]. SREBF1 is highly expressed in bovine mammary tissue, and recent investigations demonstrated that mammary expression of SREBF1 and expression of the genes for proteins involved in the activation and translocation of SREBF were reduced for both *trans*-10, *cis*-12 CLA treatment and diet-induced MFD [9]. Many lipogenic enzymes have SREBF response elements in their promoter, and, consistent with this, transcription of mammary genes involved in the complementary pathways for milk fat synthesis was coordinately downregulated during CLA- and diet-induced MFD. Collectively, these observations are consistent with SREBF1 representing a major signaling mechanism in the regulation of FA synthesis during CLA-induced MFD [6].

Only a limited number of mechanisms have been investigated in CLA-induced MFD and these predominantly at the level of gene expression. The coordinated downregulation of lipogenic enzymes during MFD is expected to involve multiple regulatory systems and the interaction of multiple signals. Mechanisms regulating lipid synthesis and SREBF1-c continue to be identified and will provide strong hypotheses to test the regulation of milk fat synthesis.

Chapter 3

Ability of Conjugated Linoleic Acid to Reduce Adiposity is Independent of Spot 14 Gene Expression

3.1 Introduction

Conjugated linoleic acid (CLA) is a generic term used to describe positional and geometric isomers of linoleic acid containing two adjacent double bonds. A number of CLA isomers are naturally produced by rumen bacteria as intermediates in the biohydrogenation of dietary polyunsaturated fatty acids (PUFA), with *cis*-9, *trans*-11 CLA being the predominant isomer found in ruminant-sourced foods [32]. CLA isomers also originate from industrial hydrogenation and *cis*-9, *trans*-11 and *trans*-10, *cis*-12 are the two CLA isomers that have been most extensively studied [4]. The interest in CLA increased dramatically when in vivo and in vitro studies documented a wide range of beneficial effects relating to cancer, atherosclerosis, and obesity [4, 48]. Investigations of the antiobesity effect of CLA have demonstrated a reduction of body fat occurred in several animal models when CLA was included at $\leq 1\%$ of the diet [144]. This CLA-mediated delipidation is mainly attributed to the *trans*-10, *cis*-12 CLA isomer and involves, but not limited to, a reduction in de novo lipid synthesis (DLS) [145]. The mechanism by which CLA affects de novo lipid synthesis (DLS) is not fully understood

but it involves a downregulation of the expression and/or activity of the key lipogenic genes and transcription factors in the process of DLS.

The thyroid hormone responsive spot 14 (*S14*) gene encodes for a small nuclear protein that is predominantly expressed in lipid synthesizing tissues like liver, adipose tissue and the lactating mammary gland [34]. Rates of lipogenesis and *S14* gene expression are highly correlated. They are increased by triiodothyronine (T3) injection [37,146,147] and feeding a high carbohydrate diet [38,148] and decreased by fasting, feeding diets high in PUFAs [149,150], and treatment with glucagon [146] or catecholamines [151]. Although the exact function of S14 is still unclear, its tissue expression profile [34,152], nuclear localization [153], rapid expression relative to other lipogenic genes [154] and impaired lipogenesis and lipogenic gene expression in primary hepatocytes transfected with *S14* antisense oligonucleotide [43], suggest that this protein might be associated with normal lipogenesis. As expected, a mouse model possessing a targeted disruption in the *S14* gene exhibits impaired DLS in the mammary gland [45]. Similarly, treatment of lactating dairy cows with *trans*-10, *cis*-12 CLA caused a reduction in de novo synthesis of milk fatty acids (FAs) accompanied by a significant reduction in the mRNA abundance for *S14* [9] and growing mice receiving dietary *trans*-10, *cis*-12 CLA had a significant reduction in body fat and reduced *S14* gene expression [9,145]. Based on the *S14* partial knockout (*S14* null) mice phenotype in mammary gland and the white adipose tissue (WAT) gene expression of mice treated with CLA, we hypothesized that the *S14* gene might be essential for normal lipogenesis in WAT. Moreover, *S14* might be essential to CLA effect on lipid metabolism. Therefore, we conducted this experiment to examine these hypotheses.

3.2 Material and Methods

3.2.1 Animals and Treatments

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee and mice were maintained in accordance with NIH Guide for the Care and Use of Laboratory Animals. Mice for the current studies were the progeny of wild type (Wt) and *S14* null C57B1/6J breeding pairs that were kindly provided by Dr. C. Mariash (University of Minnesota, Minneapolis, MN). The genotype of the progeny was determined by multiplex PCR according to Zhu et al. [155]. Mice were routinely fed ad lib a pelleted rodent diet (22% protein, 5% fat; diet #8640, Harlan Teklad, Indianapolis, IN). At 9 wk of age mice were shifted to a semipurified low fat diet (Table 3.1; Research Diets, New Brunswick, NJ).

Male Wt and *S14* null mice were assigned to one of four treatments in 2 X 2 completely randomized factorial design (10/group). For 2 wk mice received either water (control) or 40 ul of CLA supplement (Clarinol^{TM} G-80; Lipid Nutrition, Channahon IL) in equal doses administrated twice daily. The CLA supplement was in the triglycerides (TG) form with the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers representing 37% and 38% of the FAs, respectively. Other FAs in the supplement included 16:0 (4%), 18:0 (2%), 18:1 *cis*-9 (12%) and 18:2 *cis*-9, *cis*-12 (1%). The amount of the CLA supplement given to the animals was chosen to provide a daily total CLA of approximately 1% of the diet based on pretrial feed intake.

3.2.2 Data and Sample Collection

The body weight (BW) and feed intake of the mice were measured between 10:00 and 11:00 h on days 1, 2 and 14 of the experiment. On day 14 of the experiment, mice were euthanized at 14:00 h by CO_2 inhalation followed by cervical dislocation. Liver and WAT depots (epididymal (EPF), mesentric (MEF) , subcutaneous (SCF) and retropritoneal (RPF)) were immediately dissected, blotted dry, weighed and snap frozen in liquid nitrogen; samples

Table 3.1: **Composition of the Experimental Diet**

Variable	**g/100 g**	**kcal%**
Ingredient		
Soybean oil	2.37	5.55
Sunflower oil[1]	1.90	4.44
Casein	18.96	19.72
Corn starch	47.39	49.30
Maltodextrin	9.48	9.86
Sucrose	9.48	9.86
Celluolose	4.74	0.00
L-Cysteine	0.28	0.30
Mineral mix[2]	0.95	0.00
Vitamin mix[3]	0.95	0.99
Choline bitartarate	0.19	0.00
Dicalcium phosphate	1.23	0.00
Calcium carbonate	0.52	0.00
Analysis [4]		
Protein	19.2	23.7
Carbohydrate	67.3	41.4
Fat	4.2	23.6

[1] Trisun high oleic sunflower oil
[2] Standard salt mix (S100026)
[3] Standard vitamin mix (V10001)
[4] Analysis calculated based on nutrient content of individual ingredients. Diet energy content was 3.85 kcal/g.

were then stored at -80 ^{0}C for subsequent RNA and FA analyses.

3.2.3 Sample Analysis

3.2.3.1 Fatty Acid Analysis

FA methyl esters of the CLA supplement were prepared by mild acid methylation using 1% sulfuric acid in methanol according to Christie [156]. Dietary lipids were extracted and determined by the method of Sukhija and Palmquist [157]. The lipid extraction from liver and EPF was according to Hara and Radin [158] as modified by Castaneda-Gutierrez et al. [159]. Methylation of FAs in the lipid extracted from diet and tissues was performed by base-catalyzed transmethylation according to Christie [160] as modified by Chouinard et al. [161]. The FA methyl esters were quantified using gas liquid chromatography (GCD system HP G1800 A; Hewlett Packard, Avondale, PA) equipped with a CP-Sil 88 capillary column (100 m x 0.25 mm i.d. with 0.2-m film thickness; Varian Instruments, Walnut Creek, CA). Gas chromatographic conditions for separation of the FA methyl esters were described by Castaneda-Gutierrez et al. [159]. FA peaks in chromatograms were identified and quantified using pure methyl ester standards (GLC569 and GLC60, NuChek Prep, Elysian, MN). The FA profile of the diet is presented in Table 3.2. Oleic acid (45.9%) and linoleic acid (30.0%) were the major FAs in the semipurified diet fed throughout the experimental period.

3.2.3.2 RNA Extraction and Real-Time PCR

Total RNA was extracted from ~ 30 mg of liver and ~ 70 mg of EPF using the RNeasy Lipid kit (Qiagen, Valencia, CA). Potential genomic DNA contamination was excluded through on-column DNase treatment (RNase-Free DNase Set; Qiagen). Moreover, RNA concentration and quality were determined by a 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was reverse transcribed using SuperScript III First Strand Synthesis kit (Invitrogen) with random primers. Quantitative real-time PCR (qRT-PCR) assays were devel-

Table 3.2: **Fatty Acid Composition of the Experimental Diet**[1]

Fatty Acid	g/100 g
8:0	0.03
10:0	0.07
12:0	2.25
14:0	4.06
14:1, *cis*-9	0.01
16:0	7.12
16:1, *cis*-9	0.11
18:0	3.01
18:1, *cis*-9	45.93
18:2, *cis*-9, *cis*-12	30.01
18:3, *cis*-9, *cis*-12, *cis*-15	2.64
others	4.76

[1] Does not include CLA supplement

oped for genes of interest (Table A.1). Briefly, primers were designed using Primer3Plus [162] to span the exon boundaries when possible and the optimal primers were selected. qRT-PCR reaction included Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and 400 nmol/L of gene-specific forward and reverse primers (Invitrogen, Carlsbad, CA). Twenty five ng cDNA was amplified using a 2-step program (95°C for 15 s and 60°C for 60 s) with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The specificity of the primers was verified by presence of a single product in the dissociation curve at the end of amplification process. A standard curve was derived from serial dilutions of pooled cDNA from adipose or liver tissues. Relative mRNA concentrations were expressed in arbitrary units and the logarithms (base 10) of concentrations were plotted against the threshold cycles (C_Ts). The resulting least square fit was used as the standard curve to derive the arbitrary concentration for each sample.

3.2.3.3 Liver Triglyceride Determination

Total liver lipid was extracted according to Folch et al. [163]. Briefly ~ 150 mg liver tissue was homognized with 3 ml of 2:1 (v/v) chloroform-methanol mixture and vortexed for 40 min. The extract was rinsed by addition of 0.6 ml of water, mixed and vortexed for 40 min followed by centrifugation for 1 min at 2500 rpm (1430g). An aliquot was removed (~ 100 ul) from the bottom phase and used to determine the TG concentration based on the Hantzsch condensation for estimating serum TG according to Fletcher [164] as modified by Foster and Dunn [165]. The reference curve was generated using glycerol trioleate (T-7140; Sigma-Aldrich Co., St. Louis, MO).

3.2.4 Statistical Analysis

Data were analyzed using ANOVA model in R statistical package [166]. The model included the fixed effect of genotype (Wt and *S14* null) and CLA treatment, and the interaction of genotype and CLA treatment. The interaction term was dropped from the model if it there

was no statistical or graphical evidence of an interaction. When the interaction effect was significant, preplanned contrasts were used to test the genotype effect within the control groups and the CLA effect within each genotype. The animal BW of the first 2 days of treatment were averaged and used as covariate for the statistical analysis of BW, feed intake, and the weights of liver and different fat depots. The covariate was removed from the model if $P > 0.3$. For gene expression analysis, two house keeping genes ((18S ribosomal RNA (*18S*) and beta-2-micro-globulin (*B2M*), Table A.1) were used to normalize gene expression data. The normalization factor was used as covariate in statistical analysis of the tested genes and it was removed from the model if the effect was $P > 0.3$. The normality and variance homogeneity assumptions of the model were verified by checking the studentized residual distribution and the plot of studentized residuals versus predicted values. Log transformation was performed to meet the model assumption for some response variables (SCF and RPF depot weights and *S14* mRNA level). Data points were considered outliers and excluded if studentized residuals were > -3 or < 3; this rarely occurred and represented one data point per response variable.

3.3 Results

3.3.1 Performance Parameters and Adipose Depots

Treatment had little effect on growth; although differences were significant, BW for the two genotypes and CLA treated groups differed by only 1 to 4% with the overall final average of 24.1 g (SEM = 0.27). Feed intake was approximately 10% lower in the CLA-treated group ($P < 0.0001$) and 5% higher ($P = 0.02$) in the *S14* null mice. *S14* null mice had about 25% less EPF than the Wt group ($P = 0.01$), but other fat depots were similar in weight between the two groups (Figure 3.1). However, CLA treatment decreased substantially the weight of all fat depots, and this was especially evident in the EPF, SCF and RPF depots where the reduction was almost 50% compared to the control mice ($P < 0.0001$). The MEF depot was the least

responsive to CLA treatment and it was decreased by less than 20% ($P = 0.02$). There was no evidence of CLA by genotype interaction on the weight of different fat depots.

3.3.2 Liver Weight and Liver Triglyceride Content

Treatment effects on liver weight and liver TG content are shown in Figure 3.2. The *S14* null mice had slightly larger livers (~ 10%) compared to Wt animals ($P < 0.001$) and CLA treatment increased liver weight ($P < 0.001$) regardless of the genotype. CLA treatment resulted in a substantial increase in the hepatic TG content in both *S14* null and Wt mice ($P < 0.0001$).

3.3.3 Tissue Fatty Acid Profile

Treatment effects on the FA profile of EPF and liver are shown in Table 3.3 and Table 3.4, respectively. The FA profile was relatively similar between the two genotypes for both EPF and liver, an exception being a reduction in the proportion of palmitic acid in both tissues for the *S14* null mice. In the case of WAT, the proportion of oleate was increased in the *S14* null mice. In CLA supplemented animals, both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers were incorporated into EPF and liver lipid ($P < 0.0001$) with the relative increase being greater for *cis*-9, *trans*-11 CLA in both tissues. In addition, CLA treatment induced extensive changes in the weight percent for many FAs. In EPF there was a decrease in the proportion of the myristate, palmitoleate and linoleate, whereas CLA treatment increased the proportion of oleate and linolenate. CLA significantly increased oleate to stearate (18:1/18:0) ratio in the EPF from *S14* null mice. In liver lipids, CLA treatment resulted in an increase in the proportion of the myristate, oleate, and linolenate. In contrast, there was a decrease in proportion of the stearate and linoleate. CLA also significantly increased the hepatic lipid ratio of oleate to stearate (18:1/18:0) in both genotypes.

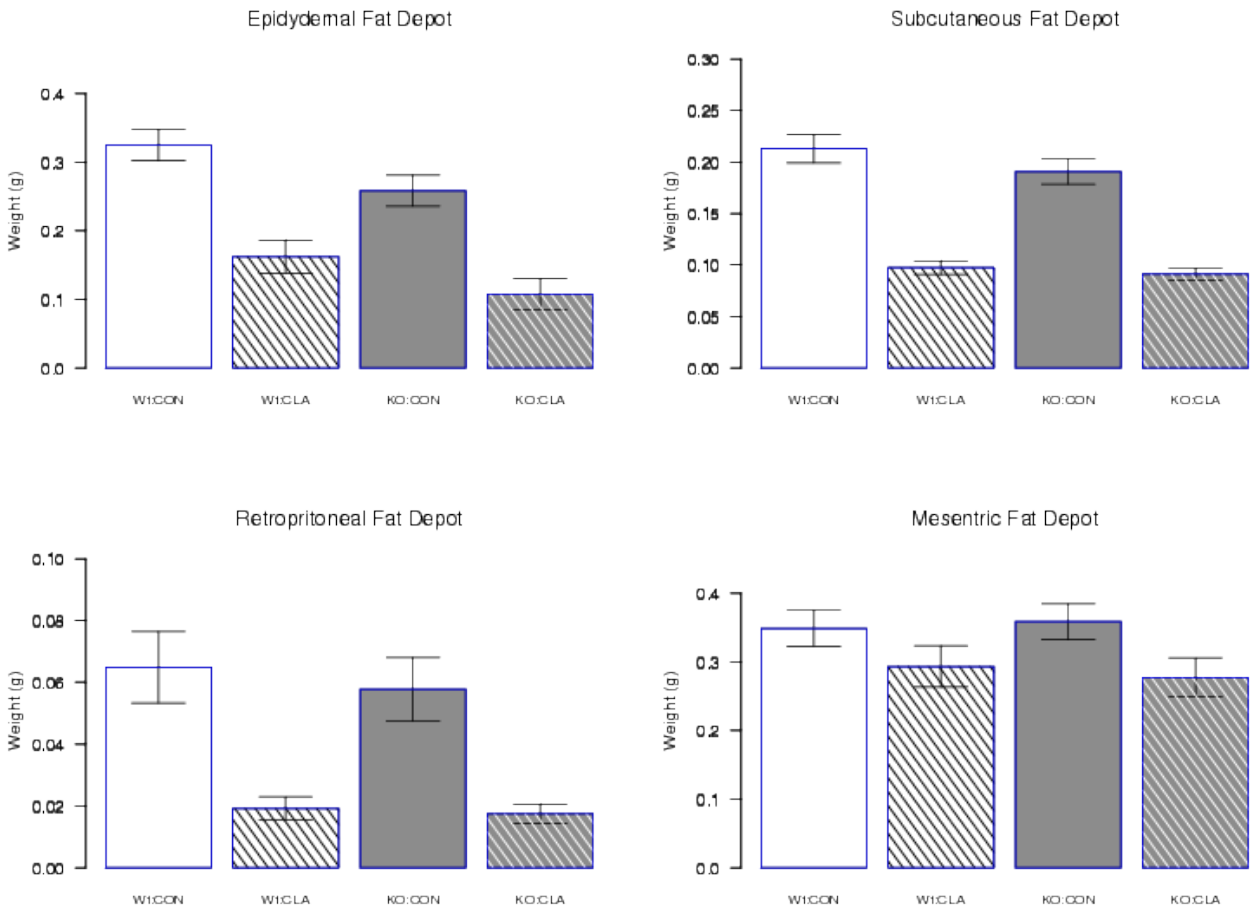

Figure 3.1: **Treatment Effect on White Adipose Tissue Deposition**. Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat diet and received either water (**CON**) or 40 ul of conjugated linoleic acid (**CLA**) as oral supplement daily for 2 wk. At the end of the study, mice were euthanized and different fat depots were dissected and weighed. Each bar represents the mean ± SE (n = 10). There was a CLA effect for all depots ($P < 0.05$), but significant genotype effect only for epididymal depot ($P = 0.01$) and no interaction effect ($P > 0.65$).

