ABSTRACT

HOUSE, RALPH LEE. Functional Genomic Characterization of the Anti-Adipogenic Effects of *trans* 10, *cis* 12-Conjugated Linoleic Acid (t10c12-CLA) in a Polygenic Obese Line of Mice. (Under the direction of Jack Odle)

We analyzed gene expression during t10c12-CLA-induced body fat reduction in a polygenic obese line of mice. Adult mice (N=185) were allotted to a 2x2 factorial experiment consisting of a non-obese (ICR-control) and an obese (M16-selected) line of mice fed a 7% fat, purified diet containing either 1% linoleic acid (LA) or 1% t10c12-CLA. Body weight (BW) gain by day 14 was 12% lower in CLA compared to LA fed mice ($P<0.0001$). By day 14, t10c12-CLA reduced weights of epididymal, mesenteric and brown adipose tissues as a percentage of BW in both lines by 30, 27 and 58%, respectively, and increased liver weight/BW by 34% ($P<0.0001$). Total RNA was isolated and pooled (4-5 mice per composite) from epididymal adipose (day 5 & 14) and liver (day 14) of the obese mice to analyze gene expression profiles using Agilent mouse oligo microarray slides (4 per tissue•day) representing >20,000 genes. Numbers of genes differentially expressed by ≥ two fold in epididymal adipose (day 5 & 14) and liver (day 14) were 29, 125, and 80, respectively. Of particular interest in adipose, CLA putatively increased expression of the uncoupling proteins (1 and 2), carnitine palmitoyltransferase (L and M), and carnitine translocase, but decreased expression of PPAR-γ, GLUT-4, perilipin, caveolin-1, adiponectin and resistin ($P<0.01$). In conclusion, this experiment has revealed candidate genes that will be useful in elucidating mechanisms underlying the potent anti-adipogenic effects of t10c12-CLA.

FUNCTIONAL GENOMIC CHARACTERIZATION OF THE ANTI-ADIPOGENIC EFFECTS OF *trans* 10, *cis*12-CONJUGATED LINOLEIC ACID (t10c12-CLA) IN A POLYGENIC OBESE LINE OF MICE

by

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DEDICATION

This body of work is equally dedicated to the following people:

- My daughter, Lily, for being a continual source of motivation and joy. I only hope that I may forever reciprocate in kind.
- My wife, Sheetal, whose love and understanding has never faltered, no matter the situation. This along with the value of your personal and scientific advice has contributed greatly to the success of this work.
- My parents, Lee and Fadia, for always having the right advice and whose sacrifice has made this work possible. No words can describe the fortune and pride that I feel to have you as parents.
- My brothers, Alan and Paul, for being good friends as well as family, and whose companionship provided me much happiness, when no more work could be done.
- My good friend and co-worker, Dr. Lin Xi, for teaching me how to become truly skillful in the laboratory and never hesitating to take time to meticulously explain concepts and experimental rationale. Your invaluable contribution to my success and growth in the laboratory will be forever appreciated.
- My mentor, Dr. Jack Odle, for never turning me away from your office, always listening intently, and helping me through many a storm, both personal and professional. You have and will continue to be an eternal source of inspiration, to be a good leader and scientist, and to live fully to one's potential. Any success that I have or will receive in my journey through science will perpetually be dedicated, in part, to you.

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House, R.L., J.P. Cassady, E.J. Eisen, T.E. Eling, and J. Odle. 2004. Functional genomic characterization of polygenic obese mice fed *trans* 10, *cis* 12 conjugated linoleic acid. FASEB J. 18:4 (abst).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Conjugated linoleic acid (CLA); Body mass index (BMI); National Health and Nutrition Examination Survey (NHANES); quantitative trait loci (QTL); free fatty acid (FFA); fatty acid oxidation (FAO); triglyceride (TG); glucose transporter-4 (GLUT-4); 5'-AMP-activated protein kinase (AMPK); peroxisome proliferator activated receptor (PPAR); peroxisome proliferator response element (PPRE); 15 Deoxy-Δ12,14 - prostaglandin J2 (PGJ2); thiazolidinedione (TZD); adipocyte protein 2 (aP2); phosphoenolpyruvate carboxykinase (PEPCK); CCAAT/enhancer-binding protein α (C/EBPα); low density lipoprotein (LDL); Zucker diabetic fa/fa rats (ZDF); saturated fatty acids to monounsaturated fatty acids (SFA:MUFA); uncoupling proteins (UCPs); tumor necrosis-α (TNF-α); acyl-CoA binding protein (ACBP); interleukin (IL); mitogen-activated protein kinase kinase/ extracellular signal-related kinase (MEK/ERK); lipoprotein lipase (LPL); carntine palmitoyl transferase (CPT); acetyl-CoA carboxylase (ACC); regulatory binding protein-1 (SREBP-1); fatty acid synthase (FAS); peroxisomal acyl CoA oxidase (ACO); cytochrome P450A1 (CYP4A1); stearoyl-CoA desaturase-1 (SCD-1); stearoyl-CoA desaturase-1 (SCD-2); minimum information about microarray experiments (MIAME); caveolin 1 (cav-1); caspase 3; (casp-3); real time reverse transcriptase-polymerase chain reaction (QRT-PCR); retinoic acid receptor (RXR); phosphofructokinase-2 (PFK-2); pyruvate dehydrogenase (PDH); diacylglycerol acyltransferase (DGAT); B-cell lymphoma 2 (Bcl-2); cytochorome-c (cyt-c); eukaryotic translation initiation factor 4E binding protein 1(Eif4ebp1); eIF4E-binding protein-1 (4EBP1); PPAR-γ co-activator-1 (PGC1); phosphatidylinositol 3-kinase (PI3K)

CHAPTER I

LITERATURE REVIEW

CONJUGATED LINOLEIC ACID (CLA): DE-LIPIDATION THROUGH GENETIC MODULATION OF LIPID METABOLISM IN LIVER AND ADIPOSE TISSUE

Obesity and Society:

General Facts:

Over the past two decades the prevalence of obesity has risen to epidemic proportions in the United States and the rest of the world. A recent study reported that in 2000, there were 435,000 tobacco-related deaths and 400,000 deaths associated with poor diet and physical inactivity, which includes obesity as a subcategory (Mokdad et al. 2004). However, this report has been controversial and obesity is still currently considered the second leading cause of preventable deaths in the U.S. (Marshall 2004). The 1999-2000 National Health and Nutrition Examination Survey (NHANES) reported that approximately 64% of the U.S. population of adults ≥ 20 years old are overweight or obese (www.cdc.gov/nchs/products/pubs/pubd/hestats/obese/obse99.htm) (Hill et al. 2003). More specifically, 31% of U.S. adults are considered obese (Body-mass index ≥ 30 (BMI (weight (kg)/height^2 (m^2))), an increase from the 22.9% reported in NHANES III (1988-1994) (Flegal et al. 2002). Amongst the population of U.S. children, 15.5% between ages 12 and 19, 15.3% between ages 6 and 11, and 10.4% between ages 2 and 5 are overweight (BMI ≥ 25) (Ogden et al. 2002). Additionally, the 1999-2000 NHANES reported a 4% increase in

number of overweight children between 6 and 19 from 11% reported in the 1988-1994 NHANES III. (www.cdc.gov/nchs/products/pubs/pubd/hestats/overwght99.htm). Prevalence of extreme obesity (BMI ≥ 40) quadrupled between 1986 and 2000 from 1 in 200 adults to 1 in 50 (Sturm 2003). There was also an increase by a factor of 5 within the same period of Americans with a BMI ≥ 50, from 1 in 2000 to 1 in 400 (Sturm 2003). It seems apparent that prevalence of clinically severe obesity will soon add significantly to the gravity of the obesity epidemic. On a global scale, the World Health Organization reported that in 1995 there were approximately 200 million obese adults in the world. By 2000, this number increased to over 300 million (www.who.int/nut/obs.htm). Whilst the majority came from industrial nations, interestingly, 115 million were from developing countries.