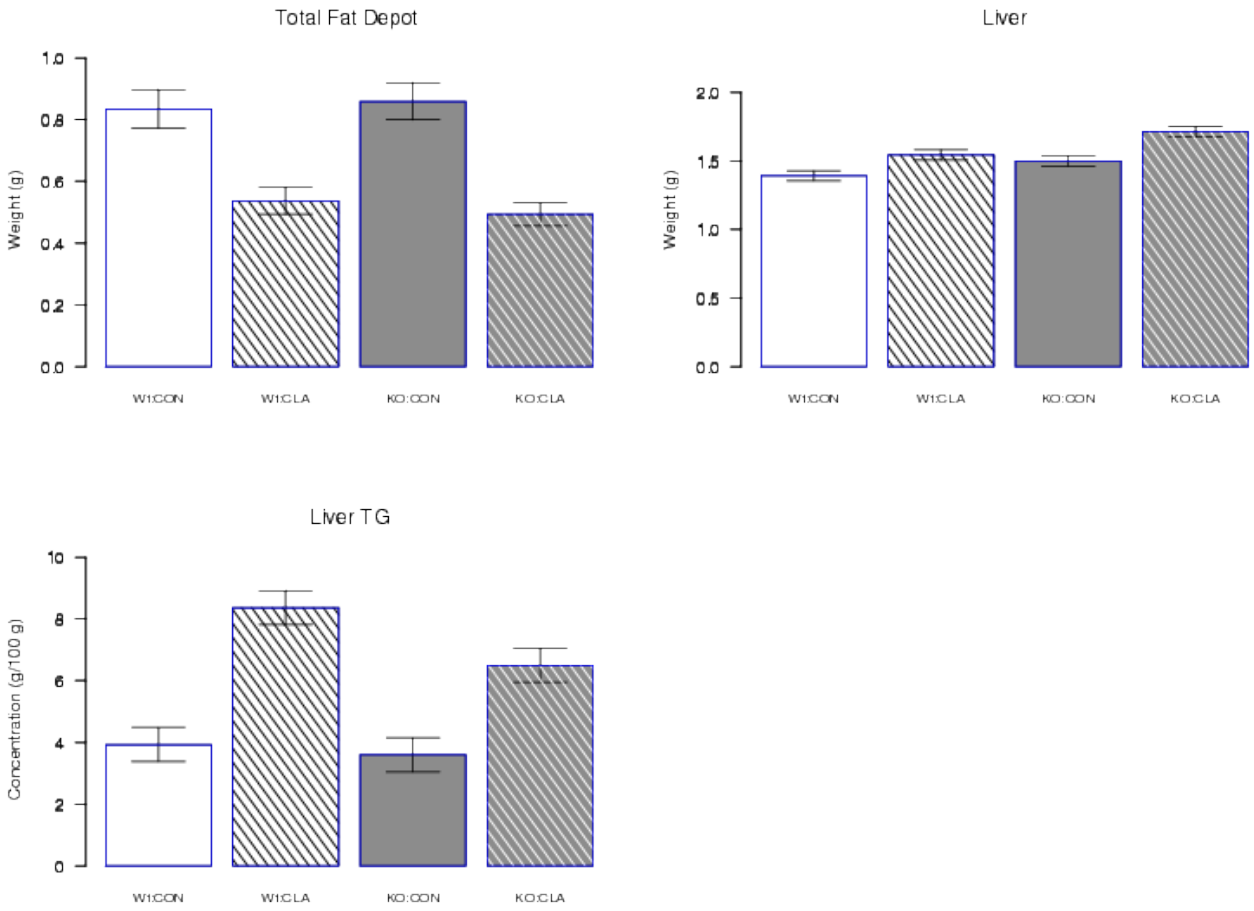

Figure 3.2: **Treatment Effect on Total fat, Liver Weight and Liver Triglyceride Concentration**. Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat diet and received either water (**CON**) or 40 ul of conjugated linoleic acid (**CLA**) as oral supplement daily for 2 wk. At the end of the study, mice were euthanized, liver and different fat depots were weighed and liver TG concentration was determined. Each bar represents the mean ± SE (n = 10 for total fat and liver and n = 4 for TG). There were CLA and genotype effects for liver weight ($P < 0.001$), and CLA effect ($P < 0.0001$), but no genotype effect ($P = 0.68$) for liver TG. There was CLA effect ($P < 0.0001$) but no genotype for total fat weight.

Table 3.3: **Treatment Effects on Adipose Fatty Acid Profile**[1]

	Wt		KO		*P* Value		
FA (% of total FAs)	CON	CLA	CON	CLA	Genotype	CLA	Interaction
14:0	1.10±0.13	0.79±0.12	1.32±0.11	0.98±0.17	0.10	<0.05	0.92
16:0	18.39±0.47	17.91±0.51	16.04±1.00	16.43±0.39	<0.01	0.93	0.36
16:1, *c-9*	5.97±0.40	3.29±0.19	5.48±0.21	3.41±0.14	0.50	<0.01	0.29
18:0	1.89±0.13	1.84±0.03	1.82±0.18	1.62±0.03	0.13	0.18	0.45
18:1, *cis*-9	35.69±0.62	41.57±0.89	37.86±0.54	43.74±1.19	0.02	<0.01	1.00
18:2, *cis*-9, *cis*-12	25.74±0.40	23.04±1.00	25.11±2.00	21.64±1.82	0.49	0.05	0.80
CLA, *cis*-9, *trans*-11	ND[2]	1.15±0.10	ND	1.09±0.12	0.77	<0.0001	NA[3]
CLA, *trans*-10, *cis*-12	ND	0.54±0.06	ND	0.46±0.04	0.31	<0.0001	NA
18:3	0.68±0.03	0.78±0.02	0.83±0.05	0.99±0.05	< 0.01	<0.05	0.52
20:4	0.16±0.01	0.18±0.01	0.15±0.01	0.17±0.01	0.55	0.15	0.81
22:6	0.17±0.01	0.10±0.01	0.14±0.02	0.08±0.02	0.19	< 0.01	0.89
	—	—	—	—	—	—	—
18:1/18:0	19.08±1.06	22.65±0.38	19.34±1.30	27.03±0.97	< 0.05	< 0.01	0.07

[1] Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat and received either water **(CON)** or 40 ul of conjugated linoleic acid **(CLA)** as oral supplement daily for 2 wk. At the end of the study, mice were euthanized, epididymal WAT samples were collected and fatty acids were extracted and quantified using gas liquid chromatography. Values represent observed mean ± SE (n = 4).

[2] ND = Not detected (< 0.05 % of total FAs)

[3] NA = Not applicable

Table 3.4: **Treatment Effects on Hepatic Fatty Acid Profile**[1]

	Wt		KO		*P* Value		
Fatty acid variable	CON	CLA	CON	CLA	Genotype	CLA	Interaction
14:0	0.37±0.06	0.48±0.04	0.43±0.05	0.59±0.08	0.17	0.05	0.70
16:0	24.20±1.05	25.18±0.20	21.56±0.96	22.40±0.51	<0.01	0.26	0.93
16:1, *cis*-9	3.15±0.20	2.94±0.34	2.69±0.06	2.79±0.47	0.35	0.87	0.62
18:0	8.68±0.43	6.14±0.92	9.53±0.13	7.12±1.2	0.27	<0.01	0.93
18:1, *cis*-9	23.77±1.77	31.94±2.46	21.18±0.72	30.06±2.99	0.32	<0.01	0.87
18:2, *cis*-9, *cis*-12	10.50±1.18	8.54±0.70	12.09±0.24	8.75±0.63	0.26	<0.01	0.38
CLA, *trans*-10, *cis*-12	ND[2]	0.16±0.02	ND	0.14±0.03	0.53	<0.0001	NA[3]
18:3	0.59±0.05	0.93±0.12	0.60±0.04	0.97±0.12	0.83	<0.01	0.87
20:4	5.01±0.43	3.04±0.57	6.10±0.12	3.67±0.65	0.10	<0.01	0.65
22:6	5.82±0.64	3.68±0.69	6.84±0.06	4.74±0.93	0.14	<0.01	0.98
	—	—	—	—	—	—	—
18:1/18:0	2.78±0.84	5.71±0.84	2.23±0.84	4.79±0.84	0.39	<0.01	0.83

[1] Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat and received either water **(CON)** or 40 ul of conjugated linoleic acid **(CLA)** as oral supplement daily for 2 wk. At the end of the study, mice were euthanized, liver samples were collected and fatty acids were extracted and quantified using gas liquid chromatography. Values represent observed mean ± SE (n = 4).
[2] ND = Not detected (< 0.05 % of total FAs)
[3] NA = Not applicable

3.3.4 Adipose Gene Expression

Treatment effects on mRNA abundance of adipose genes are presented in Figure 3.3. As expected, *S14* mRNA was negligible in the *S14* null mice. The mRNA levels of acetyl-coenzyme A carboxylase alpha (*ACACA*) ($P = 0.08$), stearoyl-coenzyme A desaturase 1 (*SCD1*) ($P = 0.04$) and lipoprotein lipase (*LPL*) ($P = 0.07$) were upregulated in the *S14* null mice compared to Wt. CLA treatment decreased the *S14* mRNA abundance in the Wt mice by almost 75% ($P = 0.03$). Similarly, CLA treatment downregulated the mRNA abundance of fatty acid synthase (*FASN*) ($P < 0.01$) and *ACACA* ($P = 0.02$) by about 60% and sterol regulatory element binding transcription factor 1-c (*SREBF1-c*) ($P = 0.04$) by almost 40% in both genotypes. On the other hand, CLA significantly decreased the mRNA level of *SCD1* in *S14* null mice ($P = 0.03$) and numerically decreased *SCD1* in Wt mice (non-significant). Moreover, CLA treatment increased the abundance of carnitine palmitoyltransferase 1a (*CPT1a*) gene regardless of the genotype ($P < 0.0001$) and there was no CLA effect on the mRNA level of *LPL* ($P = 0.52$).

3.3.5 Liver Gene Expression

Treatment effects on mRNA abundance of hepatic genes are presented in Figure 3.4. The CLA treatment had no effect on the mRNA abundance of *S14* gene in the Wt mice ($P = 0.32$) or the mRNA abundance for *SREBF1-c* ($P = 0.84$) in either genotype. However, CLA treatment upregulated the mRNA abundance of *FASN* ($P = 0.02$) and *SCD1* ($P < 0.01$) but only for the *S14* null mice. On the other hand, CLA increased the mRNA abundance of peroxisome proliferator-activated receptor alpha (*PPARa*) transcription factor in the *S14* null mice and its target gene *CPT1a* in both genotypes ($P < 0.01$). Moreover, CLA treatment increased the expression of fatty acid translocase (*CD36*) by more than two-fold ($P < 0.01$) and increased the mRNA abundance of of fatty acid binding protein 1 (*FABP1*) ($P < 0.01$) for both genotypes.

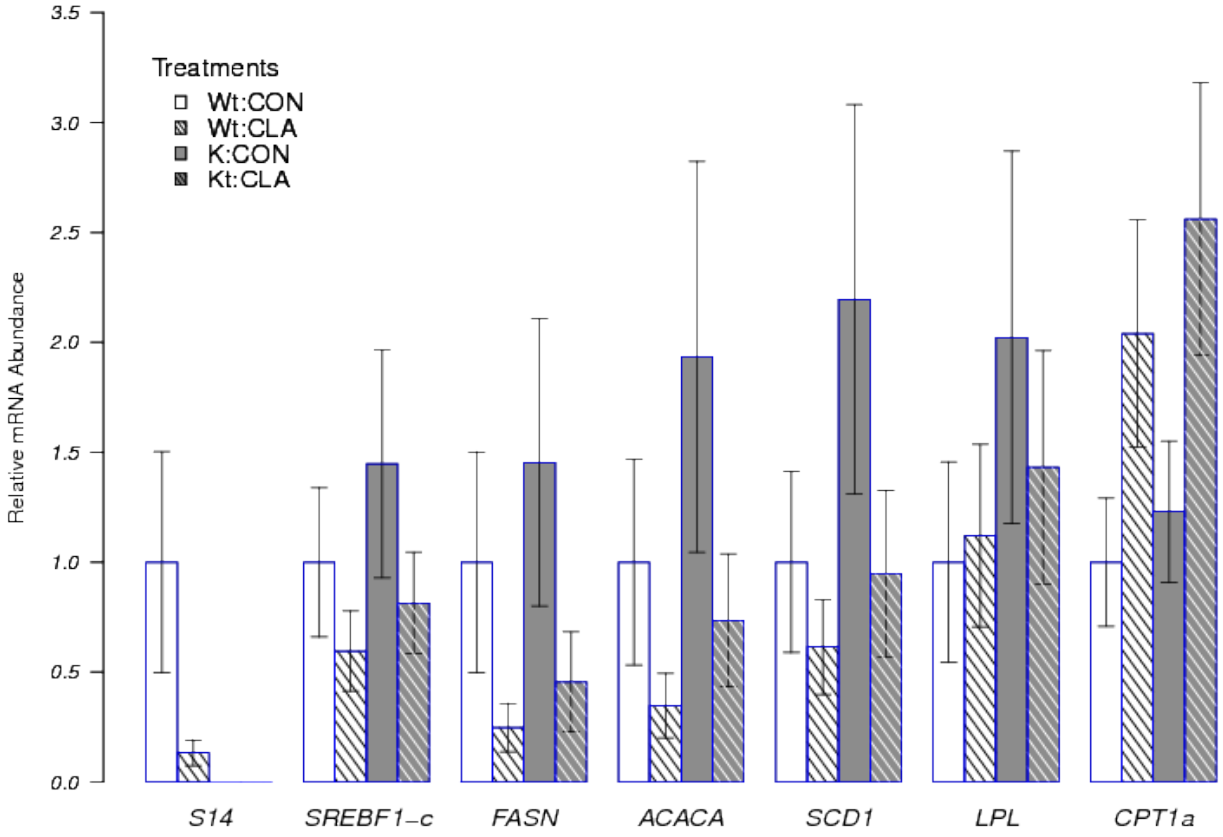

Figure 3.3: **Treatment Effect on Adipose Gene Expression**. Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat diet and received either water **(CON)** or 40 ul of conjugated linoleic acid **(CLA)** as oral supplement daily for 2 wk. At the end of the study, mice were euthanized and epididymal fat samples were collected and total RNA was analyzed by RT-PCR for the mRNA abundance of indicated genes: ***S14*** = thyroid hormone responsive spot 14, ***SREBF1-c*** = sterol regulatory element binding factor 1-c, ***FASN*** = fatty acid synthase, ***ACACA*** = acetyl-coenzyme A carboxylase alpha, ***SCD1*** = stearoyl-coenzyme A desaturase, ***LPL*** = Lipoprotein lipase, ***CPT1a*** = carnitine palmitoyltransferase 1a. Each bar represents the mean ± SE (n = 7). Significant CLA effect for all genes except *LPL*, and genotype effect for *ACACA* ($P = 0.08$), *SCD1* ($P = 0.05$) ($P = 0.08$) and *LPL* ($P = 0.07$).

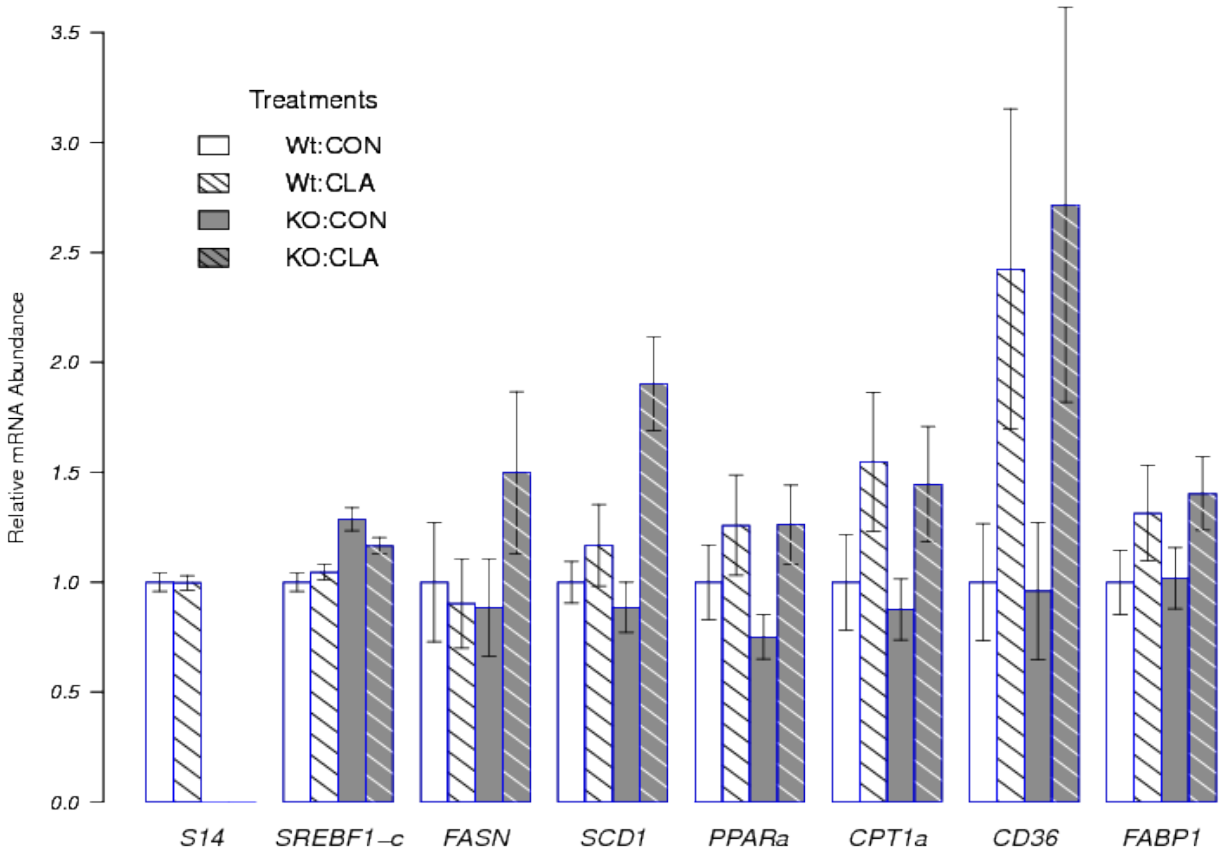

Figure 3.4: **Treatment Effect on Hepatic Gene Expression**. Nine wk-old male C57BL/6J wild type **(Wt)** and *S14* null **(KO)** mice were fed a semipurified low fat diet and received either water **(CON)** or 40 ul of conjugated linoleic acid **(CLA)** as oral supplement daily for 2 wk. At the end of the study, mice were euthanized and liver samples were collected and total RNA was analyzed by RT-PCR for the mRNA abundance of indicated genes: ***S14*** = thyroid hormone responsive spot 14, ***SREBF1-c*** = sterol regulatory element binding factor 1-c, ***FASN*** = fatty acid synthase, ***SCD1*** = stearoyl-coenzyme A desaturase, ***PPARa*** = peroxisome proliferator activated receptor alpha, ***CPT1a*** = carnitine palmitoyltransferase 1a, ***CD36*** = fatty acid translocase, ***FABP1*** = fatty acid binding protein 1. Each bar represents the mean ± SE (n = 7). Significant CLA effect for *FASN*, *SCD1* and *PPARa* in *S14* null mice ($P < 0.05$) and for *CPT1a*, *CD36* and *FABP1* in both genotypes ($P < 0.01$). There was no genotype effect for any gene.

3.4 Discussion

Due to the substantial rise in the prevalence of obesity over the past 30 years, interest in CLA as a weight loss treatment has increased [112,167]. The first report that suggested a potential CLA antiobesity effect was published in 1997, when mice were fed a diet supplemented with 0.5% CLA had a 60% decrease in body fat after 4–5 wk [113]. Supplementation with a CLA mixture (equal concentrations of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 isomers) or the *trans*-10, *cis*-12 CLA isomer alone decreased body fat mass in many species and several mechanisms have been proposed to explain the CLA-induced reduction in fat deposition [51,114]. One of these mechanisms involves decreased lipid accumulation of adipocytes and downregulation in the expression of enzymes involved in lipogenesis. For example, *ACACA*, *FASN* and *SCD1* are all decreased in the WAT of mice when the diet is supplemented with mixed CLA isomers or *trans*-10, *cis*-12 CLA [118,168–170].

As discussed in the introduction, several lines of evidence suggest that S14 protein may be involved in the regulation of lipogenesis. Moreover, reports have shown that *S14* gene has been linked to the development or maintenance of obesity in humans; a 48-h fast results in minimal downregulation of *S14* mRNA in WAT of obese patients as compared with non-obese patients [171]. The objective of the current study was to examine lipogenesis in *S14* null mice and to determine the effect of knocking out the *S14* gene on the antiobesity mechanism of CLA. To address our objective, *S14* null and Wt mice were fed a semipurified low fat diet (4% fat w/w) and treated with CLA for 14 days in a 2 X 2 randomized factorial design. We observed that knocking out the *S14* gene reduced the EPF by 25% (P = 0.01) and the SCF by 13% (P = 0.15) with the total fat mass being reduced by almost 8% compared to the Wt mice. A similar phenotype has been reported previously where deletion of the *S14* gene decreased fat mass by almost 45% in 29-wk-old male mice [172]. The young age of the mice used in the present study (11 vs 29 wk of age), the relatively short term observation period (2 vs 29 wk), and the method used to determine the degree of fatness (manual dissection vs DEXA scan) may be the basis for the less dramatic reduction observed in the present study. Nevertheless,

results suggest that that S14 does play a role in fat deposition.

The role of S14 in fat deposition was further investigated by examining the mRNA abundance of key lipogenic enzymes in WAT of *S14* null mice. In contrast to the observed reduction in fat deposition, *SREBF1-c* and *FASN* mRNA abundance were not reduced in the WAT from the *S14* null mice. Interestingly, *ACACA* and *SCD1* mRNA levels were significantly increased in the epididymal WAT of the *S14* null mice. Although unexpected, Zhu et al. [45] observed a similar paradox in the milk fat synthesis of *S14* null mice. They reported that TG levels in the milk and mammary tissue were reduced by 28% and the rate of mammary DLS was decreased by 62% in *S14* null mice when compared with Wt. Nevertheless, the *S14* null mutation had no effect on mRNA abundance of *FASN* and *ACACA* [45]. Moreover, they reported that ACACA enzyme activity was significantly increased in the lactating mammary gland of the *S14* null mice [45]. Based on these findings, Zhu et al. [45] suggested that the regulatory effect of S14 on lipogenesis does not involve alteration of the transcription of the rate-limiting lipogenic enzymes in the mammary gland; we conclude the same for WAT in the present study. Indeed, malonyl-CoA, the substrate of the FASN reaction, has been shown to be increased in the mammary gland of the *S14* null mice; this suggests that the defect in lipogenesis may be related to an in vivo reduction in FASN activity and that the S14 protein may act as an allosteric regulator of FASN in vivo activity [8].

In the present study, knocking out the *S14* gene resulted in a modest (10%) increase in liver weight ($P < 0.001$) whereas hepatic TG concentration was unchanged. This suggests that S14 protein may be not required for hepatic lipogenesis under the current experiment conditions. Zhu et al. [155] reached a similar conclusion based on their studies showing that knocking out the *S14* gene did not affect hepatic lipogenesis when the mice were acutely (24 h) treated with T3, fed a high carbohydrate diet, or administrated T3 plus fed a high carbohydrate diet [155]. Hepatic mRNA levels of several lipogenic genes including *FASN* were not different between the Wt and *S14* null mice 24 h after being given T3 [155]. Likewise, in the present study there were no differences in the hepatic mRNA abundance of *SREBF1-c*, *FASN*

or *SCD1* between the *S14* null and Wt mice. One explanation for the failure of the *S14* null mice to have a reduction in hepatic lipogenesis is that another related protein might compensate for the lack of S14 [8]. Indeed, a paralog of *S14* has been identified and given the name S14 related protein (*MIG12*) [45]. The *MIG12* gene has many similarities to the *S14* gene; it is well expressed in lipogenic tissues (liver and adipose tissue), and it is regulated in liver by carbohydrate feeding in vivo, and by glucose metabolism in cultured hepatocytes [173]. Furthermore, just as for *S14*, a carbohydrate response element (ChRE) was detected in the *MIG12* promoter where the hetero-dimer of carbohydrate response element binding protein (ChREBP) and MAX-like protein X (MLX) bind [173]. These similarities raises the possibility that MIG12 functions similarly to S14 in such tissues [173] and may compensate for the absence of S14 in the *S14* null mouse. Moreover, *MIG12* is highly expressed in WAT compared to liver and mammary tissue (almost 5-fold greater when compared with liver) [45]. Moreover, in *S14* null mice, the *MIG12* mRNA abundance is greater than that in the Wt mice (although non significant) [45]. Therefore, the increased expression of the *MIG12* in the null mice might explain the greater expression of both *ACACA* and *SCD1* in the *S14* null mice compared to the Wt. Interestingly, *MIG12* was less abundant in mammary gland compared with liver which may explain the contrast in the lipogenic phenotype between the two tissues in the *S14* null animal [45]. Using small interfering RNA to simultaneously reduce levels of *S14* and *MIG12* in cultured primary hepatocytes, the rates of lipogenesis were decreased by approximately 65% in cells treated with insulin and high glucose. Furthermore, expression of either *S14* or *MIG12* gene products was sufficient to fully restore normal lipogenesis [36].

In the present study, there was no genotype effect on the live BW, and this is in agreement with the findings of Anderson et al. [172]. They reported that first generation backcross (N1) *S14* null and Wt mice born to homozygous dams and maintained on a 4% fat diet from the time of weaning showed no significant differences in BW at 8 wk of age. Moreover, N11 backcross *S14* null mice fed the 4% fat diet was not different in BW compared to Wt mice at 31 wk of age.

Dietary supplementation with CLA significantly reduced EPF, SCF and RPF depots by almost 50% in both Wt and *S14* null mice with no evidence of a CLA by genotype interaction. The total dissected fat tissue, which include the MEF depot, was decreased by almost 40% in the CLA supplemented group. Similar effects of CLA on fat deposition have been reported previously in mice and other animal models [114]. The CLA supplement used in the current study was a mixture of the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA. The *trans*-10, *cis*-12 CLA isomer was first shown to inhibit milk fat synthesis in dairy cows [135] and subsequent studies have verified that the *trans*-10, *cis*-12 CLA was the specific CLA isomer responsible for the delipidative effects in growing animals [168, 174, 175]. As low as 0.1% of the diet as *trans*-10, *cis*-12 CLA was able to decrease the gonadal fat pad [176] and 0.2% *trans*-10, *cis*-12 CLA decreased significantly both RPF and EPF depots when given for 4 wk [177].

In the current study, the fact that CLA-induced a delipidative effect regardless of the genotype indicates that expression of the *S14* gene is not essential for the CLA mechanism. The mechanistic details of CLA on adiposity are not yet fully understood but CLA treatment has been shown to decrease the mRNA abundance and/or the activity of lipogenic enzymes in WAT [118, 168–170, 178]. In the current study, the mRNA abundance of *SREBF1-c*, *FASN* and *ACACA* decreased by almost 30% - 70% regardless of the genotype. However, DLS is relatively less important in fat deposition when fat is provided in the diet. In this case the dietary supply of preformed FAs would be the major source of FAs for body fat accretion. CLA reduces body fat even in situations where animals have been fed a high fat diet, so the mechanism must involve more than just effects on DLS synthesis. Indeed, results from various studies have suggested that in addition to DLS, CLA effects can involve reduction of energy intake, increase in energy expenditure, inhibition of adipogenesis, induction of inflammation, stimulation of lipolysis and induction of apoptosis [112]. However, most investigations have utilized *trans*-10, *cis*-12 CLA doses of 0.5% of diet or greater and a recent study demonstrates this may complicate evaluation of mechanism. Foote et al. [176] showed that a CLA dose of 0.5% *trans*-10, *cis*-12 isomer substantially reduced mammary and gonadal

fat pads in growing mice and this was associated with an increase in the expression of the inflammatory markers; mRNA abundance of chemokine monocyte chemoattractant protein-1, tumor necrosis factor alpha, interleukin 6 and others were increased by 3- to 6-fold [176]. The implication was that inflammatory markers were a component of the mechanism whereby CLA induced a reduction in body fat. However, when Foote et al. [176] used lower doses of *trans*-10, *cis*-12 CLA effects on mammary and gonadal fat were still observed but there were no effects on inflammatory factors; they concluded these latter effects were a consequence of an excessive dose of CLA and not essential components of the CLA mechanism for reducing fat accretion.

In conclusion, while a modest reduction in fat accretion occurred in *S14* null mice, there was no corresponding reduction in the expression of key lipogenic enzymes. Although a similar phenotype has been reported in mammary gland of *S14* null mice [45], this suggests , in contrary to our hypothesis, that the regulation of lipogenesis by S14 protein might not take place through the transcriptional regulation of lipogenic enzymes. A compensatory mechanism in response to decreased lipogenesis might be involved to maintain or upregulate the lipogenic gene expression. However, the decrease in fat accretion and in mammary lipogenesis in the *S14* null mice might indicate other roles by which S14 protein regulates lipogenesis that are not likely to be compensated by other mechanisms in mammary and adipose tissues. Indeed, recent data has confirmed the ability of S14 to form hetero-dimers with other proteins in the cytoplasm including MIG12 which regulate the activity of ACACA [179]. Moreover, in the mammary gland of *S14* null mice, there was an indication of reduction in the activity of FASN [45]. Altogether these data confirm that S14 might play different roles in the regulation of lipogenesis and more research is required to explore this possibility. On the other hand, and in contrary to our hypothesis, S14 protein does not seem to be a component in the mechanism of the CLA delipidative effect; in the present study a reduction in fat accretion by CLA was clearly evident whether the genotype was Wt or *S14* null.

Chapter 4

Effect of Fish Oil Supplementation on Mammary Lipogenesis in Lactating Mice

4.1 Introduction

The long chain ω-3 polyunsaturated fatty acids (LC ω-3 PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are prevalent in marine fish oil (FO) and represent a unique class of fatty acids (FAs) that show a wide range of functions in biological systems. These effects include the ability to modulate gene expression [52], inflammatory processes [53] and cellular membrane structure and function as well as signaling pathways involved in normal and pathological cell functions [54]. Recent epidemiological and clinical studies with humans and biomedical studies using animal models indicate that FO may be useful for reducing the risk of coronary heart disease and atherosclerosis [180], treating inflammatory conditions [181], and preventing certain types of cancer [5].

The ability of LC ω-3 PUFAs to affect lipid metabolism in liver and to a lesser extent adipose tissue, has been of a considerable interest. Studies involving oral administration of FO or purified ethyl esters of EPA and DHA in both experimental animals and humans report a decrease in serum and hepatic levels of triglycerides (TG), cholesterol and phospholipid [10, 182–187], and a reduction in body fat deposition [11, 62]. Although mechanistic details are not fully understood, the effects of LC ω-3 PUFAs in liver involve the regulation of the

activity of metabolic nuclear receptors which results in repartitioning of metabolic fuel (i.e, FAs) away from TG synthesis and storage toward oxidation, thereby decreasing the substrate available for very low density lipoprotein synthesis and secretion [63, 182–184].

The effect of LC ω-3 PUFAs on the biology of mammary gland, the major lipid synthesizing organ during lactation, has not been investigated extensively and to the best of our knowledge the effects on mammary enzymes involved in lipogenesis per se have not been reported. Mammary lipogenesis is nutritionally regulated and highly responsive to dietary FA composition [188]. It is also regulated by bioactive FAs. For example, dietary supplements of *trans*-10, *cis*-12 conjugated linoleic acid (CLA) were able to induce milk fat depression (MFD) in lactating ruminants and non-ruminants [6]. Although the mechanisms are not completely elucidated, the CLA-induced MFD involves a coordinated downregulation of mammary lipid synthesis characterized by a decrease in de novo FA synthesis and a corresponding downregulation in the expression of lipogenic enzymes [6].

Investigations of the effect of FO or LC ω-3 PUFAs on the milk FA composition in lactating mice indicate a trend toward an increase in the proportion of medium chain fatty acids (MCFAs) in milk fat relative to the control diets [64–68]. Most diets are devoid of MCFAs so their presence in milk fat is generally indicative of mammary de novo lipogenesis (DNL). However, some fish oils do contain modest amounts of MCFAs, especially myristic acid. Nevertheless, results from the above investigations provide no evidence that the rate of mammary de novo FA synthesis is reduced by feeding FO or ω-3 PUFAs. However, these experiments were not designed specifically to address effects on de novo lipid synthesis and such an effect would be contrary to the well established ability of ω-3 PUFAs to downregulate hepatic lipid synthesis. Therefore, the objective of the present study was to examine the effects of FO supplementation on mammary lipogenesis as well as the expression of lipogenic genes and transcription factors in mammary and hepatic tissues of lactating mice.

4.2 Material and Methods

4.2.1 Animals

All experimental procedures related to animals were approved by Cornell University Institutional Animal Care and Use Committee. Eight wk old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, MA) and housed in group cages under controlled temperature (22-26 ^{0}C) and a 12 h light-dark cycle. Mice were fed ad lib a pelleted rodent chow diet containing 5% fat and 22% protein (diet #8640; Harlan Teklad; Harlan Teklad, IN). At wk 10 of age, breeding was initiated (1 male/4 females) and once gestation was confirmed females were housed individually until the end of the study. On d 3 of lactation, litter size was adjusted to 7 pups per dam through cross fostering.

4.2.2 Design

One wk before parturition, mice were shifted to a semipurified pelleted diet containing 5% oil. Then on d 6 of lactation, two groups of the dams were randomly switched to semipurified pelleted diets containing 10% oil that were enriched with either oleic acid (high fat control (HFC)) or FO (n = 8). To account for the effect of dietary oil level, a third group of mice continued on the semipurified diet containing 5% oil (low fat control (LFC)). The experimental diets were based on a widely used nutritionally balanced rodent diet (AIN-76A) and were mixed to our specifications by Research Diets Inc.(New Brunswick, NJ) (Table 4.1). Dietary oil sources and proportions were chosen so that the HFC diet serves as a control for FO diet at the high oil level (10%) while the LFC diet accounts for effect of dietary oil level in comparison with the FO diet. Safflower oil (high oleic acid) and palm oil were provided by Research Diets Inc. and the menhaden oil (Virginia Prime Gold^{TM}) was provided by Omega Protein Corp. (Houston, TX).

Table 4.1: **Experimental Diet Composition**

	Diet[1]		
Variable	**LFC**	**HFC**	**FO**
Ingredient	g/100 g		
Oil	5	10	10
Safflower oil [2]	0.5	4.0	1.0
Menhaden oil [2]	-	-	9.0
Palm oil [2]	4.5	6.0	-
Casein	20.0	21.2	21.2
DL-Methionine	0.3	0.3	0.3
Corn starch	10.0	10.6	10.6
Maltodextrin	5.0	5.3	5.3
Sucrose	50.0	42.4	42.4
Celluolose	5.0	5.3	5.3
Mineral mix [3]	3.5	3.7	3.7
Vitamin mix [4]	1.0	1.1	1.1
Choline bitartarate	0.2	0.2	0.2
Analysis [5]			
Protein	20	22	22
Carbohydrate	66	59	59
Fat	5	10	10

[1] Diet abbreviations were as follows: **LFC**, low fat control; **HFC**, high fat control; **FO**, fish oil.
[2] The safflower oil contained 78% oleic acid; menhaden oil contained 12.7% EPA and 12.3% DHA and palm oil contained 38% palmitic acid and 42% oleic acid.
[3] Standard salt mix (S10001).
[4] Standard vitamin mix (V10001).
[5] Calculated analysis based on individual ingredients. Diet energy value (kcal/g) were 3.9, 4.1, and 4.1 for LFC, HFC, and FO, respectively

4.2.3 Data and Sample Collection

Dams, pups, and feed were weighed daily between 0930 and 1030 h. After 7 d on the experimental diets, pups were euthanized by CO_2 at 1130 h and stomach milk clots were collected and pooled for each litter, and stored at -20^{0}C for subsequent analysis. Dams were anesthetized with ketamine-zylazine (1 and 0.1 mg/10 g body weight (BW), respectively) and then euthanized by cervical dislocation while under anesthesia. One #4 mammary gland was placed in ice-cold isotonic tris-sucrose buffer (0.25 M sucrose, 30 mM Tris, 1m M glutathione and 1 mM EDTA; pH 7.3) and immediately used to determine lipogenic capacity. The other #4 mammary gland in addition to liver and fat depots (gonadal , mesentric and perirenal) were rapidly dissected, weighed, and snap frozen in liquid nitrogen and then stored at -80 ^{0}C until further analysis.

4.2.4 Sample Analysis.

4.2.4.1 Mammary Lipogenic Capacity Assay

The incorporation of ^{14}C glucose by mammary tissue explants was determined according to Bauman et al. [189]. Briefly, mammary tissue explants were prepared using a Stadie-Riggs hand microtome, and ~150 mg tissue explants were incubated in triplicate in 3 mL of a modified Krebs-Ringer bicarbonate buffer solution (pH 7.4) that also contained 25 mM glucose, 0.5 μCi/ml glucose (D-[^{14}C(U)]; PerkinElmer,Waltham, MA) and 0.1 unit insulin. Vials were gassed with a mixture of O_2:CO_2 (95:5), sealed, and incubated for 3 h at 37° C in a shaking water bath. After the incubation was terminated, tissue was rinsed, saponified, and lipids obtained by petroleum ether extraction [190]. One mL aliquots of the petroleum ether extract together with 10 ml of scintillation fluid (5 g 2,5-diphenyloxazolc per liter toluene) were assayed for radioactivity in a Packard Tri-Carb 2200CA Liquid Scintillation Counter.

4.2.4.2 Fat and FA Profile

Milk clot samples were freeze dried overnight, thoroughly mixed and ~20 mg were analyzed for fat concentration and FA profile according to Harvatine [191]. Briefly, internal standards (triheptadecanoin [C17:0 methyl ester] and trinonadecanoin [C19:0 methyl ester] were added and the milk clot fat was extracted according to Hara and Radin [158]. The fat was methylated overnight at 40^0C in 1% methanolic sulfuric acid and subsequently transmethylated [191]. The methyl esters were then quantified by gas chromatography using a fused-silica capillary column (CP-Sil 88; 100 m x 0.25 mm (i.d.); Varian Inc., Santa Clara, CA) and conditions as described by Perfield et al. [192]. FA peaks on the chromatograms were identified and quantified using pure methyl ester standards (GLC569, GLC60; NuChek Prep, Elysian. MN). A butter reference standard (CBM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was used to validate recoveries and correction factors for individual FA. Milk clot fat concentration was determined based on dilution of the internal standards.

Dietary FAs were extracted according to Hara and Radin [158]. Methylation and gas chromatography analysis was as described for milk clot samples. The FA composition of the diets is presented in Table 4.2 with each diet having a distinct profile related to the oil source used in the diet formulation.

4.2.4.3 RNA Extraction and Real-Time PCR

Total RNA was extracted from ~30 mg of mammary tissue or liver using RNeasy Lipid kit (Qiagen, Valencia, CA). The potential genomic DNA co-extraction with RNA was excluded through on-column DNase treatment (RNase-Free DNase Set; Qiagen). RNA concentration and quality were determined by 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) and samples with RNA Integrity Number (RIN) less than 8 were excluded from downstream reactions. Total RNA was reverse transcribed using High Capacity cDNA Reverse Transcrip-

Table 4.2: **Dietary Fatty Acid Profile**

	Diet[1]		
Fatty acids[2]	**LFC**	**HFC**	**FO**
	g/100 g		
12:0	0.25	0.18	0.35
14:0	3.45	2.85	10.28
16:0	38.13	32.31	19.73
16:1, ω-7	0.44	0.43	11.69
18:0	5.30	4.71	4.60
18:1, ω-9	35.64	45.05	10.88
18:1, ω-7	0.79	0.76	2.76
18:2, ω-6	13.94	11.76	8.88
18:3, ω-3	0.52	0.53	1.59
20:4, ω-6	-	-	1.08
20:4, ω-3	-	-	1.28
20:5, ω-3 , EPA	-	-	10.86
22:5, ω-3	-	-	1.92
22:6, ω-3, DHA	-	-	5.93
Others	1.50	1.42	8.09

[1] Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8).