Obesity is an independent risk factor for a number of health problems such as diabetes mellitus, hypertension, coronary heart disease, elevated cholesterol levels, depression, musculoskeletal disorders, gallbladder disease, and several cancers (Kortt et al. 1998; Stunkard et al. 2003). In 1995 the total cost for treating obesity in the U.S. was $51.64 billion (direct medical costs) (Wolf and Colditz 1998). In 2003 the total had increased to $75 billion, half of which was paid for by Medicare and Medicaid (Finkelstein et al. 2004). Concern within the government was seen clearly in 2003, when 140 bills aimed directly at obesity were filed in the U.S. legislature, almost double the 72 filed in 2002 (Connolly 2003).

Therapeutic Options:

Many drugs that were or are available for the treatment and prevention of obesity have adverse side effects; for example, the addictive nature of amphetamines and problems with valvular heart disease using phentermine-fenfluramine (fenfluramine was withdrawn

on September 15, 1997) (Wadden et al. 1998; Pi-Sunyer 2003; Wyatt and Hill 2004). At the time of writing this review, two drugs that have FDA approval, Meridia (sibutramine hydrochloride) and Xenical (orlistat) have been shown to help in losing and maintaining weight loss (Wyatt and Hill 2004). However, between 1998 and 2001, 150 people taking Meridia were hospitalized, 29 of whom died (Gura 2003). Patients taking Xenical experience side effects such as cramping and severe diarrhea (Gura 2003). However, prescribing these drugs is justifiable if the risk of the treatment is not as severe as the risk of the condition left untreated, a judgment that must be made by one's physician (Wyatt and Hill 2004).

The Foundation of Obesity:

It now seems apparent that there is no single contributing factor associated with obesity; rather it is a multi-factorial problem spanning environment, physiology and genetics (Wyatt and Hill 2004). Given the drastic increase in the prevalence of obesity within the past two to three decades, it is probable that environment is a significant driving force (Hill et al. 2003). Two main factors considered responsible are high caloric intake and decreased physical exercise (Hill and Peters 1998; French et al. 2001; Peters et al. 2002). It should be noted, however, that the impact of genetics and its interactions with these environmental factors should not be underestimated. Studies analyzing effects of genetics and environment on BMI in identical or fraternal twins reared apart or together have shown that genetics contributes substantially to the similarity of BMI between twins, whilst the environment has little, if any effect (Stunkard et al. 1990; Allison et al. 1996; Maes et al. 1997; Comuzzie and Allison 1998; Friedman 2003; Schousboe et al. 2004). Similar results have been observed comparing adoptees with their biological parents and

full siblings (Stunkard et al. 1986; Maes et al. 1997; Sorensen et al. 1998; Comuzzie and Allison 1998). From an evolutionary perspective, food has not historically been as readily available or abundant as it is today, especially in industrialized societies. Our genetic makeup has evolved in an environment that encourages storage of fat to better survive periods of famine and starvation, but has not had to develop a strong mechanism to control excessive reserves (Pi-Sunyer 2003). The epidemic proportions of obesity in the U.S. and the world coupled with a deficiency in adequate medication emphasize the importance of researching and elucidating mechanisms associated with obesity, so that we may better formulate methods of treatment and prevention.

Genetics Associated with Obesity:

Interplay between the contributing factors of genetics, behavior and environment have made obesity a complex phenotype (Comuzzie and Allison 1998). Gene elucidation is further complicated by the fact that, unlike simple Mendelian disorders (single locus mutation), many common diseases in humans, including obesity, are multifactorial and polygenic in nature (Williamson and Kessling 1990; Pomp 1997; Rocha et al. 2004b). There have been several genetic disorders described in humans that result in an obesity phenotype. Examples of a few include syndromes such as Prader-Willi, Alstrom and Bardet-Biedl. In these diseases, obesity is part of a complex disorder that includes several other maladies that arise from genetic abnormalities (Bardet 1920; Biedl 1922; Prader et al. 1956; ALSTROM et al. 1959; Goldstein and Fialkow 1973; Steiner 1990; Holm et al. 1993).

Use of animal models, particularly mice, has proven particularly useful in characterizing phenotypic and genotypic traits associated with obesity. Single-gene

mutants expressing the obesity phenotype, such as the obese (*ob/ob*), agouti, fat (*fat/fat*) and tubby (*tub/tub*) mouse, were reported as early as the 1920s (agouti) (Danforth 1925; Danforth 1927; Ingalls et al. 1950; Coleman and Eicher 1990). However, genes encoding these phenotypes (obese (*ob*), agouti (A^Y), fat (*cpe*), and tubby (*tub*)) were not characterized until recently (Bultman et al. 1992; Zhang et al. 1994; Naggert et al. 1995; Noben-Trauth et al. 1996).

A mutation in the *ob* gene (as seen in the *ob/ob* mouse) has been identified as the cause of obesity in some humans, and is characterized by morbid obesity with low levels of serum leptin (Zhang et al. 1994; Montague et al. 1997; Farooqi et al. 2002). Leptin is a circulating hormone that is secreted by adipose tissue, it has been shown to decrease feed intake and increase energy expenditure, resulting in de-lipidation of adipose tissue when administered to *ob*/*ob* mice by subcutaneous injection (Pelleymounter et al. 1995; Halaas et al. 1995; Campfield et al. 1995; Clement et al. 1998). Similarly, it was shown that obesity in human subjects was ameliorated by subcutaneous injection of recombinant human leptin, causing a drastic reduction in feed intake (Farooqi et al. 2002). In one example, a boy weighing 42kg (about 93 lbs) at three years of age lost 10 kg (about 22 lbs) after 48 months of treatment (Farooqi et al. 2002). Other monogenic diseases have been described (see Farooqi and O'Rahilly (2004) for further review); however, only 5 to 6% of children considered obese suffer from a mutation at a single locus, and a majority of cases are polygenic in nature (Friedman 2003; Farooqi and O'Rahilly 2004). Development of polygenic mouse models, such as the mouse line M16 (described below), more closely resemble the major human obesity condition and may, therefore, prove particularly useful

in characterizing the traits associated with obesity (Eisen and Leatherwood 1978a; West et al. 1994; Warden et al. 1995; Rocha et al. 2004b).

The Polygenic Obese Line of Mice, M16:

The polygenic obese line of mice M16 (and an inbred line M16i formed from M16), was developed at North Carolina State University by Dr. Eugene J. Eisen. The M16 line was selected for rapid postweaning gain from an outbred ICR (Institute for Cancer Research; developed by T.S. Hauschka in 1948 at Fox Chase Cancer Center, Philadelphia PA) albino population by selecting the highest 25% (M=maximum) of males and females within full-sib families, from a population size consisting of 16 parental pairs (hence the nomenclature M16) (Hanrahan et al. 1973; Hanrahan and Eisen 1973; Eisen 1975). Selection was conducted over more than 27 generations (Eisen 1975).

The M16 line exhibits positive correlated responses in body weight and percent body fat. The mice are moderately obese and hyperphagic, and grow rapidly between 4 and 6 weeks of age (Eisen and Leatherwood 1978b; Pomp 1997). Compared to the ICR control line, M16 mice have 60% greater body weight and larger organs, indicating a positive correlation between these two traits (Eisen 1986; Pomp 1997) (Figure 1.1). The M16 line also displays greater adipocyte hypertrophy and hyperplasia compared to the ICR line, and the mice are hyperglycemic, hyperinsulinemic and hypercholesterolemic (Robeson et al. 1981). These results with M16 mice were recently verified, indicating that genetic drift and/or inbreeding had not altered these traits (Allan et al. 2004). The M16 line also had a higher water intake and higher plasma levels of leptin, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Allan et al. 2004). Restricting pre-weaning energy intake by

manipulating the number of pups nursed, showed that the genetic line effect was the predominant cause of obesity, with only a minor contribution arising from pre-weaning energy intake (Eisen and Leatherwood 1978a; Smith et al. 1983). Limiting post-weaning energy intake during the 4 to 6 week rapid growth period decreased body fat and adipose cellularity in restricted animals (Eisen and Leatherwood 1978b; Smith et al. 1983). However, at the end of the restricted feeding period, the M16 line had similar body weights compared to the control ICR line, but had increased body fat and less water content (Eisen and Leatherwood 1978b). Also at this time period, M16 mice were allowed to feed *ad libitum* and by 16 weeks of age had attained similar body weights and fat content as mice fed *ad libitum* throughout the duration of the trial (Eisen and Leatherwood 1978b).

A recent study crossed the M16 line with a low body weight (L6) line and analyzed the resulting F2 generation to identify quantitative trait loci (QTL) for complex traits such as body weight and body fatness (Rocha et al. 2004a; Rocha et al. 2004b). Their results found QTL for fatness on Chromosomes (Chr) 2, 7, 15 and 17, with the largest effects emanating from Chr 2 (Rocha et al. 2004b). Projects such as these, utilizing a polygenic obese model that more closely resembles the human obesity condition will certainly make major contributions towards furthering our understanding of the genetic complexity associated with a polygenic trait such as obesity. The M16 line of mice is an effective animal model that will surely make a valuable contribution in furthering this endeavor.

Adipose Tissue, More Than Just Lipid Storage:

General Function:

A loose connective tissue that is made up of cells called adipocytes, adipose tissue is surrounded by blood vessels, collagen fiber and immune cells (Ahima and Flier 2000). In a situation where energy intake exceeds energy expenditure, adipocytes can store a seemingly unlimited amount of energy as lipid in triglyceride form; however, adipose tissue can increase in size as a result of hyperplasia as well as hypertrophy. Upon energy consumption and digestion, glucose is released into the bloodstream, which triggers the release of insulin from pancreatic β-cells. In addition to promoting the storage of glucose in the form of glycogen in the liver, insulin binds to its respective receptor on the surface of the adipocyte, which triggers the migration of glucose transporter-4 (GLUT-4) to the cell surface and promotes glucose uptake into the adipocyte. Carbon obtained from the glucose molecule by the process of glycolysis can then used to make triglycerides through the process of lipogenesis. However, in some species lipogenesis occurs predominantly in the liver. In the event that there is a demand for energy exceeding intake, such as in a fasted state, triglycerides undergo lipolysis (driven primarily by hormone sensitive lipase) and are released into circulation in the form of free fatty acids (FFAs). Once released, FFAs may be oxidized and used by various tissues and organs as a source of energy.

Adipokines:

Traditionally thought of solely as a site for lipid storage, adipose tissue has recently gained recognition as an endocrine organ responsive to both central and peripheral metabolic signals (Rajala and Scherer 2003). A transgenic line of mice whose white adipose tissue had been ablated developed diabetes with fatty liver and enlarged organs, as

well as low fertility and premature death (Moitra et al. 1998). This emphasized the vital role that adipose tissue plays in growth, reproductive function, glucose metabolism and fasting tolerance (Moitra et al. 1998). A review written by Ahima and Flier (2000) provides a comprehensive list of proteins secreted from adipose tissue. Recently, the hormones secreted by adipose tissue, called adipokines, in particular leptin, adiponectin and resistin have received much attention for their potential roles in the mechanisms associated with obesity.

Leptin:

Briefly described earlier in this review, leptin is predominantly expressed in white adipose tissue and controls satiety through interactions with the hypothalamus. In a fed state, leptin concentrations increase, resulting in signals that decrease feed intake and increase energy expenditure (Pelleymounter et al. 1995; Halaas et al. 1995; Campfield et al. 1995; Clement et al. 1998). However, leptin seems to work over a long- term period and is not solely responsible for cessation of energy intake (Maffei et al. 1995). Upon its release, leptin interacts predominantly with leptin receptors in the arcuate nucleus, ventral premamillary nucleus, ventromedial, dorsomedial and lateral hypothalamic nuclei (Schwartz et al. 1996; Mercer et al. 1996; Fei et al. 1997; Elmquist et al. 1998). These are areas in the brain known to control feed intake and body weight.

There is a direct correlation with circulating leptin levels and BMI, and it follows that levels tend to be high in many cases of obesity, but with no effect on energy intake (Maffei et al. 1995; Frederich et al. 1995; Considine et al. 1996); only rare cases of obesity arise due to a leptin deficiency (Farooqi et al. 2002). It has been proposed that this may be due to leptin resistance; however, mechanisms that would be involved are not yet clearly

understood (see Chehab et al. (2004) for detailed review) (Ahima and Flier 2000; Chehab et al. 2004).

Adiponectin:

Adiponectin is a hormone that is expressed solely in adipose tissue and is the most abundant mRNA transcript therein (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996). Contrary to leptin, adiponectin is negatively correlated with BMI and its mRNA and plasma concentrations are predominantly reduced in obesity (Hu et al. 1996; Arita et al. 1999; Hotta et al. 2000; Weyer et al. 2001). By increasing fatty acid oxidation (FAO), adiponectin decreases insulin resistance by decreasing triglyceride (TG) concentration in liver and muscle of obese mice (Hu et al. 1996; Arita et al. 1999; Hotta et al. 2000; Yamauchi et al. 2001; Weyer et al. 2001; Fruebis et al. 2001). A QTL analysis located a marker for insulin resistance on human chromosome 3q27, approximating the region that codes for adiponectin (Kissebah et al. 2000). By directly activating 5'-AMP-activated protein kinase (AMPK), it has been proposed that adiponectin directly regulates insulin sensitivity and glucose metabolism (Yamauchi et al. 2002). This was recently verified with identification of two adiponectin receptors (AdipoR1 and AdipoR2) expressed in skeletal muscle (predominantly AdipoR1) and liver (AdipoR2) (Yamauchi et al. 2003; Debard et al. 2004). Expression or suppression of these receptors showed they mediate an increase in AMPK, FAO and glucose uptake (Yamauchi et al. 2003).