[2] FA profile of the complete diet which included the supplemented oils.

tion kit (Applied Biosystems, Carlsbad, CA) with random primers. Quantitative real-time PCR (qRT-PCR) assays were developed for genes of interest (Table A.1). Briefly, primers were designed using Primer3Plus [162] to span the exon boundaries when possible and the optimal primers were selected. Quantitative real-time PCR (qRT-PCR) reaction included Power SYBR® Green PCR Master Mix (Applied Biosystems) and 400 nmol/L of gene-specific forward and reverse primers (Invitrogen, Carlsbad, CA). Twenty five ng cDNA was amplified using a 2-step program (95°C for 15 s and 60°C for 60 s) with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The specificity of each primer was verified by the presence of a single product in the dissociation curve at the end of amplification process. A standard curve was derived from serial dilutions of pooled cDNA from mammary or liver tissues. Relative concentrations were expressed in arbitrary units and the logarithms (base 10) of concentrations were plotted against crossing points. The resulting least square fit was used as the standard curve to derive the arbitrary concentration for each sample.

4.2.5 Statistical Analysis.

Data were analyzed using ANOVA model in R statistical package [166]. The average of d 5 and d 6 data was used as co-variant in the analysis of dam feed intake and BW gain. A normalization factor [193] was generated from three house keeping gene (*18S*, *B2M* and ribosomal protein S20 (*RPS20*)) and used as co-variate in the analysis of gene expression data. Co-variates were removed from the model when $P > 0.3$. Data points with studentized residuals outside the range of 2.5 to -2.5 were considered outliers and excluded from analysis. Few points were excluded in analysis and rarely more than one per response variable.

4.3 Results

4.3.1 Performance-Related Variables

The effect of dietary treatment on performance parameters of the experimental animals is shown in Table 4.3. Feed intake was similar for dams receiving the LFC and HFC diets, but the FO supplemented group had a lower total ($P < 0.001$) and average daily ($P = 0.01$) feed intake. There were no significant treatment effects on lactating dam BW at the end of the study ($P = 0.26$) or on BW gain ($P = 0.22$). Similarly, there were no significant treatment effects on litter growth parameters in terms of final litter weight ($P = 0.70$) or weight gain ($P = 0.59$) during the 7-d experimental period.

4.3.2 Fat Deposition

In general, the three adipose tissue depots that were quantified followed a similar pattern for treatment effects. Weights for the gonadal, mesentric and perirenal fat depots were lowest in the LFC group, intermediate for the HFC group and highest for the FO group (Table 4.3). Combining these depots, the dissected fat mass was 32% and 14% greater for the FO treatment as compared to the LFC and HFC, respectively.

4.3.3 Milk Clot FA Composition

Treatment effects on milk clot FA profile are shown in Table 4.4. In the present study there was a distinct treatment effect on the milk fat concentration of FAs that reflected the unique FA profile for each diet (Table 4.2). Milk fat from the HFC group had an elevated oleic acid content consistent with that diet having a greater oleic acid content. Likewise, EPA and DHA were found almost exclusively in the milk fat from the FO group ($P < 0.001$), and this was accompanied by higher levels of palmitoleic acid ($P < 0.001$) and lower oleic acid ($P < 0.001$) compared with the other groups.

Table 4.3: **Treatment Effect on Dam Parameters and Litter Weight**

		Treatment[1]			
Variable		**LFC**	**HFC**	**FO**	*P* Value
Weight	Final (g)	27.0±0.5	28.0±0.4	27.8±0.4	0.26
	Gain[2] (g)	0.6±0.5	1.8±0.5	1.4±0.5	0.22
Feed Intake	Total (g)	63.0±1.7^{a}	60.0±1.6^{a}	51.0±1.7^{b}	<0.001
	Daily (g)	8.9±0.2^{a}	8.6±0.2^{ab}	7.7±0.3^{b}	0.01
Liver	Weight (g)	2.36±0.08^{a}	2.20±0.07^{a}	1.79±0.07^{b}	<0.001
	Lipid (mg/g)	38.0±2.2^{a}	34.1±2.6^{a}	18.3±1.06^{b}	<0.001
Fat depot	Gonadal (g)	0.24±0.02^{b}	0.29±0.02^{ab}	0.34±0.02^{a}	0.01
	Mesentric (g)	0.39±0.03^{b}	0.42±0.02^{ab}	0.47±0.03^{a}	0.12
	Perirenal (g)	0.05±0.01^{b}	0.07±0.01^{a}	0.08±0.01^{a}	0.02
Litter	Final weight (g)	39.3±0.6	39.6±0.6	38.9±0.6	0.70
	Weight gain[2] (g)	19.7±0.6	19.9±0.6	19.1±0.6	0.59

[1] Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). On d 13, dams and their litter were weighed and then euthanized where liver and different fat depots were dissected and weighed. Values represent LS mean ± SE.

[2] Dams' and litters' weight gain represent the difference between d 6 and d 13 weight records.

[ab] Values with different letters differ statistically with *P* values as indicated.

Of particular interest was the effect of treatment on de novo synthesized fatty acids (DS-FAs). Comparisons of these FAs indicate there were no treatment effects on proportion of short and medium chain FA (<C16; $P = 0.21$), although the concentration of C:14 and C:14.1 was slightly lower in milk fat from the HFC treatment group. Moreover, the HFC treatment had a higher proportion of preformed FAs (> C16) compared with both LFC and FO treatment ($P = 0.04$).

4.3.4 Mammary Lipogenic Capacity and Gene Expression

Mammary tissue lipogenic capacity was determined by measuring the incorporation of radio-labeled glucose into lipids. Observed rates were similar to those reported previously for lactating rodents [190]. Of special interest, we found no dietary treatment effects on the lipogenic capacity of the mammary explants (Figure 4.1).

Treatment effects on mammary lipogenic gene expression are presented in Table 4.5. There was no significant treatment effect on mRNA abundance for acetyl-coenzyme A carboxylase alpha (*ACACA*), fatty acid synthase (*FASN*) and lipoprotein lipase (*LPL*), key enzymes in milk fat synthesis. Likewise, there was no effect of dietary treatment on mRNA abundance for transcription signaling genes associated with the regulation of lipid synthesis including the sterol regulatory element binding transcription factor 1-c (*SREBF1-c*), thyroid hormone responsive spot 14 (*S14*), insulin induced gene 1 (*INSIG1*), insulin induced gene 2 (*INSIG2*), SREBF chaperone (*SCAP*) or membrane-bound transcription factor peptidase, site 1 (*MBTPS1*) (Table 4.5). However, the FO diet did result in a significant reduction in mammary mRNA abundance for stearoyl-coenzyme A desaturase 1 (*SCD1*) and diacylglycerol O-acyltransferase 1 (*DGAT1*).

4.3.5 Liver Weight and Hepatic Gene Expression

The FO treatment resulted in dams with livers that were smaller and contained less fat. Liver weights for dams on the FO treatment were 19 to 24% lower compared with LFC and HFC

Table 4.4: **Treatment Effect on Milk Clot FA Profile**

	Treatment[1]			
	LFC	**HFC**	**FO**	*P* Value
FA (%)[2]				
10:0	2.51±0.21	2.69±0.29	3.00±0.29	0.50
12:0	10.66±0.37	10.01±0.34	9.69±0.34	0.17
14:0	15.85±0.49^{a}	14.03±0.64^{b}	15.90±0.46^{a}	0.02
14:1	0.17±0.01^{a}	0.13±0.01^{b}	0.18±0.01^{a}	<0.01
16:1, ω-7	2.44±0.11^{b}	1.64±0.10^{c}	4.97±0.10^{a}	<0.001
18:0	2.29±0.05	2.18±0.05	2.14±0.05	0.96
18:1, ω-9	22.91±0.91^{b}	28.62±0.77^{a}	12.77±0.77^{c}	<0.001
18:2, ω-6	2.79±0.15^{c}	3.40±0.14^{b}	4.30±0.15^{a}	<0.001
20:5, ω-3, EPA	0.07±0.11^{b}	0.09±0.10^{b}	3.08±0.11^{a}	<0.001
22:6, ω-3, DHA	0.04±0.01^{b}	0.06±0.01^{b}	3.34±0.21^{a}	<0.001
Total by source (%)[3]				
<16 carbons	29.20±1.00	26.86±0.94	28.77±0.94	0.21
16:0	29.41±0.54^{a}	26.71±0.50^{b}	25.96±0.56^{b}	<0.01
>16 carbons	37.33±1.39^{b}	42.16±1.19^{a}	38.47±1.19^{b}	0.06

[1] Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). On d 13, pups were euthanized and stomach milk clots were collected and pooled for each litter.

[2] Calculated as % of total FA. Values represent LS means ± SE.

[3] FA <16 carbons originate from *de novo* synthesis, FA >16 carbons originate from extraction from plasma, and 16 carbon FA originate from both extraction from plasma and de novo synthesis.

[ab] Values with different letters differ statistically at the indicated *P* values.

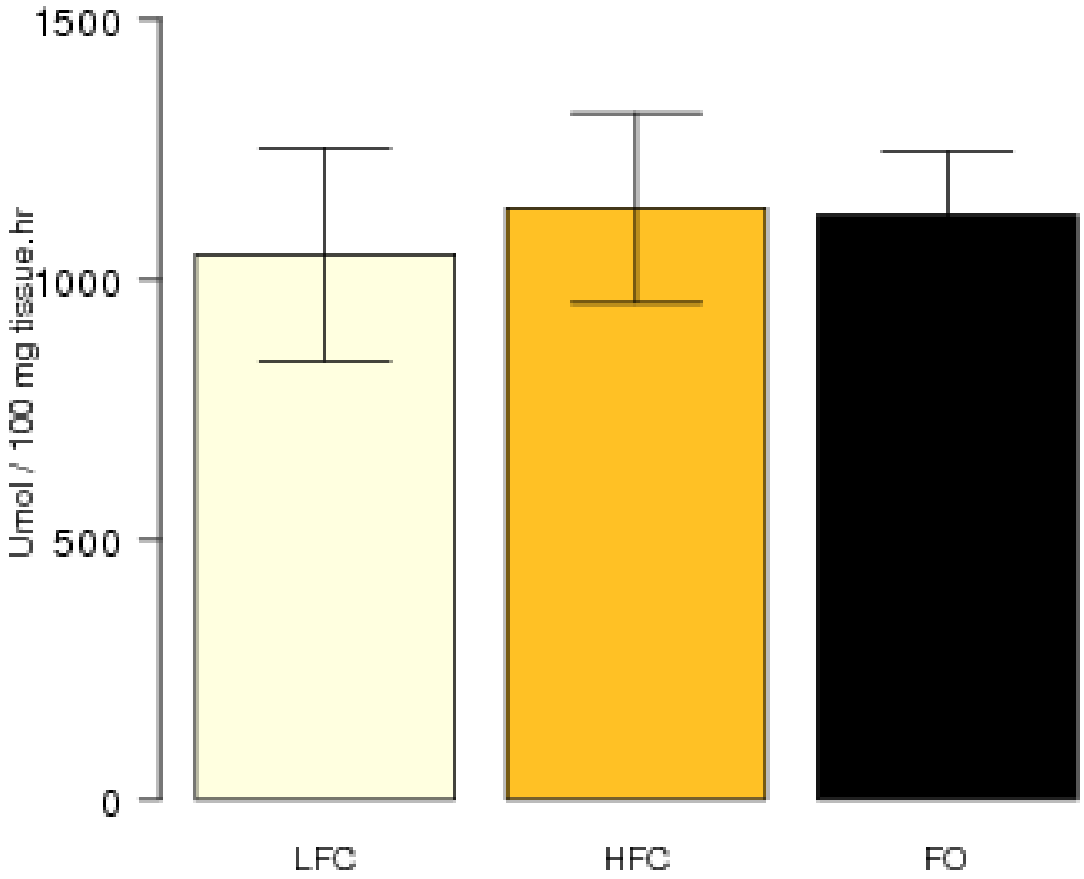

Figure 4.1: **Treatment Effect on ^{14}C Glucose Incorporation Rate by Mammary Explants**. Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). Mammary tissue explants were prepared and ~150 mg tissue explants were incubated in triplicate in 3 mL of a modified Krebs-Ringer bicarbonate buffer solution (pH 7.4) that also contained 25 mM glucose, 0.5 μCi/ml ^{14}C glucose and 0.1 unit insulin. Vials were gassed with a mixture of O_2:CO_2 (95:5), sealed, and incubated for 3 h at 37 °C in a shaking water bath. Each bar represents the mean ± SE (n = 8) expressed as μmole of glucose incroporated into lipids per 100 mg tissue.hr.

Table 4.5: **Treatment Effect on mRNA Abundance of Mammary Genes**[1]

		Treatment			
		LFC	**HFC**	**FO**	*P* Value
Lipid Synthesis	*ACACA*	1.00±0.24	1.03±0.21	0.81±0.20	0.53
	FASN	1.00±0.34	1.15±0.35	0.65±0.22	0.25
	DGAT1	1.00±0.07^{a}	0.71±0.06^{b}	0.65±0.06^{b}	<0.001
	LPL	1.00±0.13	1.20±0.14	0.98±0.21	0.54
	SCD1	1.00±0.09^{a}	0.81±0.08^{a}	0.49±0.07^{b}	<0.001
	S14	1.00±0.19	1.13±0.21	0.97±0.19	0.70
SREBF related	*SREBF1-c*	1.00±0.28	0.96±0.28	0.71±0.24	0.55
	SCAP	1.00±0.19	1.06±0.19	1.19±0.21	0.59
	INSIG1	1.00±0.21	1.04±0.22	0.93±0.21	0.87
	INSIG2	1.00±0.11	0.90±0.10	1.02±0.11	0.56
	MBTPS1	1.00±0.11	1.02±0.10	0.87±0.10	0.31

[1] Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). The fourth abdominal mammary gland was collected at d 13 and total RNA was analyzed by real time PCR for the mRNA abundance of indicated genes. Gene abbreviations are: ***ACACA*** = acetyl-coenzyme A carboxylase alpha, ***FASN*** = fatty acid synthase, ***DGAT1*** = diacylglycerol O-acyltransferase 1, ***LPL*** = Lipoprotein lipase, ***SCD1*** = stearoyl - coenzyme A desaturase, ***S14*** = thyroid hormone responsive, ***SREBF1-c*** = sterol regulatory element binding transcription factor 1-c, ***SCAP***= SREBF chaperone, ***INSIG1*** = insulin induced gene 1, ***INSIG2*** = insulin induced gene 2, ***MBTPS1*** = membrane-bound transcription factor peptidase, site 1. Values represent LS means expressed relative to LFC diet ± SE (n = 6).

(P<0.001, Table 4.3). This was accompanied by a concomitant reduction in hepatic lipid concentration by approximately 52% and 35% when compared with the LFC and the HFC, respectively (Table 4.3).

Hepatic expression of several lipogenic genes was reduced; mRNA abundance for *FASN*, *ACACA*, glycerol-3-phosphate acyltransferase (*GPAM*) and *SCD1* were lower by 50 to 80% as compared to the LFC and HFC treatments ($P < 0.001$, Figure 4.2). Among genes related to the regulation of the transcription of lipogenic genes, there was no treatment difference in the mRNA abundance for *SREBF1-c* ($P = 0.39$); however, the mRNA abundance for *S14* and *INSIG1* was 60 - 75% lower for hepatic tissue from FO group as compared to the lactating dams on the LFC and the HFC treatments (Figure 4.2 and 4.3; $P < 0.001$). The mRNA abundance for *SCAP* also tended to be lower (32%; $P = 0.1$) for the FO group. On the other hand, there was no treatment effect on the mRNA abundance of genes involved in FA oxidation including peroxisome proliferator-activated receptor alpha (*PPARa*), carnitine palmitoyltransferase 1a (*CPT1a*), acyl-Coenzyme A oxidase 1, palmitoyl (*ACOX1*), fatty acid translocase (*CD36*) and fatty acid binding protein 1 (*FABP1*) (Figure 4.4).

4.4 Discussion

Dietary PUFAs, particularly the LC ω-3 PUFAs, have several unique metabolic effects including effects on lipid metabolism. These effects have been most extensively investigated in the liver of rodents where the LC ω-3 PUFAs have been shown to suppress hepatic lipogenesis and TG synthesis while inducing peroximal and microsomal FA oxidation [63, 182–184, 186, 194]. Although liver is considered to be central body regulator of lipid metabolism, the relative contribution of the lipogenic tissues to total FA synthesis depends on the species and the physiological status of the animal. For example, liver and adipose tissue contribute about equally to total FA synthesis in the virgin mouse, but by mid-lactation the mammary gland accounts for approximately 75% of the total rate of FA synthesis [20]. Moreover, in

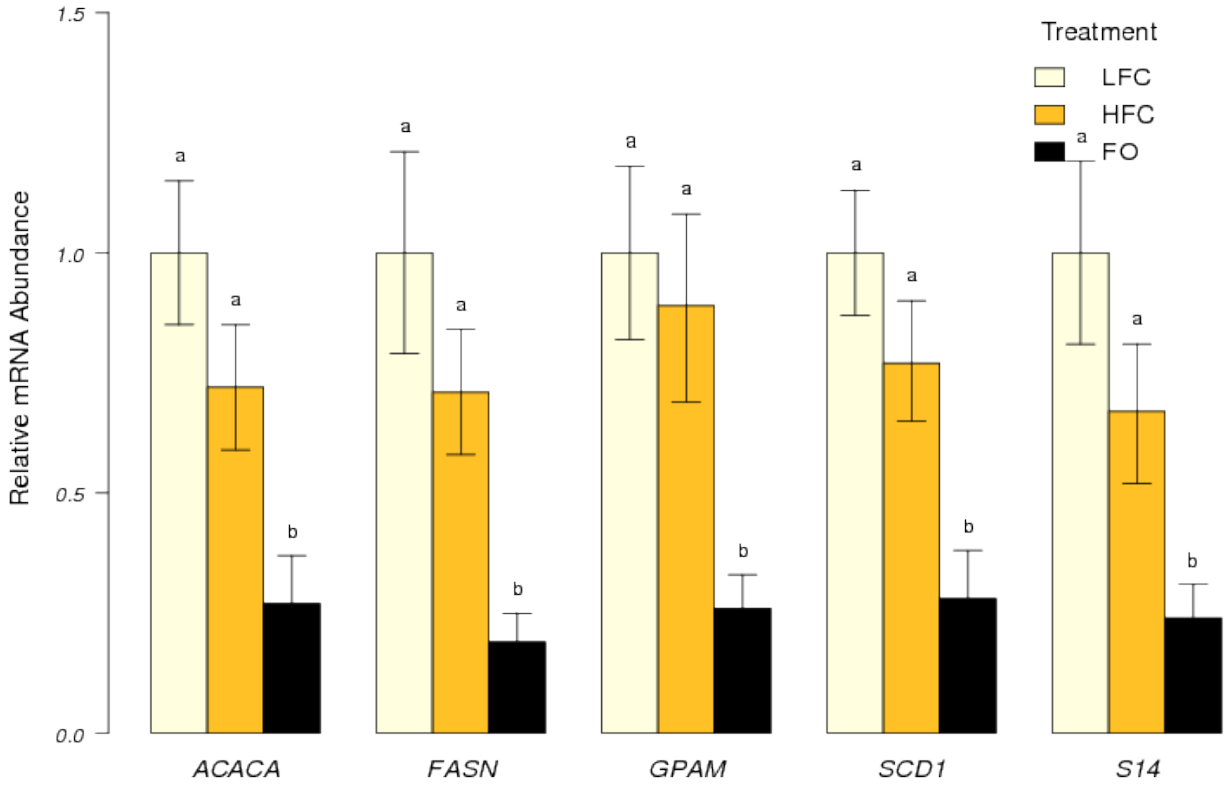

Figure 4.2: **Treatment Effect on Expression of Genes Involved in Hepatic Lipid Synthesis**. Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). Liver samples were collected at d 13 and total RNA was analyzed by real time PCR for the mRNA abundance. Gene abbreviations are: ***ACACA*** = acetyl-Coenzyme A carboxylase alpha, ***FASN*** = fatty acid synthase, ***SCD1*** = stearoyl-coenzyme A desaturase, ***GPAM*** = glycerol-3-phosphate acyltransferase, mitochondrial, and ***S14*** = thyroid hormone responsive spot 14. Each bar represents the mean ± SE (n = 6). Significant statistical differences are indicated by different letters among treatment for a specific gene; $P < 0.01$.