A recent study analyzed serum adiponectin and its relation to lipid and insulin resistance profiles in young healthy men (Kazumi et al. 2004). The researchers suggested that low circulating levels of serum adiponectin were not as closely related to insulin resistance as to adiposity and dyslipidemia. Several independent experiments using

adiponectin knockout mice have yielded conflicting results. Two studies showed that mice were insulin resistant when fed a high fat (Maeda et al. 2002) or regular diet (Kubota et al. 2002). However a third study did not detect any changes on insulin action under either diet condition (Ma et al. 2002). It has been proposed that this discrepancy may indicate that genetic background may effectively make up for insulin resistance due to low adiponectin levels (Havel 2004). There is further discrepancy in the literature as to whether insulin stimulates (Bogan and Lodish 1999; Halleux et al. 2001) or inhibits (Fasshauer et al. 2002; Tsuchida et al. 2004) adiponectin expression or secretion. It therefore remains unclear how adiponectin is regulated and its precise mechanism of action. Tsuchida et al. (2004) showed that insulin has a negative effect on adiponectin and expression of the adiponectin receptors. Their results further indicate that obesity-linked insulin resistance may cause downregulation of the adiponectin receptors, thereby leading to adiponectin resistance (Tsuchida et al. 2004). They suggest that this may be occurring through the PI3-kinase/Foxo1 dependent pathway (Tsuchida et al. 2004). Adiponectin's relation to insulin resistance makes it an intriguing target for pharmacological therapy against diabetes and obesity, making it an adipokine that is and will continue to be the subject of intense research.

Resistin:

Resistin was independently identified as an adipocyte secreted hormone in murine adipose tissue by three different groups (Holcomb et al. 2000; Steppan et al. 2001; Kim et al. 2001). In mice, the resistin gene (*retn*) is predominantly expressed in white adipose tissue and the protein is found in blood and adipose tissue (Steppan et al. 2001). However, in humans the levels of resistin in adipose tissue are controversial. Some groups report

almost undetectable levels (Savage et al. 2001; Nagaev and Smith 2001; Janke et al. 2002), while another group reports the opposite (McTernan et al. 2002a; McTernan et al. 2002b). High levels have also been reported in human preadipocytes (Janke et al. 2002) and bone marrow (Patel et al. 2003). In mice, resistin levels were almost undectable in preadipocytes (Steppan et al. 2001), indicating that resistin localization may differ between species. Several groups have reported that there is no correlation between BMI or body fat and levels of resistin (Savage et al. 2001; Janke et al. 2002; Heilbronn et al. 2004), while others have reported the contrary (Zhang et al. 2002; McTernan et al. 2002b; Degawa-Yamauchi et al. 2003; Azuma et al. 2003; De Courten et al. 2004).

Resistin's role in obesity and insulin resistance is also controversial. In their initial characterization, Steppan et al. (2001) reported increased levels of circulating resistin in an obese model of mice, and observed that administration of recombinant resistin to normal mice caused insulin resistance. However, in contrast to these findings, a study using several different obese models of mice (*ob/ob, db/db, tub/tub*, and KKA^{y}) showed a significant decrease in resistin expression (Way et al. 2001). It may be that serum resistin levels do not correlate with mRNA levels (Rajala and Scherer 2003). When antibodies that target resistin were administered to a dietary induced insulin resistant line of mice, insulin sensitivity was improved (Steppan et al. 2001). Additionally, when 3T3-L1 adipocytes were exposed to resistin, insulin-stimulated glucose uptake was reduced (Steppan et al. 2001). A transgenic mouse line with impaired resistin function showed improved glucose tolerance and insulin sensitivity (Kim et al. 2004). Further studies in humans also potentially link resistin with obesity and insulin resistance (Degawa-Yamauchi et al. 2003; Azuma et al. 2003; Smith et al. 2003), whilst others do not (Heilbronn et al. 2004). A

recent study conducted on Pima Indians concluded that resistin was associated with obesity but not insulin resistance (De Courten et al. 2004).

Given these results, it is evident that much of the data that has arisen from studies researching resistin is conflicting (reference Steppan and Lazar (2004) for in depth review). A major reason may be because the methodology is still fairly new and has not yet been standardized (Steppan and Lazar 2004). Further experimentation will shed light on resistin's mechanism and potential as a pharmacological target against obesity.

The Peroxisome Proliferator Activated Receptor (PPAR):

The PPAR family of transcription factors is part of the nuclear receptor superfamily and consists of three members, PPAR-α, PPAR-δ (also called PPAR-β) and PPAR-γ. Interestingly, they were discovered when peroxisome proliferation was induced using synthetic substances, and currently, more is known about interactions with synthetic ligands than natural ligands (Issemann and Green 1990; Dreyer et al. 1992; Kliewer et al. 1994; Krey et al. 1997; Desvergne and Wahli 1999; Ferre 2004). PPARs heterodimerize with retinoic acid receptor (RXR) before binding to a sequence on DNA termed the peroxisome proliferator response element (PPRE) (Kliewer et al. 1992), which consists of a direct repeat separated by one nucleotide (AGGTCANAGGTCA) (Tugwood et al. 1992). Each member is distributed in different tissues (Braissant et al. 1996; Su et al. 1998). PPAR-δ is expressed ubiquitously, and PPAR-α is predominantly expressed in hepatocytes, cardiomyocytes, kidney proximal tubule cells, skeletal muscle and enterocytes. PPAR-γ is expressed predominantly in adipose tissue, and will therefore be the PPAR member discussed further in this review.

The Peroxisome Proliferator Activated Receptor - Gamma (PPAR-γ):

Due to alternative splicing and promoter usage, there are three PPAR-γ transcripts (PPAR-γ1, PPAR-γ2 and PPAR-γ3) (Zhu et al. 1995). PPAR-γ1 and PPAR-γ3 code for the same protein, differing only by alternate promoters (Fajas et al. 1998). All three have 6 exons in common. PPAR-γ1 is comprised of eight coding exons, and PPAR-γ2 is comprised of seven. They have differing 5' untranslated regions, and PPAR-γ2 contains an additional 30 amino acids on the N-terminus between the second exon of PPAR-γ1 and the first common exon (Tontonoz et al. 1994; Zhu et al. 1995; Desvergne and Wahli 1999). The PPAR-γ2 isoform is predominant in rodent adipose tissue, and PPAR-γ1 is predominant in human adipose tissue (Ferre 2004).

Natural ligands that bind PPAR-γ include unsaturated fatty acids such as oleate, linoleate, cis-parinaric, eicosapentanoic and arachidonic acids; the most potent natural ligand that binds to PPAR-γ is the prostanoid 15 Deoxy-Δ12,14 - prostaglandin J2 (PGJ2) (Kliewer et al. 1995; Forman et al. 1995; Krey et al. 1997; Palmer and Wolf 1998; Ferre 2004). Unsaturated fatty acids bind at relatively low affinities and PGJ2 has not definitively been shown to exist *in vivo*. Therefore, finding an endogenous ligand to PPAR-γ is still a topic of heavy research (Rosen and Spiegelman 2001). Synthetic ligands that bind specifically to PPAR-γ belong to a class of drugs originally derived from clofibric acid, used to treat diabetes, called thiazolidinediones (TZDs) (Lehmann et al. 1995; Kliewer et al. 2001). These include troglitazone, pioglitazone, ciglitazone and rosiglitazone; currently pioglitazone and rosiglitazone are being used to treat type 2 diabetes (Desvergne and Wahli 1999; Ferre 2004).