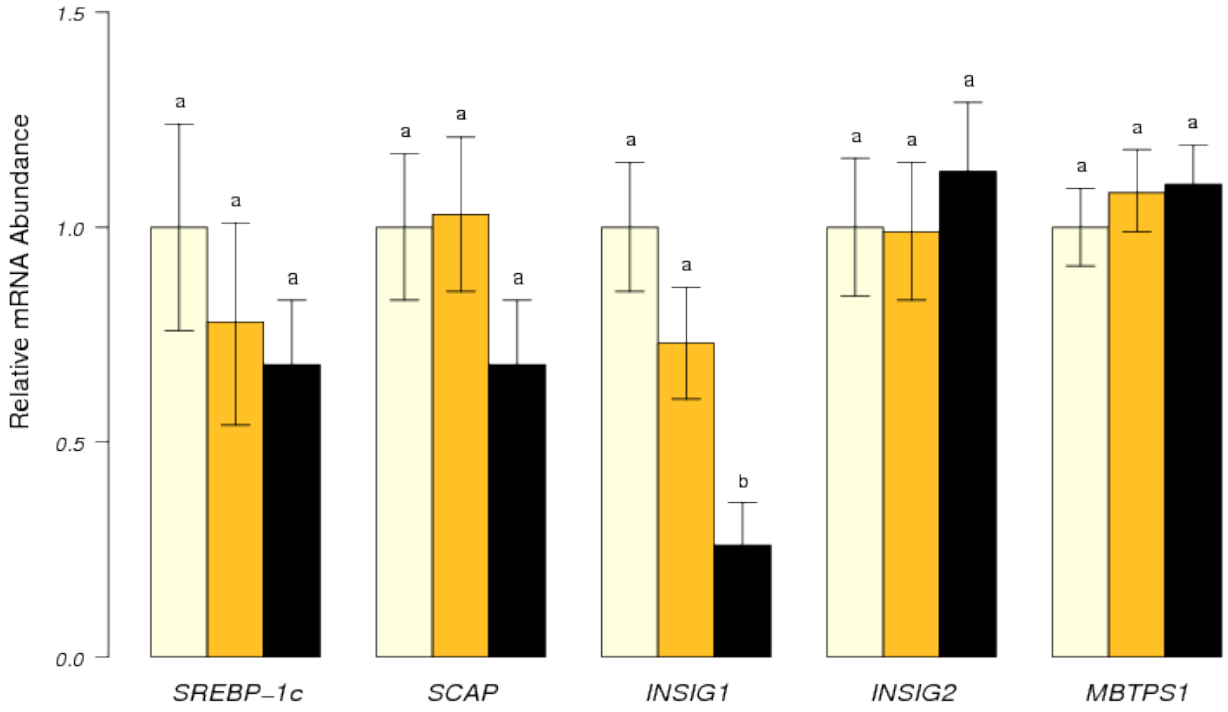

Figure 4.3: **Treatment Effect on Expression of Genes Involved in Transcription of Hepatic Lipogenic Genes**. Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). Liver samples were collected at d 13 and total RNA was analyzed by real time PCR for the mRNA abundance. Gene abbreviations are: ***SREBF1-c*** = sterol regulatory element binding transcription factor 1-c, ***SCAP*** = SREBF chaperone, ***INSIG1*** = insulin induced gene 1, ***INSIG2***= insulin induced gene 2, and ***MBTPS1*** = membrane-bound transcription factor peptidase, site 1. Each bar represents the mean ± SE (n = 6). Significant statistical differences are indicated by different letters among treatment for a specific gene with *P* values as follows: *SREBF1-c* = 0.39 , *SCAP* = 0.1, *INSIG1* = <0.001, *INSIG2* = 0.62, *MBTPS1* = 0.62.

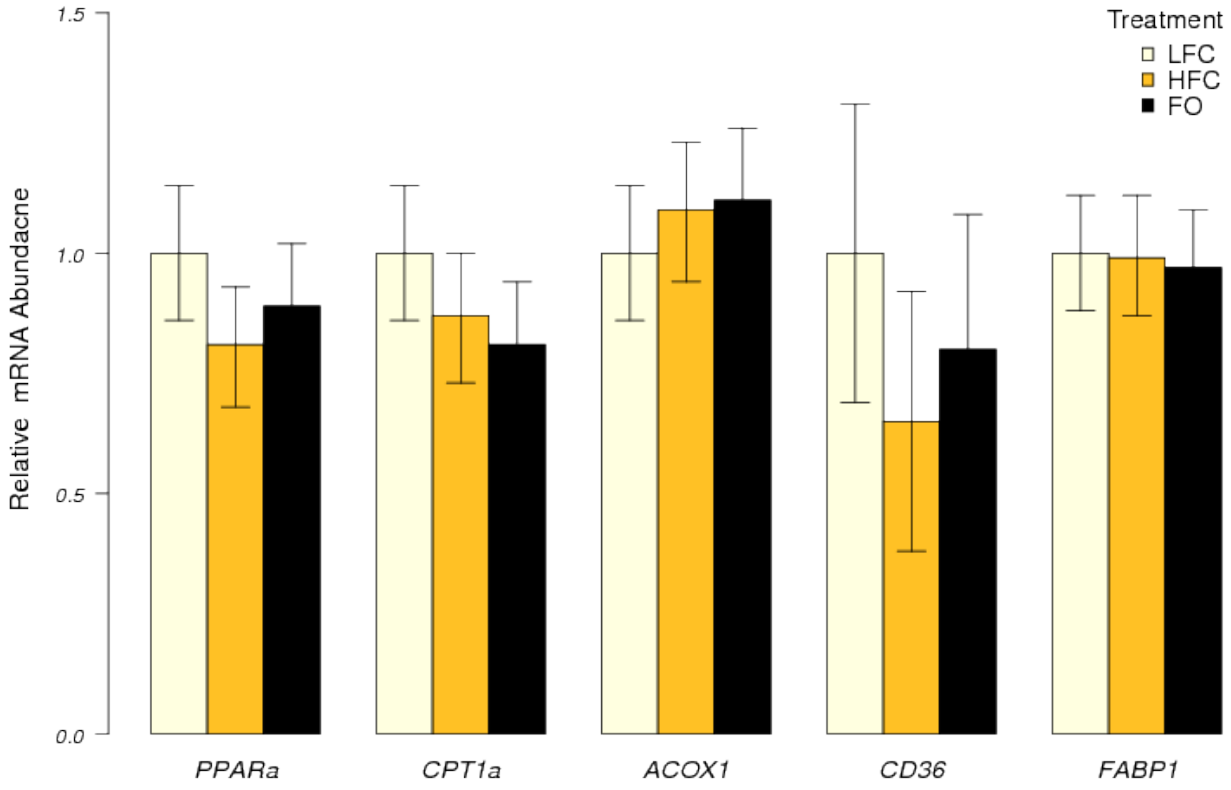

Figure 4.4: **Treatment Effect on Expression of Genes Involved in Hepatic FA Oxidation**. Female C57BL/6J mice received either semipurified, low fat control diet containing 0.5% safflower oil and 4.5% palm oil (**LFC**); high fat control oleic acid enriched diet containing 4% safflower oil and 6% palm oil (**HFC**); or fish oil enriched diet containing 1% safflower oil and 9% menhaden oil (**FO**). Diets were fed from d 6 to d 13 of lactation (n = 8). Liver samples were collected at d 13 and total RNA was analyzed by real time PCR for the mRNA abundance. Gene abbreviations are: ***PPARa*** = peroxisome proliferator activated receptor alpha, ***CPT1a*** = carnitine palmitoyltransferase 1a, ***ACOX1*** = acyl-Coenzyme A oxidase 1, palmitoyl, ***CD36*** = fatty acid translocase, and ***FABP1*** = fatty acid binding protein 1. Each bar represents the mean ± SE (n = 6). There were no significant statistical differences; $P > 0.4$.

lactating mice the mammary gland is capable of synthesizing an amount of TG equivalent to the entire weight of the animal in a 20-d lactation cycle causing Rudolph et al. [195] to refer to the mammary gland as a "lipid synthesizing machine". However, little attention has been focused on the effects of dietary LC ω-3 PUFAs on mammary lipogenesis, although milk fat synthesis is known to be regulated by other bioactive FA [6]. Therefore, the objective of the current experiment was to investigate the effect of dietary LC ω-3 PUFAs on lipogenesis in the mammary gland of lactating mice. To achieve this goal, a FO supplement was used as a dietary source of LC ω-3 PUFAs and we determined its effect on the proportion of milk FA synthesized within the gland, the in vitro mammary lipogenic capacity of the mammary gland and the mRNA abundance of lipogenic genes in the mammary gland. Therefore, lactating mice were fed a diet containing 10% total oil and compared the FO diet as source of LC ω-3 PUFAs with a control diet consisting of a vegetable oil blend enriched in oleic acid. Because mammary lipogenesis might be naturally depressed by high dietary oil level, we also included a third treatment group where mice were fed a low fat diet (5% vegetable oil blend).

In the current study, the lipogenic capacity of mammary explants was measured and results demonstrated that rates of ^{14}C glucose incorporation into lipids were similar among treatment groups ($P = 0.97$). To confirm these results, the FA composition of the milk fat was analyzed to determine the contribution of DSFAs. Milk short-chain (4 to 8 carbons) and MCFAs (10 to 14 carbons) are generally not supplied by the diet so these arise almost exclusively by de novo synthesis from glucose within the mammary epithelial cell as a result of tissue-specific modification of the universal FASN reaction [14]. Consequently their proportion in milk can be taken as a proxy for the rate of mammary de novo lipid synthesis compared to other sources [132]. Comparison of the FA profile showed no differences among treatments in the proportion of FA less than C16 ($P = 0.21$). Fat is the major energy component of milk and the growth rate of the nursing pups is correlated with milk fat production [196–198]; we observed no difference among dietary treatments for litter weight ($P = 0.70$) or weight gain

(P = 0.59) during the 7-d experimental period. Others have also observed that FO supplementation to lactating rodents had no negative effect on litter growth rates [65, 68]. Taken together the results in the current study suggest neither the rates of mammary de novo lipid synthesis nor milk production parameters were affected by dietary FO supplementation.

The effect of LC ω-3 PUFAs on lipogenic genes has not been examined previously for lactating mammary tissue, but in hepatic tissue the mechanism involves effects on the SREBF system with corresponding decreases in the mRNA abundance and the activity of lipogenic genes [63,185,186,199]. In the present study we observed that the gene expression profile for lactating mammary tissue showed no treatment effect for the mRNA abundance of *ACACA* (P = 0.53) and *FASN* (P = 0.25), two key enzymes in the FA synthesis pathway. Moreover, *DGAT1* mRNA abundance did not differ between FO and HFC group. Thus, there was no evidence that dietary supply of LC ω-3 PUFAs resulted in downregulation of the expression of lipogenic genes, and these results are consistent with the absence of dietary effects on the growth rates of the nursing pups, the in vitro lipogenic capacity of mammary explants and the milk fat profile of de novo synthesized FA. Nevertheless, the LC ω-3 PUFAs supplied by the FO diet were taken up and utilized by the mammary epithelial cells as evident by increases in the milk fat content of EPA and DHA.

The LC ω-3 PUFAs in FO have been well established as negative regulators of hepatic FA biosynthesis in non-lactating rodents. Investigations have consistently shown effects of FO or EPA and DHA on the SREBF system and corresponding decreases in mRNA abundance and activity for key lipogenic enzymes [63, 185, 186, 199]. In the present study there were distinct treatment effects on lipid metabolism in the liver from the lactating dams which differed markedly from the results with mammary tissue. FO treatment decreased liver weight by about 20 to 25% compared to the other two dietary groups ($P < 0.001$) and this decrease coincided with an approximate 50% reduction in hepatic lipid concentration in the FO-treated mice ($P < 0.001$). FO supplementation has been reported previously to decrease hepatic lipid deposition and that was attributed to several mechanisms including downregulation of de

novo lipid synthesis and/or enhanced FA oxidation [63, 183, 185, 186, 194]. In the present study the reduction in hepatic fat deposition paralleled a significant decrease in mRNA levels of the key enzymes in the de novo FA synthesis pathway. The mRNA abundance for *ACACA*, *FASN* and *GPAM* as well as *SCD1* and *S14* were decreased in the FO treatment group by 70% to 80% and 50% to 70% compared to LFC and HFC groups, respectively.

The LC ω-3 PUFAs also enhance hepatic FA oxidation in non-lactating rodents and this coincided with increases in the mRNA abundance for *PPARa* and genes involved in FA trafficking and oxidation in mitochondria and peroxisomes [182–184, 186, 187, 200]. However, alterations in FA oxidation do not appear to play a significant role in the hepatic lipid attenuating effect of FO we observed for lactating mice. The mRNA abundance of *CPT1a* and *ACOX1*, key enzymes in the mitochondrial and peroxisomal β-oxidation, respectively, was unaffected by the FO diet. Likewise the mRNA levels for *CD36* and *FABP1*, the FA transporter and trafficking proteins in liver, did not differ among dietary treatments. These genes are regulated primarily by the PPARa nuclear receptor which functions as a hepatic lipid sensor responding to the influx of FA by stimulating the transcription of PPARa-regulated genes [201] and we observed no effect of the FO diet on *PPARa* mRNA abundance. For a complete evaluation, quantitation of protein levels and enzyme and transcription factor activity data would be required; nevertheless, in the current study the absence of a FO effect on the expression level of *PPARa* and its regulated genes in the liver from lactating mice suggests that the *PPARa* and the FA oxidation pathway were not activated.

Overal, the downregulation of hepatic lipogenesis, but not an enhanced FA oxidation, appears to be the determining factor in the lipid ameliorating effect of FO supplementation in the current study. In an effort to outline the probable mechanism of LC ω-3 PUFAs on hepatic lipid metabolism, Harris and Bulchandani [182] evaluated 42 rat studies where the effects of LC ω-3 PUFAs (FO, EPA or DHA individually) were tested on hepatic lipid metabolism. The clearest signal from the reviewed studies was that LC ω-3 PUFAs consistently downregulated hepatic lipogenesis; lipogenesis was inhibited every time ω-3 PUFAs

were fed to rats. Stimulation of β-oxidation may also be relevant but only 33 out of 47 experiments found it to be increased; thus the inconsistency in these findings call this particular pathway into question. In agreement with the present study, Tanaka et al. [186] reported that EPA treatment markedly attenuated liver TG level in mice and significantly decreased the mature SREBF1 levels and lipogenic gene expression; however, there were no effects on hepatic peroxisome proliferation or mitochondrial β-oxidation. Also, Ukropec et al. [202] reported that the enzyme activity of CPT-1a was not effected by ω-3 PUFAs supplementation. Moreover, Arai et al. [187] found no effect of menhaden oil or tuna oil on expression level of two PPARa target genes, *ACOX1* and UCP2. However, these results contradict other reports of significant upregulation of the mitochondrial and peroxisomal palmitoyl-CoA oxidation rates and activity levels of various FA oxidation enzymes in rats fed FO or purified EPA and DHA [183, 184, 200].

The lipogenic enzymes are known to be regulated primarily at the transcription level under the control of SREBF1-c, the master regulator of lipid homeostasis [203], and their activities are strongly correlated with mRNA abundance [183]. SREBF1-c belongs to the SREBFs family of membrane-bound transcription factors that regulate enzymes responsible for cholesterol, FA, and TG synthesis [104]. We examined the effect of dietary treatment on mRNA of *SREBF1-c* and interestingly the expression was similar among treatments in both mammary (P = 0.55) and liver (P = 0.43). Nevertheless, there was clear downregulation in the mRNA abundance of SREBF-1c target genes in the liver of the FO group. SREBFs are synthesized as precursors bound to the endoplasmic reticulum (ER) and nuclear envelope; upon activation, SREBFs are released from the membrane and travel to the golgi where a mature protein is released by a sequential two-step cleavage process [203]. This mature SREBF then enters the nucleus where it binds to the sterol regulatory elements [204, 205] in the promoter region of target genes thereby increasing their expression levels. Therefore, results in the present study are consistent with FO regulating the SREBF1-c expression posttranslationally by decreasing the amount of its active nuclear form. Indeed, Yahagi et al. [104]

demonstrated that feeding mice FO (sardine oil, rich in EPA, or tuna oil, rich in DHA) for 7 d caused a significant decrease in the amount of hepatic nuclear SREBF (nSREBF1) protein compared with feeding either saturated (tristearin) or monounsaturated (triolein) fat. Interestingly, they observed no effect on either *SREBF1-c* mRNA or membrane-bound precursor protein levels. Thus, Yahagi et al. [104] and Tanaka et al. [186] concluded that LC ω-3 PUFAs appear to regulate abundance of nSREBF protein mainly at a post-translational level.

To test the hypothesis that LC ω-3 PUFAs regulates the nSREBF1-c abundance, we determined the mRNA level of key genes in the SREBF processing pathway. INSIG1 and INSIG2 are membrane proteins of the ER that play a central role in the feedback control of lipid synthesis in animal cells. INSIG's regulate lipid synthesis by binding to SCAP, an escort protein required for the cleavage and activation of the SREBF family. Binding of INSIG's to SCAP leads to ER retention of the SCAP and SREBFs complexes, preventing the proteolytic generation of the transcriptionally active nSREBFs, thereby limiting transcription of SREBF target genes [206]. In the present study, there was no treatment effect on *INSIG2* mRNA in either liver (P = 0.62) or mammary tissue (P = 0.56). However, with FO treatment hepatic level of *INSIG1* mRNA abundance decreased by almost 75% and 65% compared to LFC and HFC respectively ($P < 0.001$). A similar effect of LC ω-3 PUFAs on hepatic expression of *INSIG1* has been reported previously [186, 187]. In contrast, there was no effect on *INSIG1* in the mammary gland (P = 0.87). Theoretically the reduction in hepatic *INSIG1* would not be expected given the strong downregulation in the SREBF1-c target genes in the liver which is believed to be due to reduced nSREBF1-c level. In a study done by Engelking et al. [207], nSREBF1 levels increased by 2-fold and the hepatic content of total cholesterol and TG increased by 4- and 6- fold, respectively, in liver of *INSIG1* and *INSIG2* double knockout mice compared with the control. Moreover, in the liver of transgenic mice over-expressing human *INSIG1* the content of all nSREBFs was reduced with a marked reduction in the levels of mRNAs encoding enzymes required for synthesis of cholesterol, FA, and TG [206]. However, *INSIG1* itself was identified as a SREBF target gene using micro-array analysis of mRNA from the

mouse liver [208] whereas *INSIG2* was not [208,209]. *INSIG1* mRNA is expressed at high levels when nSREBF levels are high as a result of sterol deprivation and the expression declines drastically when cells are overloaded with sterols [209]. This provides a feedback mechanism to decrease or increase the SREBF cleavage and hence the nSREBF abundance. Therefore, the downregulation of in the current study might provide a feedback mechanism to increase SREBF cleavage and hence can be seen within the context of the reduced abundance effect of nSREBF1-c which add support to FO downregulating effect on the nSREBF1-c and its target genes in the liver.