In addition to having a role in type 2 diabetes, insulin sensitivity, atherosclerosis and cancer (see Rosen and Spiegelman 2001 for in depth review), PPAR-γ has been implicated in an essential role in differentiation of adipose tissue (adipogenesis) and lipid metabolism. Experiments have shown it binds the 5'-flanking region of the adipocyte protein 2 (aP2) gene (codes for adipose fatty acid binding protein), and induces expression of phosphoenolpyruvate carboxykinase (PEPCK) (Tontonoz et al. 1994; Tontonoz et al. 1995; Olswang et al. 2002). Interestingly, PEPCK has a PPRE that is functional only in adipose tissue (Devine et al. 1999; Ferre 2004). Numerous experiments conducted *in vitro* have shown that PPAR-γ is associated with differentiation of adipose tissue (Tontonoz et al. 1994; Chawla et al. 1994; Lehmann et al. 1995; Bastie et al. 1999; Gurnell et al. 2000) through interaction with the transcription factor CCAAT/enhancer-binding protein α (C/EBPα) (Wu et al. 1999). A study conducted using PPAR-$\gamma^{+/+} \leftrightarrow$ PPAR-$\gamma^{-/-}$ chimeric mice allowed fat cells to develop from either PPAR-$\gamma^{+/+}$ or PPAR-$\gamma^{-/-}$ cells, and showed that adipogenesis derived from the wildtype (PPAR-$\gamma^{+/+}$) cells, with little to no contribution from the null (PPAR-$\gamma^{-/-}$) cells (Rosen et al. 1999). This showed that PPAR-γ was essential for differentiation of adipose tissue *in vivo*. PPAR-γ is also involved in expression of resistin (Song et al. 2002; Patel et al. 2003). Experiments with PPAR-γ have established that it plays an essential role in adipocyte biology, but distinct mechanisms still remain unclear. The contributions that have been made with TZDs against diabetes and the promise that PPAR-γ can be utilized as a pharmacological target against cancer and atherosclerosis, make elucidating its mechanism a subject of intense research.

In Search for a Cure:

Given the multi-factorial nature of obesity, it is understandable that research for a cure or preventative has been approached from many different directions. Whilst environment and lifestyle play crucial roles, it has been shown that there are many physiological and genetic mechanisms that can be effectively targeted. A significant discovery from research responding to this epidemic is the de-lipidative effects of conjugated linoleic acid (CLA). Beneficial effects of CLA have been reported in cancer, atherosclerosis, diabetes and obesity, and have been heavily researched. Its effects are not only being studied for use as a pharmacological agent, but also as a tool to further our understanding of the mechanisms associated with obesity. Whilst effects of CLA are well documented, the mechanisms by which it exerts these effects remain unclear. The purpose of the remainder of this review will be to describe research that has been conducted on CLA, with a focus on its effects in obesity.

Conjugated Linoleic Acid; General Information:

The CLA family consists of several conjugated and stereoisomeric variations of linoleic acid (*cis, cis*-$\Delta^{9,12}$-octadecadienic acid), of which 16 have currently been identified (Sehat et al. 1998a; Sehat et al. 1998b; Rickert et al. 1999). Natural forms of CLA can be found predominantly in ruminant products, such as milk, cheese and beef (Ha et al. 1987; Chin et al. 1992; Parodi 1997; Lavillonnière et al. 1998; Sehat et al. 1998a), and exist primarily (80-90%) in the *cis*-9, *trans*-11 isoform (c9t11), also known as rumenic acid (Parodi 1977; Chin et al. 1992; Ma et al. 1999; Martin and Valeille 2002). Within ruminants, lamb contains the highest amount of CLA, and veal the lowest (Chin et al.

1992). Measurements of c9t11-CLA in human adipose tissue have found that its presence is highly correlated with milk fat intake (Jiang et al. 1999) and amounts to about 200 mg/d for men and 150mg/d for women (Ritzenthaler et al. 2001). Anaerobic ruminant bacteria, such as *Butyrivibrio fibrisolvens*, produce CLA (predominantly c9t11-CLA) through biohydrogenation of linoleic acid and α-linolenic acid obtained from plant material (Kepler et al. 1966; Pariza et al. 2001; Martin and Valeille 2002). A recent study has shown that endogenous synthesis is responsible for more than 91% of the c9t11-CLA present in milk fat (Kay et al. 2004). Upon synthesis, CLA is either absorbed or further metabolized to vaccenic acid (*trans*-11-octadecenoic acid) (Kepler et al. 1966), which can be converted back to c9t11-CLA by the enzyme Δ9 desaturase (also called stearoyl-CoA desaturase) (Pollard et al. 1980; Pariza et al. 2001).

Several methods are currently available to chemically synthesize CLA (Bretillon et al. 2003; Delmonte et al. 2003; Delmonte et al. 2004). Current CLA food supplements are synthesized by alkaline isomerization of linoleic acid enriched vegetable oils (e.g. safflower and sunflower oil) and are mostly available in a 1:1 ratio of c9t11- and t10c12-CLA (Martin and Valeille 2002; Terpstra 2004). Several companies offer dietary supplements containing CLA; examples include Your Life®, Natrol® and Nature's Way® (Figure 1.2) (Larsen et al. 2003). Recently, Loders Croklaan Lipid Nutrition reported they obtained self-affirmed GRAS status for their product Clarinol, potentially leading to its use in functional foods in the U.S. (Patton 2004). Cognis, a competing company, expects that their product Tonalin, will receive GRAS status later this year (2004) (Patton 2004). However, it should be noted that GRAS status is "self affirmed," and does not represent approval from the Food and Drug Administration. Even though CLA supplements are readily available, specific

benefits for humans appear to be relatively small and any detrimental effects are subject to further investigation (Reference Terpstra 2004 for detailed review).

The Effects of CLA in the Body:

In 1987 Ha et al. reported that CLA present in fried ground beef reduced tumor incidence in mice chemically treated to induce epidermal neoplasia (Ha et al. 1987); their results initiated a flurry of research (reference www.wisc.edu/fri/clarefs.htm for a list of publications on CLA since 1987) (Pariza et al. 2001). To date a majority of the experiments using CLA have used an isomeric mix containing approximately a 1:1 ratio of c9t11- and t10c12-CLA (approximately 40.8-41.1% and 43.5-44.9% respectively) (Pariza et al. 2001). These experiments, both *in vivo* and *in vitro*, have reported that CLA has beneficial effects against cancer, atherosclerosis, diabetes and obesity. Studies have also reported that results vary between species (Table 1.1), and that the c9t11- and t10c12-CLA isomers have differing effects on body composition. However, it now seems apparent that the t10c12-CLA isomer is responsible for changes in body composition and adipocyte morphology (Park et al. 1999; de Deckere et al. 1999; Pariza et al. 2001; Hargrave et al. 2002; Clement et al. 2002; Evans et al. 2002b). Described below is a brief synopsis of observations in cancer, atherosclerosis and diabetes, followed by a detailed review of CLA's effects on obesity; specifically focused on changes in gene expression and physiology of liver and adipose tissue.

Cancer:

The anti-carcinogenic effects of CLA were first observed in a mouse model treated with 7,12-dimethylbenz (a) anthracene to induce epidermal neoplasia (Ha et al. 1987). In

the same experiment, Ha et al. confirmed their observations by treating mice with CLA synthesized from linoleic acid by base isomerization. They also showed that CLA inhibited mutagenesis in bacteria (Ha et al. 1987). The group later described that CLA had antioxidant properties and inhibited tumorigenesis in the forestomach of mice (Ha et al. 1990). A similar effect was observed in rat mammary tissue; however in this study, maximum anti-oxidant activity was reported with 0.25% CLA supplementation and maximum anti-carcinogenic activity was with 1% supplementation, indicating that there may be different mechanisms associated with each effect (Ip et al. 1991). Studies conducted on human transplanted mammary and prostate cancer cells in severe combined immunodeficient mice, showed that CLA was effective in inhibiting metastasis and growth of the cancer cells independent of the immune system (Visonneau et al. 1997; Cesano et al. 1998; Belury 2002b). Effects of CLA on colon carcinogenesis in rats suggested that CLA was acting in a way other than direct interaction with the pro-carcinogen (Liew et al. 1995).

Analysis of liver and mammary tumor extracts showed that only the c9t11-CLA isomer was present, indicating that this was the isomeric form predominantly involved in the anti-carcinogenic effect (Ip et al. 1991). They later showed that butter fat (predominantly c9t11-CLA) was effective in reducing rat mammary carcinogenesis (Ip et al. 1999). However, an *in vitro* study conducted on human colorectal and prostatic cancer cells showed that the t10c12-CLA isomer had a large effect against colorectal cancer by activating caspase dependent apoptosis and decreasing bcl-2 (B-cell lymphoma 2) gene expression (Palombo et al. 2002; Ochoa et al. 2004). Recent studies have shown that CLA may exert its anti-carcinogenic effects through the cell cycle (Ip et al. 2001; Futakuchi et al. 2002; Majumder et al. 2002; Cao et al. 2004) and apoptotic pathway (Ip et al. 2000; Park et

al. 2001; Oh et al. 2003; Park et al. 2004; Ochoa et al. 2004). For a detailed review on potential mechanisms and CLA effects during different stages of tumor formation, cell proliferation and apoptosis, see Belury (2002b). The anti-carcinogenic properties of CLA observed both *in vivo* and *in vitro* are promising; however, there have been some experiments where an anti-carcinogenic effect was not observed (Wong et al. 1997; Petrik et al. 2000). Therefore, the use of CLA in cancer therapy is currently subject to further investigation.

Atherosclerosis:

Anti-atherosclerotic effects of CLA were initially observed in rabbits fed a diet containing 14% fat for 22 weeks. Rabbits whose diets contained CLA showed decreased total and low density lipoprotein (LDL) cholesterol and triglycerides, as well as decreased atherosclerotic plaque in the aorta (Lee et al. 1994). Later studies conducted in hamsters with diet-induced hypercholesterolemia showed similar results with reduced total plasma cholesterol (Nicolosi et al. 1997; Wilson et al. 2000). There have been conflicting observations regarding which isomers (c9t11- or t10c12-CLA) are predominantly responsible. A study conducted feeding hamsters a diet supplemented with either both isomers (c9t11- and t10c12-CLA) or pure c9t11-CLA found that the mixed isomers reduced plasma lipids, but c9t11-CLA did not (Gavino et al. 2000). A recent study reported opposite effects; hamsters that were on a c9t11-CLA supplemented high cholesterol diet showed a decrease in plasma cholesterol levels, while the mixed isomer had little effect (Valeille et al. 2004). Differences have also been observed between species. In C57BL/6 mice, CLA was reported to increase aortic fatty streak formation (potential early lesion of atherosclerosis) (Munday et al. 1999), but in humans no differences were observed in

platelet aggregation, thrombosis, plasma cholesterol, LDLs or TGs (Benito et al. 2001a; Benito et al. 2001b). In contrast, Truitt et al. (1999) showed that CLA did have an effect on human plate aggregation and suggested that it may also have anti-thrombotic properties (Truitt et al. 1999). Additionally, in a recent study with apoE$^{-/-}$ mice with pre-established atherosclerotic lesions, Toomey et al. (2003) showed that CLA prevented development of and caused degeneration of lesions (Toomey et al. 2003). Collectively, results from these studies are conflicting and a clear conclusion regarding the effects of CLA on atherosclerosis cannot be made.

Diabetes:

The anti-diabetogenic effects of CLA differ markedly between species, ranging from beneficial effects in rats to detrimental effects in mice and humans. In 1998 Houseknecht et al. showed that CLA was able to improve hyperinsulinemia and normalize glucose tolerance in Zucker diabetic fa/fa (ZDF) rats. They also reported an increase in aP2 mRNA levels and activation of PPAR-γ *in vitro*, indicative of modulation through this transcription factor (see below for further review on CLA's interactions with PPAR-γ) (Houseknecht et al. 1998). Interestingly, in contrast to this observation, Granlund et al. (2003) recently showed that t10c12-CLA did not activate PPAR-γ and selectively inhibited TZD-induced PPAR-γ activation, implicating t10c12-CLA as a PPAR-γ agonist (Taylor and Zahradka 2004). A later study conducted by Ryder et al. (2001) confirmed CLA's effects on glucose tolerance and insulin action. They reported improved insulin-stimulated glucose tolerance and glycogen synthase activity in soleus muscle of ZDF rats. They further showed that these effects were predominantly exerted by the t10c12-CLA isomer (Ryder et al. 2001). Similar studies with fa/fa Zucker rats have confirmed these results, and attributed

t10c12-CLA's anti-diabetogenic effects to reduced oxidative stress and muscle lipid levels (Henriksen et al. 2003; Taylor and Zahradka 2004). A recent study by Nagao et al. (2003b) showed that CLA increased adiponectin gene expression and plasma levels in ZDF rats. They proposed that this may also be a potential mechanism by which CLA reduces hyperinsulinemia (Nagao et al. 2003b). Opposite effects to those observed in rats have been reported in mice and humans. Several studies have shown that t10c12-CLA supplementation leads to insulin resistance in mice (DeLany et al. 1999; Tsuboyama-Kasaoka et al. 2000; Clement et al. 2002; Roche et al. 2002) and humans (Riserus et al. 2002; Riserus et al. 2004b); this may be due to a decrease in plasma leptin levels (Wang and Jones 2004), or an increase in triacylglycerol levels in the muscle (Terpstra 2004). The paradoxical effect of CLA in diabetes is a dramatic example of the species specific differences that exist with supplementation of this fatty acid.