In conclusion, the present study demonstrates that dietary FO supplementation had no effect on different mammary lipogenic parameters; mammary lipogenic capacity, level of DSFAs and mammary lipogenic gene expression were not significantly affected by FO treatment. In contrast, the FO effect on hepatic lipogenesis was obvious and resulted in impressive decrease in hepatic lipid accumulation and downregulation of lipogenic gene expression. Our investigation is among the first to examine this at the cellular level in lactating animals but others have observed the same downregulation of lipogenesis in liver of growing rodents fed a diet high in LC ω-3 PUFAs. Thus, the present study highlights the importance of the tissue specific effects of LC ω-3 PUFAs on lipogenic pathway. The exact mechanism behind the differential effects of LC ω-3 PUFAs remains to be established.

Chapter 5

CLA Effect on Gene Expression of CLA-Induced MFD in Lactating Ewe

5.1 Introduction

Conjugated linoleic acid (CLA) is a generic term used to describe positional and geometric isomers of linoleic acid. A number of CLA isomers are naturally produced by rumen bacteria as intermediates in the biohydrogenation of dietary polyunsaturated fatty acids (PUFA), with *cis*-9, *trans*-11 CLA being the predominant isomer found in ruminant-sourced foods [32]. CLA isomers also originate from industrial hydrogenation and *cis*-9, *trans*-11 and *trans*-10, *cis*-12 are the two isomers that have been most extensively studied [4]. Research over the last decade has established the CLAs as unusual bioactive fatty acids (FAs) that exert a range of biological effects in different tissues and species including antiobesity, anticarcinogenic, antidiabetigenic, and antiatherogenic effects [4,48]. Baumgard et al. [135] were the first to demonstrate that *trans*-10, *cis*-12 CLA resulted in a reduction in milk fat synthesis in lactating dairy cows, and this discovery provided a basis to explain the cause of diet-induced milk fat depression (MFD) , a syndrome in lactating cows that had perplexed dairy producers and scientists for over a century [132].

The molecular mechanism behind CLA-induced MFD is not completely resolved; however, the phenotypic characterization provides key insight into the functional mechanism.

In CLA-induced MFD in dairy cows, fat is the only milk component inhibited with *trans*-10, *cis*-12 CLA treatment. Furthermore, the reduction in milk fat secretion involves FAs of all chain lengths, but effects are particularly pronounced for de novo synthesized fatty acids (DSFAs) [6]. Although few studies have examined the effects of CLA on mammary lipid metabolism at the cellular level, results have clearly shown a coordinated downregulation in transcript abundance and/or enzymatic activity for lipogenic enzymes involved in the uptake, de novo synthesis, desaturation, and esterification of FAs in the mammary gland of lactating cows [9, 136, 210, 211] and laboratory animals [212–215]. Molecular mechanisms mediating the inhibitory effect of *trans*-10, *cis*-12 CLA on mammary lipogenesis have not been extensively investigated, but results support a central role for sterol regulatory element binding transcription factor (SREBF) family [9, 143, 210].

CLA-induced MFD has also been observed in small ruminants including sheep [12, 216] and goats [217, 218]. Although ruminants share similarities in many aspects, distinct differences exist related to ruminal lipid metabolism and the relative sensitivity of mammary lipogenic processes [219], and this might modify the mammary response to CLA treatment. For example, in lactating goats diet-induced MFD resulted in 18 to 32% reduction in the DSFAs with effects being independent of mammary expression or activity of acetyl-coenzyme A carboxylase alpha (ACACA) and fatty acid synthase (FASN) [220–222].

Dairy ewes may represent a good model to examine the mechanism of CLA-induced MFD. They are relatively available, cost effective, manageable in size, and daily milking allows a quantitative evaluation of treatment effects on milk fat yield and FA composition. Furthermore, the relationship between *trans*-10, *cis*-12 CLA dose and the reduction in milk fat output is similar to cows when dose is expressed on a metabolic body weight (BW) basis [12, 216]. To date, the molecular basis for MFD, whether induced by diet or CLA supplementation, has not been investigated in lactating ewes. Therefore, the objective of the current study was to investigate the molecular mechanism mediating MFD in lactating ewes fed a CLA supplement containing *trans*-10, *cis*-12 CLA isomer. For this purpose, we used tissue

samples obtained from lactating ewes that were fed a rumen-protected CLA supplement for 10 wk. A companion paper reports results for CLA effects on performance, organ weight and carcass composition [223].

5.2 Material and Methods

5.2.1 Animals and Treatments

All experimental procedures involving lactating ewes were conducted at Harper Adams University College, (Edgemond, UK) in accordance with the UK Animals (Scientific Procedures) Act 1986. Detailed procedures are reported in the companion publication [223]. Briefly, at d 16 ± 1.6 (mean ± SE) postpartum multiparous Friesland and British Milk Sheep ewes were randomly allocated to two treatments (8/treatment) based on their breed, milk yield and milk fat yield as measured in the previous 7 d, BW and body condition score. Ewes were milked twice daily and fed a basal ration (0.55 : 0.45 concentrates to forage ratio, dry matter basis) that was composed mainly of hay, rolled barley, and dried molassed sugarbeet feed [223]. The dietary metabolizable energy and crude protein averaged (per kg DM) 10.9 Mj and 156 g, respectively, with fresh feed offered once per day at 1.05x ad libitum intake.

Treatments for the present study involved diets that were supplemented with CLA at two levels: no CLA (Control; CON) or 15 g/d of CLA supplement (+CLA). The CLA was provided by a lipid-encapsulated CLA supplement that contained two CLA isomers in equal proportions, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 (Lutrell®; BASF SE, Ludwigshafen, Germany). The CLA supplement provided 1.5 g/d of *trans*-10, *cis*-12 and an equal amount of *cis*-9, *trans*-11 CLA. Ewes received the two experimental treatments throughout a 10 wk period in a randomized block design. In the companion publication there was a third treatment (40 g/d CLA supplement) which was not included in the present study. With the exception of milk fat, the phenotype between the CON and +CLA treatments was comparable after 10 wk of treatment, whereas the 40 g/d CLA group differed in milk yield, milk protein yield

and BW change.

At the end of the experimental period, ewes were slaughtered over a 72 h period by stunning and exsanguination. Subsamples of mammary secretory tissues from the left side of the mammary gland were immediately dissected and cubes were prepared. The cubes (≤0.5 cm) were immediately placed in a 15 ml disposable sample tube and immersed in 5 ml of RNAlater solution (RNAlater tissue collection: RNA stabilization solution, Ambion, Inc, USA). After sample tubes were stored at 4 ^{0}C for 24 h, the RNAlater solution was decanted and tissue samples were blotted to remove excess solution, transferred into a 2 ml eppendorf tube, and stored at -80 ^{0}C.

Milk was analyzed for fat and protein [216], with lipids extracted and FA methylated and analyzed by a gas chromatograph using a fused-silica capillary column [CP-Sil 88; 100m x 0.25mm (i.d.) Varian] [12]. In the calculation of yield for milk FAs, the glycerol in milk fat was accounted for according to Schauff et al. [224].

5.2.2 RNA Extraction and Real-Time PCR

Total RNA was extracted and purified from ~ 30 mg of mammary tissue following the Qiagen RNA extraction and clean-up protocol using RNeasy Lipid kit (Qiagen, Valencia, CA). The potential genomic DNA co-extraction with RNA was excluded through on-column DNase treatment (RNase-Free DNase Set; Qiagen). Purity of total RNA from each sample was verified using 260 nm/280 nm absorbance ratio with background correction at 320 nm using Beckman DU-640 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). RNA concentration and quality were determined by 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). Two μg total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, California) with random primers. Quantitative real-time PCR (qRT-PCR) assays were developed for genes of interest (Table A.2). qRT-PCR reaction included Power SYBR® Green PCR Master Mix (Applied Biosystems) and 400 nmol/L of gene-specific forward and reverse primers (Invitrogen, Carlsbad, Cali-

fornia). Twenty five ng cDNA were amplified using a 2-step program (95°C for 15 s and 60°C for 60 s) with an ABI PRISM 7000 sequence detection system (Applied Biosystems). The specificity of the primers was verified by presence of a single product in the dissociation curves at the end of amplification process. A standard curve was derived from serial dilutions of pooled cDNA from mammary or liver tissues. Relative mRNA concentrations were expressed in arbitrary units and the logarithms (base 10) of concentrations were plotted against the threshold cycles (C_Ts). The resulting least square fit was used as the standard curve to derive the arbitrary concentration for each sample.

All primers were designed from ovine specific sequence data in the International Sheep Genomics Consortium (ISGC) [225] and National Center for Biotechnology Information (NCBI) data bases if available. Care was taken to design primers pairs that amplify both bovine and ovine sequences to add extra layer of confirmation. For gene sequences that are not available for sheep, primers were designed from closely related species including cow, horse, mice and human. Briefly, Nucleotide sequences for cDNA of genes of interest across species were aligned using Clustal W running under Bioedit (version 7.0.9) [226] and highly conserved sequences were selected for primer designing using Primer3Plus [162]. Primers were designed to span exon boundaries when possible. The primer sequence used for the expression of mRNA are presented in Table A.2.

5.2.3 Statistical Analysis

Data were analyzed by Student's or unequal variance unpaired t-test in R statistical package [166]. A normalization factor [193] was generated from five house-keeping genes (18S ribosomal RNA (*18S*), actin, beta (*ACTB*), ribosomal protein S13 (*RPS13*), ribosomal protein S15 (*RPS15*) and ribosomal protein L13a (*RPL13A*), Table A.2) and used to normalize gene expression data. Few points were considered outliers and excluded in analysis and rarely more than one per response variable. In a few cases data were log transformed to meet the statistical test assumptions.

5.3 Results

5.3.1 Performance-Related Variables

Effects of dietary treatment on performance parameters of the lactating ewes at wk 10 of treatment are presented in Table 5.1. There were no treatment effects on feed intake, BW or body condition score between animals in the control group and those receiving the CLA supplemented diet. Similarly, there were no treatment effects on milk yield or milk composition for protein or lactose at wk 10 of the study. In contrast, CLA treatment significantly decreased both milk fat concentration (g/100 g) and milk fat yield (g/d) by almost 22% when compared to the control group.

A comparison of effects of CLA supplement on the FA composition of milk fat demonstrated that the proportions of several FAs were altered (Table 5.2). Specifically, proportions of C6:0, C8:0, C10:0 and C12:0 were reduced for the CLA supplemented treatment whereas C18:0, *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA were increased. Effects of CLA treatment were further examined by grouping milk FA according to source and comparing the profile (Figure 5.1) and yield (Figure 5.2). CLA treatment had the greatest effect on de novo synthesized FAs (<16 carbons) with significant decreases in the proportion (15%; $P = 0.04$) and the daily yield (27%; $P < 0.04$). CLA treatment resulted in an increase in the proportion of FAs $> C16$ (27%, $P = 0.04$) and numerical decreases (non-significant) in the yields of 16 carbon FAs (15%; $P = 0.29$) and the yield of >16 carbon FAs (6%; $P = 0.71$).

5.3.2 Gene Expression

The effect of dietary treatment on the expression of genes involved in mammary lipid synthesis is presented in Figures 5.3 and 5.4. The expression of several lipogenic genes were strongly reduced by CLA treatment with the mRNA abundance of *ACACA*, *FASN* and stearoyl-coenzyme A desaturase 1 (*SCD1*) being lower by 35 to 55% in the CLA treated group as compared to the control. Similarly, CLA treatment decreased the mRNA abundance of

Table 5.1: **Treatment Effect on Performance Parameters in Dairy Ewes**

	Treatment[1]		
Variable	**CON**	**+CLA**	*P* Value
Body Weight (kg)	63.05±2.83	60.03±3.18	0.49
Dry Matter Intake[2] (kg/d)	2.18±0.08	2.09±0.06	0.40
Milk Yield (g/d)	1028±155	1015±147	0.95
Milk Fat			
g/100 g	6.15±0.35	4.83±0.19	<0.01
g/d	61.43±8.10	48.26±6.23	0.07
Milk Protein			
g/100 g	4.94±0.16	4.82±0.11	0.57
g/d	49.83±7.12	48.09±6.01	0.75
Milk Lactose			
g/100 g	4.70±0.06	4.77±0.06	0.47
g/d	48.8±7.78	48.55±7.15	0.97

[1] Lactating ewes received either control (**CON**; no supplement) or conjugated linoleic acid supplement (**+CLA**) for 10 wk starting on day 16 postpartum. During treatment, wk 10 BW and milk yield were determined and milk samples were collected and analyzed. Values represent means and SEM for 8 ewes/treatment.

[2] Average daily dry matter intake during the 10-wk experimental period

Table 5.2: **Treatment Effect on Milk FA Profile for Dairy Ewes**[1]

	Treatment		
Variable	**CON**	**+CLA**	*P* Value
FA(g/100 g)			
4:0	2.65±0.03	2.88±0.05	<0.01
6:0	1.97±0.09	1.51±0.10	<0.01
8:0	1.82±0.12	1.23±0.12	<0.01
10:0	5.93±0.48	3.95±0.41	<0.05
12:0	3.51±0.29	2.76±0.20	<0.05
14:0	9.12±0.53	9.32±0.35	0.65
16:0	31.78±0.42	31.89±0.60	0.88
16:1, *cis*-9	1.17±0.06	0.93±0.06	<0.05
18:0	8.62±0.46	10.63±0.64	0.02
18:1, *trans*-9	0.18±0.01	0.15±0.01	0.11
18:1, *trans*-10	0.17±0.01	0.19±0.01	0.11
18:1, *trans*-11	1.05±0.08	1.13±0.06	0.24
18:1, *trans*-12	0.24±0.02	0.23±0.02	0.65
18:1, *cis*-9	19.63±0.57	20.96±0.77	0.18
18:2, *cis*-9, *cis*-12	2.08±0.10	2.16±0.11	0.61
18:3,*cis*-9,cis-12,cis-15	0.39±0.02	0.37±0.01	0.26
cis-9, *trans*-11 CLA	0.48±0.03	0.62±0.03	<0.01
trans-10, *cis*-12 CLA	>0.01±0.00	0.08±0.01	<0.001
Desaturation Index			
16:1, *cis*-9/(16:1, *cis*-9+16:0)	0.036±0.002	0.029±0.002	<0.05
18:1, *cis*-9/(18:1, *cis*-9+18:0)	0.70±0.01	0.66±0.01	0.06

[1] Lactating ewes received either control (**CON**; no supplement) or conjugated linoleic acid (**+CLA**) supplement for 10 wk starting on day 16 postpartum. During treatment, wk 10 milk samples were collected and analyzed for FA profile. Values represent means ± SE for 8 ewes/treatment.

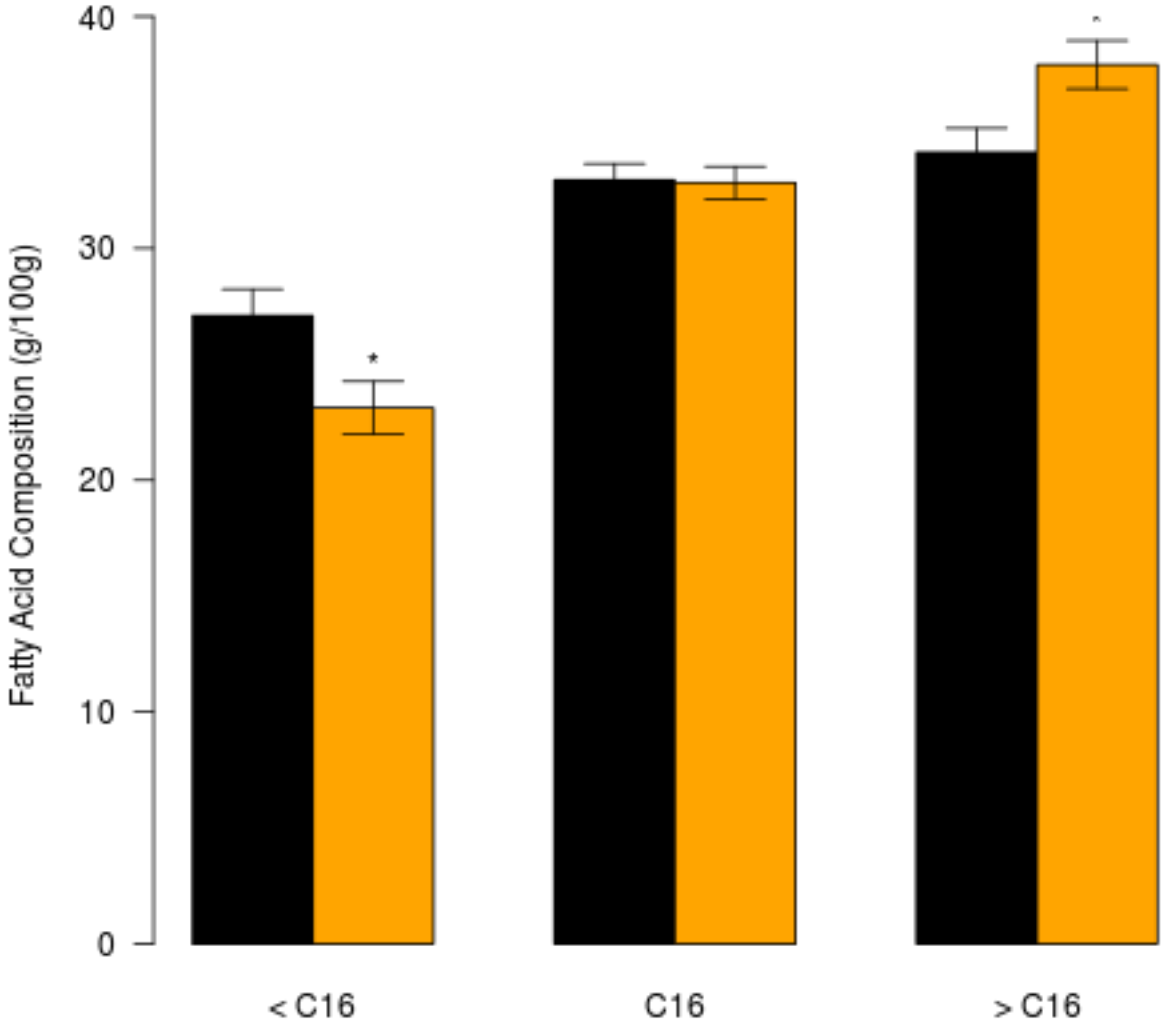

Figure 5.1: **Treatment Effect on Milk FA Composition**. Lactating ewes received either control (**CON**; no supplement; black bars) or conjugated linoleic acid supplement (**+CLA**; light bars) for 10 wk starting on day 16 postpartum. Milk samples were collected and analyzed for FA profile. FA are grouped according to source with < C16 representing de novo synthesized FA, > C16 representing preformed FA taken up from circulation and C16 being derived from both sources. Values represent means for 8 ewes/treatment with SE bars as indicated. Significant differences indicated by asterisk (*) and treatment differences were: < C16 (P = 0.04), C16 (P = 0.88) and >C16 (P = 0.04).