Obesity:

The de-lipidative effects of CLA were first observed by Park et al. (1997) in the ICR line of mice supplemented with 0.5% CLA; they reported approximately a 60% decrease in body fat after about four to five weeks of feeding (Park et al. 1997). Since then, effects of CLA on body composition in different lines of mice have been heavily investigated and have shown similar results (Pariza et al. 2001). Reductions in adiposity have been reported in Sprague-Dawley and Zucker (lean) rats; however, the effects are not as drastic as in mice (25-30%) (Azain et al. 2000; Sisk et al. 2001; Yamasaki et al. 2003). Interestingly, the effect was reversed in obese Zucker and albino rats, increasing deposition of fat (Szymczyk et al. 2000; Sisk et al. 2001). Sisk et al. (2001) showed that CLA reduced insulin levels in obese Zucker rats, and suggested that the increased fat could be attributed to a normalized

glucose tolerance, coupled with hyperphagia. A recent study conducted on rats varied the source of protein (either casein or soy) in CLA supplemented diets (Akahoshi et al. 2004). Their results showed that the de-lipidative effect of CLA was more pronounced in rats fed the soy diet, indicating that dietary protein may alter the effects of CLA and contribute to differing observations between experiments (Akahoshi et al. 2004). In swine CLA decreased fat deposition and increased lean tissue (Dugan et al. 1997; Ostrowska et al. 1999; Thiel-Cooper et al. 2001; Wiegand et al. 2002; Ostrowska et al. 2003b). Generally, experiments conducted in humans have shown that CLA does not have a significant effect on body weight (Larsen et al. 2003; Terpstra 2004; Malpuech-Brugere et al. 2004a). In a randomized double-blind trial using 60 overweight or obese people, Blankson et al. (2000) showed that CLA reduced body fat mass but had no effect on BMI (Blankson et al. 2000). Similar effects have been reported in other human trials (Smedman and Vessby 2001; Thom et al. 2001; Riserus et al. 2004a). It should be noted that the loss of body fat is 40-50% greater in mice than observed in humans (Terpstra 2004).

Feed Intake:

An issue that has remained controversial is the effect of CLA on feed intake. Several studies in mice, rats and pigs have reported that CLA has little to no effect on feed intake (Ostrowska et al. 1999; DeLany et al. 1999; DeLany and West 2000; Azain et al. 2000; West et al. 2000; Sisk et al. 2001; Thiel-Cooper et al. 2001; Terpstra et al. 2002; Wiegand et al. 2002; Yamasaki et al. 2003), while others have reported a reduction in feed intake (Dugan et al. 1997; West et al. 1998; Szymczyk et al. 2000; Miner et al. 2001; Ohnuki et al. 2001; Ryder et al. 2001; Hargrave et al. 2002; Ostrowska et al. 2003b). However, studies conducted in mice and rats with a pair-fed group on a CLA supplemented

diet confirmed a significant decrease in fat pad mass compared to controls, indicating that a reduction in feed intake could not solely account for fat mass reduction (Ryder et al. 2001; Ntambi et al. 2002; Hargrave et al. 2002). It is possible that CLA has either an averse organoleptic quality, or alters metabolism in such a way as to impart a reduction in feed intake. Given the phenotypic effects reported in the literature, the latter possibility is most probable; however, confirmation hinges upon further investigation.

Lipid Metabolism:

Several studies have shown that CLA incorporates into membrane phospholipids and alters fatty acid homeostasis (Ha et al. 1990; Belury and Kempa-Steczko 1997; Sebedio et al. 2001; Banni et al. 2001; Belury 2002a; Kelley et al. 2004). Upon absorption, CLA that is not catabolized through β-oxidation by hepatocytes is converted into a conjugated 18:3 product by Δ6 desaturase and then further elongated and desaturated into conjugated 20:3 and 20:4 (Belury and Kempa-Steczko 1997; Sebedio et al. 2001; Berdeaux et al. 2002; Gruffat et al. 2003). The physiological effects of CLA may in part be due to competition with linoleate as substrate for Δ6 desaturation. This is the rate-limiting step for arachidonate formation from linoleate, and implicates CLA in reduced arachidonate accumulation in phospholipids and subsequently, reduced eicosanoid production (Belury and Kempa-Steczko 1997; Belury 2002a; Brown et al. 2003).

In addition to adipose tissue, a major organ affected by CLA treatment is the liver. When supplemented with CLA, the liver becomes steatotic and increases in mass up to four times, an effect exerted predominantly by the t10c12-CLA isomer (Belury and Kempa-Steczko 1997; Tsuboyama-Kasaoka et al. 2000; Clement et al. 2002; Degrace et al. 2003; Kelley et al. 2004). A study in mice attributed this to an increase in liver triglycerides,

cholesterol, cholesterol esters and FFAs (Kelley et al. 2004); opposite effects on liver triglycerides have been reported in the rat (Sebedio et al. 2001). The t10c12-CLA isomer also was associated with an increase in 18:1 n-9 and a decrease in 18:2 n-6 (Kelley et al. 2004) and has been shown to alter fatty acid profiles in rats and pigs (Sebedio et al. 2001; Banni et al. 2001; Ostrowska et al. 2003a). Other experiments, conducted *in vivo* and *in vitro* have confirmed a shift in the ratio of saturated fatty acids to monounsaturated fatty acids (SFA:MUFA), in particular palmitate:palmitoleate (16:0/16:1) and stearate:oleate (18:0/18:1) with t10c12-CLA supplementation (Lee et al. 1998; Choi et al. 2000; Sebedio et al. 2001; Gatlin et al. 2002; Eder et al. 2002; Evans et al. 2002a; Brown et al. 2003) (for a review on effects between species reference Evans et al. (2002a)). This may be caused by a reduction in stearoyl-CoA desaturase-1 (discussed later in this review), an enzyme that catalyzes the biosynthesis of monounsaturated fatty acids and prefers 16:0 and 18:0 as substrates (Lee et al. 1998; Kang et al. 2004). In a recent study the early effects of CLA in mice found no consequence on the SFA:MUFA ratio after four days of supplementation (Xu et al. 2003), indicating that the change may be due to downstream, as opposed to direct effects of CLA.

How Does CLA Work? Mechanistic Elucidation:

Currently, mechanisms by which CLA imparts its dramatic effects in liver and adipose tissue are largely unknown. A review of the literature indicates that it reduces adiposity by increasing energy expenditure, apoptosis, fatty acid oxidation, and lipolysis, as well as decreasing energy intake, stromal vascular cell differentiation, and lipogenesis (Figure 1.3). In an effort to further elucidate the de-lipidative mechanisms of CLA, several

groups have conducted experiments analyzing protein and gene expression of molecules involved in metabolism of the liver and adipose tissue. A summary of this literature with respect to gene expression is provided in Table 1.2.

Adipose Tissue:

One of the methods by which CLA imparts its effects is by increasing energy expenditure (West et al. 1998; DeLany and West 2000; West et al. 2000; Ohnuki et al. 2001). Several experiments have offered insight into mechanisms by which energy is expended. Currently, the uncoupling proteins have been of particular interest in energy expenditure and oxidation. Uncoupling proteins (UCPs) are a family of several molecules that include UCP1, UCP2 and UCP3 (Adams 2000). Predominantly expressed in the mitochondrial inner membrane of brown adipose tissue, UCP1 forms a proton channel that leaks protons that would otherwise be used as substrate for ATP synthesis, resulting in the production of heat (Argyropoulos and Harper 2002). Precise functions of UCP2 and UCP3 are currently unclear, but have been implicated in regulation of insulin secretion and fatty acid metabolism, respectively (Adams 2000; Rousset et al. 2004); UCP2 is generally expressed ubiquitously, and UCP3 is predominantly expressed in skeletal muscle (Erlanson-Albertsson 2003). Upon CLA supplementation, several studies have reported an increase in UCP2 expression (Tsuboyama-Kasaoka et al. 2000; West et al. 2000; Ryder et al. 2001; Takahashi et al. 2002; Ealey et al. 2002; Kang et al. 2004) in brown and white adipose tissue and either no effect (West et al. 2000) or a decrease in UCP1 and UCP3 expression (Takahashi et al. 2002; Ealey et al. 2002). Given these results, the UCPs may not be involved in CLA mediated increased energy expenditure and de-lipidation of adipose tissue (West et al. 2000; Ealey et al. 2002).