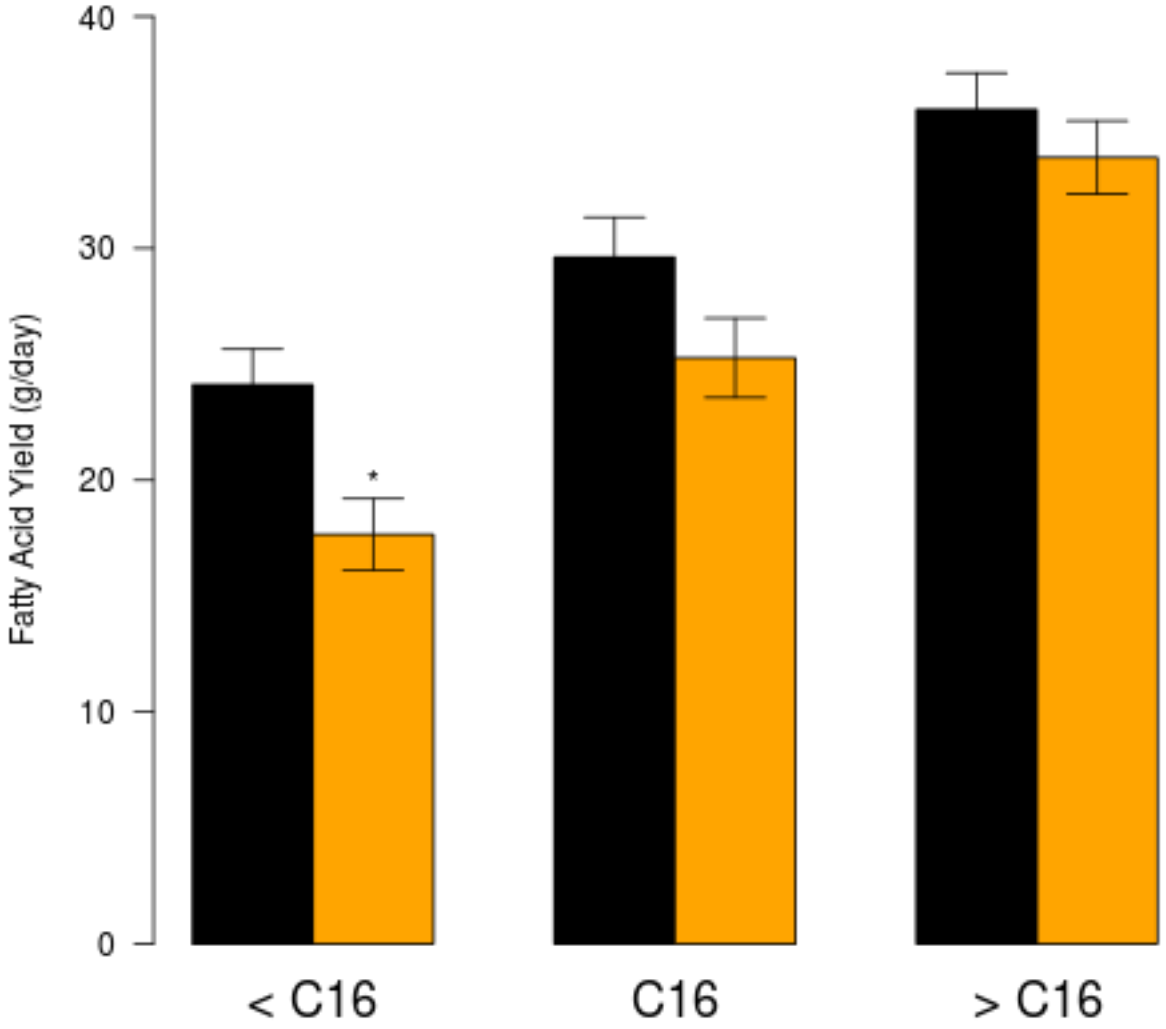

Figure 5.2: **Treatment Effect on Milk FA Yield**. Lactating ewes received either control (**CON**; no supplement; black bars) or conjugated linoleic acid supplement (**+CLA**; light bars) for 10 wk starting on day 16 postpartum. Milk samples were collected and analyzed for FA profile and yield. FA are grouped according to source with < C16 representing de novo synthesized FA, > C16 representing preformed FA taken up from circulation and C16 being derived from both sources. Values represent means for 8 ewes/treatment with SE bars as indicated. Significant differences indicated by asterisk (*) and treatment differences were: < C16 (P = 0.04), C16 (P = 0.29) and > C16 (P = 0.71).

lipoprotein lipase (*LPL*), glycerol-3-phosphate acyltransferase (*GPAM*) and diacylglycerol O-acyltransferase 1 (*DGAT1*) by about 30% compared to the control, but fatty acid binding protein 3 (*FABP3*) was unaffected. Among transcription factors related to regulation of lipogenic genes, CLA treatment decreased the mRNA abundance of the sterol regulatory element binding transcription factor 1 (*SREBF1*) and insulin induced gene 1 (*INSIG1*) by almost 60%, but there was no effect on the mRNA abundance of thyroid hormone responsive spot 14 (*S14*).

5.4 Discussion

Fat is the most variable component of milk and is especially responsive to nutrition, thereby offering a practical tool to alter its yield and composition [227]. One nutritional situation of practical and biological interest in dairy cows is diet-induced MFD. Recent work has established that diet-induced MFD is caused by biohydrogenation intermediates produced during rumen fermentation, and the most extensively investigated of these is *trans*-10, *cis*-12 CLA [6]. In lactating cows, the downregulation of mammary lipid synthesis is the most important biomarker in the CLA-induced MFD, and fat is the only milk component inhibited with *trans*-10, *cis*-12 CLA treatment [6]. While the mechanism for CLA-induced MFD is not fully understood in dairy cows [228], the ability of CLA to induce MFD has also been observed in lactating ewes [12, 216]. Further, when extrapolated to the sheep metabolic liveweight basis, the dose response relationship of daily CLA intake and MFD was similar to that reported for dairy cows. Therefore, the objective of the present investigation was to consider dairy ewes as a model and conduct an initial examination of the mechanism for CLA-induced MFD.

Consistent with previous investigations with dairy cows, CLA treatment in the current study significantly decreased both milk fat concentration and milk fat yield by almost 22%. This reduction is similar to the 17% decrease that is predicted from utilizing the milk fat

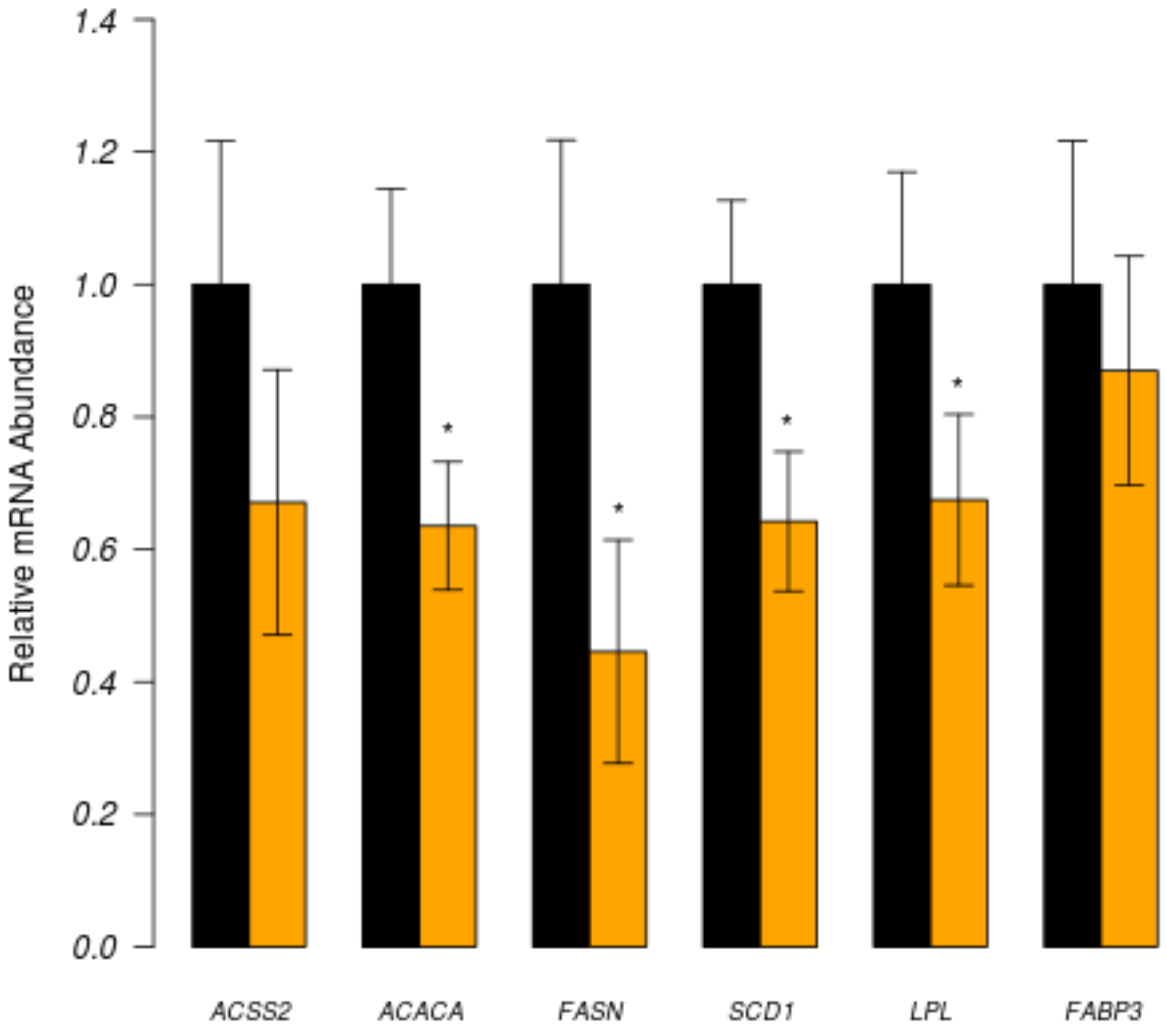

Figure 5.3: **Treatment Effects on Expression of Genes Involved in De Novo Synthesis and Uptake of Preformed FAs**. Lactating ewes received either control (**CON**; black bars; no supplement) or conjugated linoleic acid supplement (**CLA**; light bars) for 10 wk starting on day 16 postpartum. Mammary gland samples were collected at the end of the treatment and total RNA was analyzed by qRT-PCR for the mRNA abundance of indicated genes. ***ACSS1*** = acyl-CoA synthetase short-chain 2, ***ACACA*** = acetyl-coenzyme A carboxylase alpha, ***FASN*** = fatty acid synthase, ***SCD1*** = Stearoyl-coenzyme A desaturase 1, ***LPL*** = lipoprotein lipase, ***FABP3*** = fatty acid binding protein 3. Each bar represents means for 8 ewes/treatment with SE bars as indicated. Significant differences indicated by asterisk (*) and treatment *P* values were: *ACSS1* = 0.17, *ACACA* = 0.01, *FASN* = 0.02, *SCD1* = 0.01, *LPL* = 0.06, *FABP3* = 0.50.

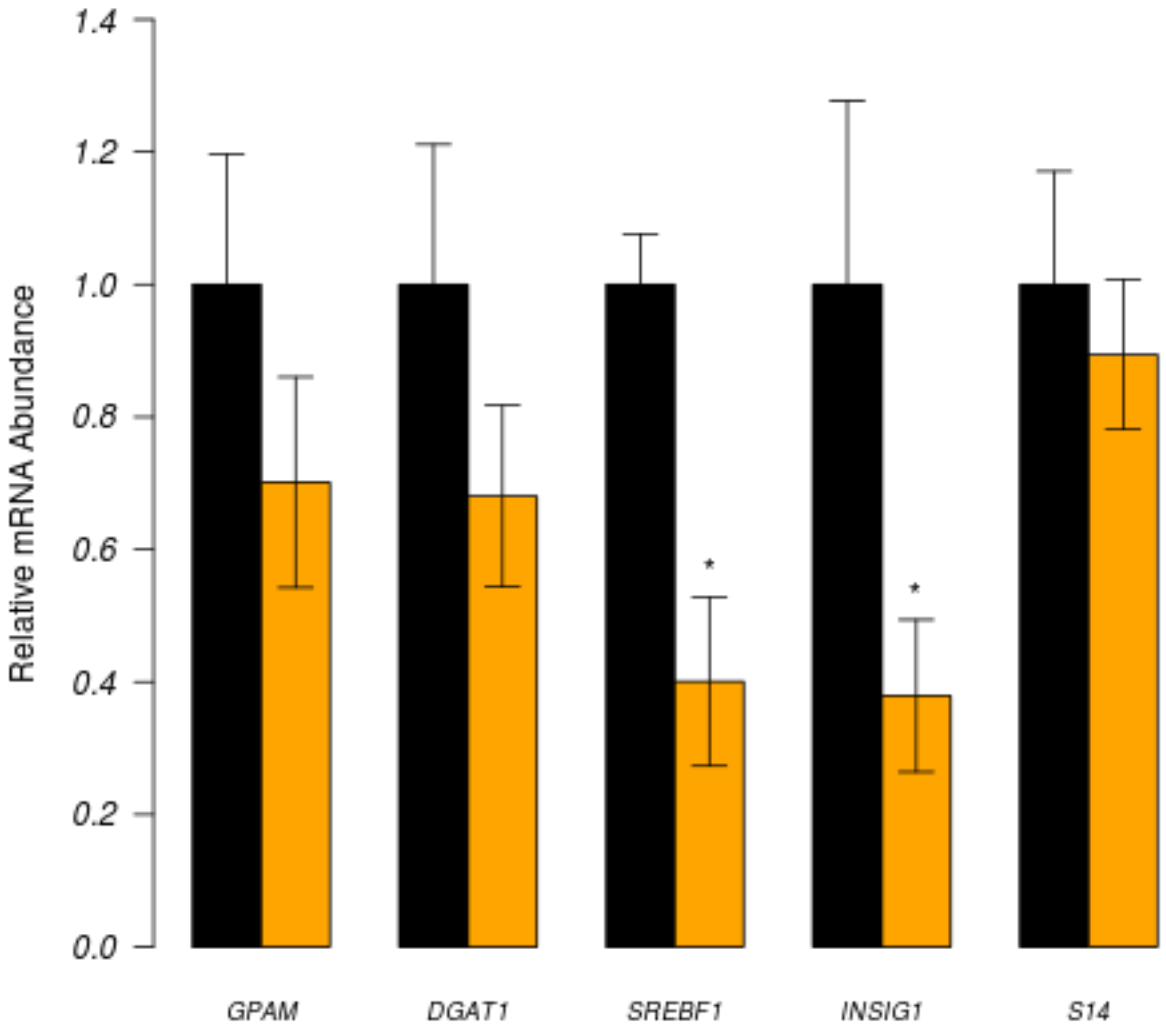

Figure 5.4: **Treatment Effect on Expression of Genes Involved in Transcription Regulation and FAs Esterification**. Lactating ewes received either control (**CON**; no supplement; black bars) or **c**onjugated linoleic acid supplement CLA; light bars for 10 wk starting on day 16 postpartum. Mammary gland samples were collected at the end of the treatment and total RNA was analyzed by qRT-PCR for the mRNA abundance of indicated genes. ***GPAM*** = glycerol-3-phosphate acyltransferase, ***DGAT1*** = diacylglycerol O-acyltransferase 1, ***SREBF1*** = sterol regulatory element-binding factor 1, ***INSIG1*** = insulin induced gene 1, ***S14*** = thyroid hormone responsive spot 14. Each bar represents means for 8 ewes/treatment with SE bars as indicated. Significant differences indicated by asterisk (*) and treatment *P* values were: *GPAM* = 0.15, *DGAT1* = 0.09, *SREBF1* = < 0.01, *INSIG1* = 0.01, *S14* = 0.72.

concentration of *trans*-10, *cis*-12 CLA we observed for CLA-treated dairy ewes in the equation relating milk fat *trans*-10, *cis*-12 CLA concentration and the percent reduction in milk fat yield developed for dairy cows abomasally infused with *trans*-10, *cis*-12 CLA [141]. Furthermore, the CLA effect in dairy ewes was specific for mammary lipid synthesis as there was no treatment effects on milk yield or milk composition of protein or lactose.

The FAs in milk fat arise from either uptake of preformed FAs from circulation or de novo synthesis within the mammary epithelial cells. For instance, short-chain FAs (4 to 8 carbons) and medium-chain FAs (10 to 14 carbons) arise almost exclusively from de novo synthesis in the mammary gland with acetate and to a lesser extent β-hydroxybutyrate serving as the carbon sources [22]. On the other hand, long-chain FAs (>16 carbons) are derived from the uptake of FAs from circulating lipoproteins (major) and non-esterified FAs (minor), while FAs of 16 carbons in length originate from both de novo synthesis and uptake of preformed FAs [132]. In ruminants, on a molar basis about one-half of the FAs in milk fat are derived from each source [22]. During CLA-induced MFD in dairy cow, the reduction in milk fat involves FAs of all chain lengths but the decrease is more substantial for those synthesized de novo [6]. A more pronounced reduction in DSFAs has also been reported for CLA-induced MFD in dairy ewes [12, 216]. In the current study, CLA treatment significantly decreased the yield of short and medium chain FAs (< 16 carbons) by 27% whereas milk fat output of 16 and >16 carbon FAs were less markedly reduced (15% and 6%, respectively; non-significant).

The response of dairy ewes and other lactating specie to *trans*-10, *cis*-12 CLA demonstrates that CLA effects on lactating mammary gland are highly specific for lipid synthesis and must include biochemical pathways of milk fat synthesis. Previous studies that have investigated the effects of the CLA on the mammary lipid metabolism have clearly shown a coordinated downregulation in the transcripts and/or enzymatic activities for genes involved in the uptake, de novo synthesis, desaturation, and esterification of FAs in mammary gland of both dairy cows [9, 136, 210, 211] and lactating rodents [212–215].

The present study extends this research to dairy ewes by investigating the mechanism of

CLA treatment on mRNA abundance of genes relating to the lipogenic pathways for mammary synthesis of milk fat. Three enzymes involved in de novo lipogenesis were examined: 1) acyl-CoA synthetase short-chain 1 (*ACSS1*), the cytosolic enzyme that catalyzes the activation of acetate, 2) *ACACA*, the biotin containing enzyme that catalyzes the formation of malonyl-CoA, and 3) *FASN* the multifunctional protein that catalyzes the use of malonyl-CoA to form saturated FAs. The mRNA abundance for *ACSS1*, *ACACA* and *FASN* was decreased by 35 to 55% in the mammary tissue from ewes receiving the CLA supplement. In the biochemical pathway for the use of preformed FAs we examined *LPL* , whose primary function is the hydrolysis of triglycerides in circulating chylomicra and very low density lipoproteins, and *FABP3* that is involved in the uptake and intracellular transport of FAs. The mammary abundance of *LPL* was reduced by 32% in the CLA treatment group whereas mRNA abundance for *FABP3* was unchanged. About two-thirds of the stearic acid taken up by the mammary gland is converted to oleic acid by the SCD1 [6] and in the present study mammary mRNA abundance of *SCD1* was reduced over 40% in the CLA treated group. The FAs in milk fat are mainly secreted as triglycerides and two key enzymes involved in the esterification were also examined, GPAM and DGAT1 [22,23]. CLA treatment decreased the mRNA abundance of *GPAM* and *DGAT1* by almost 30% compared to the control. Overall, the present study examined a number of the key enzymes involved in milk fat synthesis and observed that mRNA abundance for most was reduced as a result of the 10 wk treatment with *trans*-10, *cis*-12 CLA. Wherever values for the same enzymes have been reported, similar decreases in mammary mRNA abundance have been reported for *trans*-10, *cis*-12 CLA treatment of dairy cows [9,136,210,211] and lactating rodents [212,213,215].

The molecular mechanisms mediating the CLA-induced inhibition of milk fat synthesis are not well understood, but a role for the SREBF family of transcription factors was proposed [211], based on their function as global regulators of expression for many genes involved in lipid synthesis [203,229]. The SREBF are synthesized in the endoplasmic reticulum where they are anchored by INSIG1. To effect transcription, the SREBF must be trafficked

to the golgi where the an active N-terminal fragment is released by proteolytic cleavage thereby allowing for nuclear translocation. A role for SREBF1 was supported in studies with a bovine mammary epithelial cell line, where *trans*-10, *cis*-12 CLA decreased abundance of the nuclear active SREBF1 protein [143]. Subsequent studies showed a downregulation of *SREBF1* and *INSIG1* in milk fat depressed cows that were receiving *trans*-10, *cis*-12 CLA supplements [9, 210]. Consistent with these results, CLA treatment of lactating ewes decreased the mammary mRNA abundance of the *SREBF1* and *INSIG1* by almost 60% in the present study. However, CLA treatment has also caused a reduction in mammary mRNA for *S14* in lactating cows [9] and mice [212], but there was no effect of CLA treatment on mRNA abundance of *S14* in the present study. The exact role of S14 in the regulation of lipogenesis is unknown, but a number of reports have demonstrated that mRNA expression of *S14* is highly responsive to changes in lipogenesis in adipose tissue and liver (summarized by Cunningham et al. [230]), as well as the aforementioned studies with mammary tissue. Thus, the lack of an effect in the present study was unexpected and we have no explanation for the difference.

In summary, the present study demonstrated that CLA supplements to lactating ewes resulted in a decrease in milk fat secretion with DSFAs being most markedly affected. Consistent with this, a coordinated downregulation in the mRNA expression of key enzymes involved in de novo lipogenesis occurred. These reductions were associated with the SREBF transcription system based on the corresponding reduction in the mRNA expression of *SREBF1* and *INSIG1*, and the fact that the genes for the effected enzymes contain a sterol response element in their promoter region [77, 208]. Overall, the ewe represents a good model to examine fat synthesis in the mammary gland, and the CLA-induced milk fat depression in the lactating ewe appears to occur by mechanisms similar to other species.

Chapter 6

Integrated Discussion and Summary

Recent research has established that some nutrients can act as signaling molecules and through appropriate cellular sensing mechanisms these dietary signals are translated into changes in gene, protein, and metabolite expression [2]. Bioactive fatty acids (FAs) are among these and the information that allows nutrients to activate specific signaling pathways is contained within their molecular structure. Minor changes in this structure, e.g. the number, position and/or geometry of the double bonds, can have a profound influence on the bioactivity of the FAs and on which sensor pathways are activated. De novo lipogenesis (DNL) is a critical process that is implicated in wide range of physiological and pathological processes. As reviewed in Chapter 2, DNL from simple substrates, glucose and acetate in monogastrics and ruminant animals respectively, requires collaboration and harmony of multiple metabolic pathways. This diverse array of metabolic pathways and their key enzymes are highly active in liver, adipose tissue and the lactating mammary glands and, interestingly, in many human tumors. Although liver is considered to be central body regulator of lipid metabolism, the relative contribution of the lipogenic tissues to total FA synthesis depends on the species and the physiological status of the animal. For example, in lactating mice the mammary gland is capable of synthesizing an amount of triglyceride equivalent to the entire weight of the animal in a 20-d lactation cycle [195]. In addition to genetic, hormonal and transcriptional control, DNL is nutritionally regulated and highly responsive to dietary

FA composition. This thesis focuses on two groups of bioactive FAs, conjugated linoleic acid (CLA) and long chain ω-3 polyunsaturated fatty acids (LC ω-3 PUFAs). These FAs represent unique examples of bioactive nutrients that show a wide range of functions in biological systems including the ability to modulate gene expression [52], inflammatory processes [53] and cellular membrane structure and function as well as signaling pathways involved in normal and pathological cell functions [54]. CLA is a generic name for isomers of linoleic acid that have a conjugated pair of double bonds. They are naturally produced by rumen bacteria as intermediates in the biohydrogenation of dietary PUFAs and dietary supplementation of *trans*-10, *cis*-12 CLA markedly downregulates fat accretion in growing animals and mammary lipid synthesis in lactating animals [6, 144]. The LC ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are essential FAs important for developmental processes and the prevention of chronic diseases. Relative to DNL, the dietary LC ω-3 PUFAs are potent regulators of hepatic pathways of de novo lipid synthesis [7]. The proposed mechanisms by which these bioactive FAs effect lipid metabolism involves regulation of the abundance and activity of metabolic nuclear receptors and lipogenic enzymes. While the mechanistic details are not completely elucidated, it is clear that there are specie and tissue differences, and even differences among specific FA isomers. Therefore, the overall objective of this dissertation was to investigate the molecular mechanisms of different bioactive FAs on some aspects of lipid metabolism using different animal models; sheep and mice and physiological status; lactation and growth. The initial investigation reported in this thesis was designed to examine the role of thyroid hormone responsive spot 14 (S14) in the regulation of lipid synthesis in adipose tissue of growing mice (Chapter 3). Of particular interest was the possible role of S14 in the CLA-induced reduction in fat accretion. The S14 protein is a small acidic protein whose exact function is not yet clear. However, several lines of evidence suggested that it may play an important role in the regulation of lipogenesis. As reviewed in Chapter 2, S14 is predominantly expressed in lipid synthesizing tissues and its expression is highly correlated to rates of lipogenesis under a wide range of situations involving dietary,

hormonal, and other treatments [34, 37, 38, 43, 45, 146–154]. Likewise, a reduction in mRNA abundance for *S14* corresponds to a *trans*-10, *cis*-12 CLA induced reduction in fat synthesis in growing mice (body fat; [9, 145]) and lactating cows (milk fat; [9]). Based on this work our hypothesis was that S14 was essential for normal lipogenesis in adipose tissue and that it played a key role in the antiobesity effect of CLA. We investigated these hypotheses by utilizing a genomic approach with wild type (Wt) and *S14* null mice and treating the two groups of mice with CLA for 14 days. We observed that knocking out the *S14* gene significantly reduced the epididymal fat depot by about 25%, but there was no corresponding reduction in the mRNA abundance for lipogenic genes. Zhu et al. [45] observed a similar paradox in the milk fat synthesis of *S14* null mice where the rate of mammary DNL as well as milk and mammary tissue triglyceride content were decreased; however, they reported no effect on mRNA abundance of *FASN* or *ACACA* and, even more, the ACACA enzyme activity was significantly increased in the lactating mammary gland of the null mice [45]. Although we observed a modest reduction in fat accretion in *S14* null mice, the lack of a corresponding reduction in the expression of key lipogenic enzymes suggests that contrary to our hypothesis, the S14 protein is not involved in the transcriptional regulation of lipogenic enzymes. There could be a compensatory mechanism in response to decreased lipogenesis that might maintain or upregulate the lipogenic gene expression. However, the decrease in fat accretion (present study) and in mammary lipogenesis [45] in the *S14* null mice would be consistent with S14 protein regulating lipogenesis by other mechanisms in mammary and adipose tissues. Indeed, recent data has confirmed the ability of S14 to form heterodimers with other proteins in the cytoplasm including MIG12 which could regulate the activity of ACACA [179]. Moreover, in the mammary gland of null mice, there was an indication of reduction in the activity of FASN [45]. Altogether these data confirm that S14 might play a different role in the regulation of lipogenesis, more research is required to explore the possibility that the S14 protein may function to alter the enzyme activity of ACACA and perhaps even FASN.

In the case of CLA, we observed that *trans*-10, *cis*-12 CLA significantly reduced the total dissected fat tissue by almost 40% in both Wt and S14 null mice with no evidence of a CLA by genotype interaction. The fact that CLA-induced a delipidative effect regardless of the genotype demonstrates that expression of the *S14* gene is not essential for the CLA mechanism. In the current study, the mRNA abundance of *SREBF1-c*, *FASN* and *ACACA* decreased by almost 30% - 70% regardless of the genotype.

Therefore, contrary to our hypothesis, we found no support that S14 protein was an essential component in the mechanism of the CLA delipidative effect; rather the CLA-induced reduction in body fat accretion was clearly evident whether the genotype was Wt or S14 null.

A second focus of this thesis was to examine the effects of LC ω-3 PUFAs on lipid synthesis in lactating mice (Chapter 4). Dietary PUFAs, particularly the LC ω-3 PUFAs supplied in fish oil (FO), have several unique metabolic effects including effects on lipid metabolism. These effects have been most extensively investigated in the liver of non-lactating rodents where the LC ω-3 PUFAs have been shown to suppress hepatic lipogenesis and TG synthesis while inducing FA oxidation [63, 182–184, 186, 194]. However, the effect of LC ω-3 PUFAs on the biology of mammary gland, the major lipid synthesizing organ during lactation, has not been investigated extensively and we are not aware of any reports on effects on mammary enzymes involved in lipogenesis. The liver is also active in lipid synthesis during lactation in mice [20] and this allows the simultaneous examination of the effects of LC ω-3 PUFAs on liver and mammary tissue during this physiological state. Mice were given a diet containing 10% FO from d 6 to 13 of lactation and we found that the LC ω-3 PUFAs supplied by the FO diet were taken up and utilized by the mammary epithelial cells as evident by increases in the milk fat content of EPA and DHA. Furthermore, comparison of the FA profile for milk fat showed no differences among treatments in the proportion of de novo synthesized FAs, and the lipogenic capacity of mammary explants, measured by rates of 14C glucose incorporation into lipids, was similar among treatment groups. In addition, mRNA abundance of *ACACA* and *FASN*, two key enzymes in the FA synthesis pathway were not different among

treatments. Thus, the consistency of the mammary lipogenic gene expression profile, the absence of treatment effects on the growth rates of the nursing pups, and other mammary variables related to DNL provide strong evidence that LC ω-3 PUFAs do not adversely affect rates of mammary lipogenesis. In contrast to mammary lipid synthesis, we observed distinct treatment effects on lipid metabolism in the liver from the lactating dams. FO treatment decreased liver weight by about 20 to 25% and this decrease coincided with an approximate 50% reduction in hepatic lipid concentration in the FO-treated mice. The reduction in hepatic fat deposition paralleled a significant decrease in mRNA levels of the key enzymes in the DNL pathway. The activity of lipogenic enzymes is strongly correlated with mRNA abundance [183] and these enzymes are known to be regulated primarily at the transcription level under the control of SREBF1-c, the master regulator of lipid homeostasis [203]. In the present study there was a clear downregulation in the mRNA abundance of SREBF1-c target genes in the liver of the FO group; the mRNA abundance for *ACACA*, *FASN* and *GPAM* as well as *SCD1* and *S14* were decreased by 50% to 80% in the FO treatment group. These results are consistent with FO regulating the SREBF1-c expression posttranslationally by decreasing the amount of its active nuclear form. To confirm this point, we determined the mRNA level of key genes in the SREBF processing pathway. We found that FO treatment decreased the hepatic expression of *INSIG1* by more than 65% whereas there was no effect on *INSIG1* expression in the mammary gland and no effect on expression of *INSIG2* in either liver or mammary tissue. Overall, these results demonstrate that dietary FO supplementation to lactating mice had no effect on mammary lipogenic variables, but effects on hepatic lipogenesis were obvious and resulted in an impressive decrease in hepatic lipid accumulation and downregulation of lipogenic gene expression. One could speculate the absence of LC ω-3 PUFAs effects on mammary lipogenesis provides a mechanism to give high priority to the supply of energy (milk fat) for survival of the nursing neonates. Nevertheless, the present investigation is among the first to examine this at the cellular level in lactating animals and results highlight the importance of the tissue specific effects of LC ω-3 PUFAs

on the lipogenic pathways. The mechanism behind the differential tissue effects remains to be established but will need to accommodate the fact that the LC ω-3 PUFAs are taken up by the mammary gland and utilized in the synthesis of milk fat.

The final investigation reported in this dissertation involves the role of CLA in the regulation of milk fat synthesis (Chapter 5). Mammary lipogenesis is nutritionally regulated and highly responsive to dietary FA composition [188]. One nutritional situation of practical and biological interest in dairy cows is diet-induced milk fat depression (MFD). Recent work has established that the diet-induced MFD is caused by biohydrogenation intermediates produced during rumen fermentation, the most investigated being *trans*-10, *cis*-12 CLA [6]. Dietary supplements of *trans*-10, *cis*-12 CLA result in MFD in lactating ruminants and non-ruminants [6]. In lactating cows, the downregulation of mammary lipid synthesis is the most important biomarker in the CLA-induced MFD, and fat is the only milk component inhibited with *trans*-10, *cis*-12 CLA treatment [6]. CLA-induced MFD has also been observed in small ruminants including sheep [12,216] and goats [217,218]. Although ruminants share similarities in many aspects, distinct differences exist related to ruminal lipid metabolism and the relative sensitivity of mammary lipogenic processes [219], and this might modify the mammary response to CLA treatment. Studies of the mechanism of CLA-induced MFD have exclusively utilized the dairy cow [228], so the objective of the present study was to extend these results to lactating ewes. Of interest was whether aspects of the mechanism would be similar thereby providing an opportunity to use the lactating ewe to serve as a convenient model for future investigations. The study involved lactating dairy ewes that received a CLA supplement containing *trans*-10, *cis*-12 CLA for 10 wks. We found that the phenotypic effects of CLA treatment of lactating ewes were specific for mammary lipid synthesis, just as reported for dairy cows. CLA treatment resulted in approximately 22% decrease in both milk fat concentration and milk fat yield with no effects on milk yield or milk composition of protein or lactose. Moreover, CLA treatment significantly decreased the yield of short and medium chain FAs (< 16 carbons) which are unique products of de novo FA synthesis. To

investigate the molecular mechanism of CLA effect behind this phenotype, we examined a number of the key enzymes involved in milk fat synthesis and observed that mRNA abundance for most was reduced as a result of the 10 wk treatment with *trans*-10, *cis*-12 CLA; mRNA abundance for *ACSS1*, *ACAC*, *FASN*, *LPL*, *GPAM* and *DGAT1* were reduced by almost 30% compared to the control. The molecular mechanisms mediating the CLA-induced inhibition of milk fat synthesis are not well understood, but a role for the SREBF family of transcription factors has been proposed [211], based on their function as global regulators of expression for many genes involved in lipid synthesis [203, 229]. Consistent with these results, CLA treatment of lactating ewes decreased the mammary mRNA abundance of the *SREBF1* and *INSIG1* by almost 60%. Similar corresponding reductions in the expression of lipogenic genes and members of the SREBF1 transcription factor family have been reported previously for lactating dairy cows receiving CLA supplements [9,136,210,211] and lactating rodents [212–215]. Overall, results demonstrated that CLA supplements to lactating ewes resulted in a decrease in milk fat secretion with de novo synthesized FAs being most markedly affected. Consistent with this, a coordinated downregulation in the mRNA expression of key enzymes involved in DNL occurred. These reductions were associated with the SREBF transcription system based on the corresponding reduction in the mRNA expression of *SREBF1* and *INSIG1*, and the fact that the genes for the effected enzymes contain a sterol response element in their promoter region [77, 208]. Thus, the ewe is responding to the *trans*-10, *cis*-12 CLA in a manner similar to the cow and should represent a good model to continue mechanistic studies of the regulation of milk fat synthesis in the mammary gland.

Appendix A

Table A.1: Murine Primers Used in Real-Time PCR Analysis

Gene	Forward primer	Reverese primer
18S	GTGGGCCTGCGGCTTAAT	GCCAGAGTCTCGTTCGTTATC
ACOX1	AGTGCCACTGCGGTCCCTGA	CAGTGATGCCTGGCAGAAGCTTG
ACACA	GACCCTACACTTACTGATGAG	AAGCAATAAGAACCTGACGAG
B2M	CATGGCTCGCTCGGCGACC	AATGTGAGGCGGGTGGAACTG
CPT1a	CTCAAACCTATTCGTCTTCTG	TTGGATGGTGTCTGTCTC
DGAT1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
FASN	AGAGATCCCGAGACGCTTCT	GCCTGGTAGGCATTCTGTAGT
GPAM	CGCGGGGTCAGCACATGGTT	ACGAAGGGCCTCTTCCGGCT
INSIG1	CTCCGGGCAGAGCTCAGGATTTCT	ACCCCGCGGATCACCACGTT
INSIG1	TCACAGTGACTGAGCTTCAGCA	TCATCTTCATCACACCCAGGAC
INSIG2	GGAGTCACCTCGGCCTAAAAA	CAAGTTCAACACTAATGCCAGGA
LPL	GGACGGTAACGGGAATGTATG	ACGTTGTCTAGGGGGTACTTAAA
MBTPS1	CTGGTGGTTTTGCTCTGTGG	GGCTGTGAAGTATCCGTTGAAAG
PPARa	GTCATCACAGACACCCTC	TATTCGACATCGATGTTCAG
RPS13	TCCCTCCCAGATAGGTGTAATCC	TCCTTTCTGTTCCTCTCAAGGT
RPS20	GAGAAGGTTTGTGCGGACTTG	CCGGCTCAATACTGATGGAAG
S14	TGAGAACGACGCTGCTGAAAC	AGGTGGGTAAGGATGTGATGGAG
SCD1	TGGGAAAGTGAGGCGAGCAACTG	AGGGAGGTGCAGTGATGGTGGTG
SREBP-1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
SCAP	TGGAGCTTTTGAGACTCAGGA	TCGATTAAGCAGGTGAGGTCG

Table A.2: Ovine Primers Used in Real-Time PCR Analysis[1]

Gene	Forward primer	Reverese primer
18S	GATCCATTGGAGGGCAAGTCT	GCAGCAATTTAATATACGCTATTGG
ACTB	GCGTGGCTACAGCTTCACC	CTTGATGTCACGGACGATTTC
ACAC	TTTCCTAAATTTTTCACGTTCC	AGGCTCCAGGTGACGATAGA
ACSS1	TGAGCCTGGAACTGAAGTGA	GACTCCATACCTCTTGAGTGTGTT
DGAT1	GTTCCTCACCCAGCTCCAG	GATGAGCCAGATGAGGTGGT
FABP3	GGACAGCAAGAATTTCGATGA	CGATGATTGTGGTAGGCTTG
FASN	TGGTGACAGATGATGACAGG	GAAGAAGGAAGCGTCAAACC
INSIG1	GTCATCGCCACCATCTTCTC	GACTGTCGATGCAGGGGTA
LPL	ACCTGAAGACTCGTTCTCAGATG	GGCCTGGTTGGTGTATGTATT
MGPAT	GCATTGGTCGGTGTAAGCAT	TTCTTTCCACTTCAAGGTTGC
RPL13A	AGCCACCCTGGAGGAGAAGCG	TTTCGGCCTGCTTCCGTAGCC
RPS15	CGGCAAGATGGCGGAAGTGGAAC	TGGCGCGCGCTGTATAGCTG
RPS20	TTCACCGGATTAGGATCACC	CTTTTCCTTCGCGCCTCT
S14	CCTCACCCATCTTACCCTGA	TTGCAGGTCCAGGTCTTTCT
SCD	CATCAACCCCCGAGAGAATA	GGTGTGGTGGTAGTTGTGGA
SREBF1	AGCTCAAGGACCTGGTGGTG	GCTGAAGGAAGCGGATGTAG

[1] Primers were designed from specific ovine sequences or based on highly conserved DNA sequences among related species.

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