In addition to increasing energy expenditure, CLA reduces adipose tissue mass by initiating apoptosis and modulating differentiation of pre-adipocytes. Using the polygenic obese line of mice (M16), Miner *et al*. showed that apoptosis in retroperitoneal fat pads of mice fed CLA was 4-fold greater than observed in control mice (Miner et al. 2001). They reported approximately a 50% decrease in the weight of retroperitoneal fat pads and approximately a 40% decrease in epididymal fat pads after five days of treatment (Miner et al. 2001). In later work, Hargrave et al. confirmed that the t10c12-CLA isomer was predominantly responsible for this effect (Hargrave et al. 2002). Interestingly, they also reported that effects of t10c12-CLA were independent of genetic strain (Hargrave et al. 2002). Studies have shown that an increase in TNF-α (a cytokine that has been shown to induce leptin production, lipolysis, adipocyte de-differentiation, as well as apoptosis of pre- and mature adipocytes (Prins et al. 1997)) expression in adipose tissue resulted from CLA supplementation, further indicating that apoptosis is a probable mechanism in the de-lipidative effects of CLA (Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003).

It also has been shown that CLA imparts an inhibitory effect on stromo-vascular pre-adipocyte proliferation and differentiation (Satory and Smith 1999; Brodie et al. 1999; Evans et al. 2000a). While at first there was some conflict (Choi et al. 2000), it now seems that modulation of pre-adipocyte differentiation by CLA appears to be driven in part by downregulation of PPAR-γ expression (Brown and McIntosh 2003). CLA itself may be a ligand for this transcription factor (McNeel et al. 2003). An exception is CLA's interactions with porcine adipocytes, which have shown either an opposite or no effect on PPAR-γ (Ding et al. 2000; McNeel and Mersmann 2003). It should be noted, that McNeel

and Mersmann (2003) observed that CLA may have had an inhibitory effect on differentiation after two days of treatment.

With exception to studies in rats that have reported activation of PPAR-γ and an increase in aP2 expression (Houseknecht et al. 1998; Belury et al. 2002), both *in vivo* studies in mice (Tsuboyama-Kasaoka et al. 2000; Takahashi et al. 2002; Kang et al. 2004) and *in vitro* studies in human (Brown et al. 2003; Brown et al. 2004) and 3T3-L1 adipocytes (Evans et al. 2000b; Kang et al. 2003; Granlund et al. 2003) have confirmed that, upon CLA supplementation there is a decrease in PPAR-γ expression, indicating a reduction in differentiation of pre- to mature adipocytes. This was shown to be an effect predominantly exerted by the t10c12-CLA isomer (Kang et al. 2003; Brown et al. 2003; Granlund et al. 2003). Additionally in these species, there is a decrease in C/EBP-α and PPAR-γ target genes such as aP2, perilipin-A and acyl-CoA binding protein (ACBP) (Kang et al. 2003; Brown et al. 2003; Granlund et al. 2003). Interestingly, Brown et al. (2003) observed an increase in leptin gene expression in contrast to studies that have reported a significant decrease in mice and rats (Takahashi et al. 2002; Warren et al. 2003; Nagao et al. 2003a) and no effect, or a decrease in serum leptin levels in humans and rats respectively (Medina et al. 2000; Riserus et al. 2002; Yamasaki et al. 2003; Petridou et al. 2003). However, in a recent study Brown et al. (2004) confirmed their previous observations and went on to show that t10c12-CLA increases production and secretion of the cytokine, IL-6 and the chemokine, IL-8 from stromo-vascular cells. They propose a mechanism where, upon secretion from stromo-vascular cells, IL-6 and IL-8 bind to their respective receptors on the surface of adipocytes (Brown et al. 2004). This leads to phosphorylation of transcription factors through induction of MEK/ERK signaling, that in

turn inhibits expression of PPAR-γ and its downstream targets (Brown et al. 2004). This would then lead to decreased cellular TG content, as well as fatty acid and glucose uptake (Brown et al. 2004), which has been previously reported (Evans et al. 2000a; Evans et al. 2000b; Kang et al. 2003; Brown et al. 2003).

In agreement with observations on glucose and fatty acid uptake, there have been several studies that have reported a reduction in expression of lipoprotein lipase (LPL: fatty acid uptake) ((Park et al. 1997; Park et al. 1999; Lin et al. 2001; Kang et al. 2003; Xu et al. 2003) and GLUT-4 (Takahashi et al. 2002; Brown et al. 2003). Several studies have further analyzed the effects of CLA on energy metabolism in adipose tissue. They showed that t10c12-CLA caused an increase in fatty acid oxidation in 3T3-L1 adipocytes, and suggested that this may be one mechanism by which it lowers TG content (Park et al. 1997; Evans et al. 2002b). Similar effects have also been reported in rats (Sergiel et al. 2001). Associated with these observations, there is an increase in carntine palmitoyl transferase (CPT) activity (Park et al. 1997; Rahman et al. 2001), a mitochondrial membrane bound protein essential for shuttling long chain fatty acids into mitochondria where they undergo β-oxidation. However, Brown et al. (2004) reported a decrease in fatty acid oxidation in human adipocytes, adding to observations of differing effects between species. With exception to UCPs, there have been no studies analyzing differential gene expression response to CLA associated with fatty acid oxidation in adipose tissue.

Currently, effects of CLA on lipolysis are conflicting. Several *in vitro* studies have reported an increase in lipolysis in response to CLA (Park et al. 1997; Brown et al. 2003), while no effect has been reported *in vivo* in mice (Xu et al. 2003). Interestingly, Brown et al. (2003) previously reported a decrease in expression of hormone sensitive lipase, a key

enzyme in the lipolytic cycle, suggesting a possible reduction in lipid breakdown. Therefore, it should be noted that effects of CLA on gene expression may not accurately correspond to protein expression.

In association with a decrease in LPL activity, several studies have reported a decrease in lipogenesis (Brown et al. 2001; Evans et al. 2002b; Brown et al. 2004; Wang and Jones 2004). Two studies attribute the predominant CLA de-lipidative effect to lipogenesis inhibition, especially in early response to CLA (4 days) supplementation, instead of increased lipolysis (Brown et al. 2001; Xu et al. 2003). Lin et al. (2004) recently reported that t10c12-CLA was a more potent inhibitor of de novo lipogenesis in the mammary gland of lactating mice. At the gene expression level, there is a reported reduction in acetyl-CoA carboxylase (ACC) (Tsuboyama-Kasaoka et al. 2000; Brown et al. 2003; Peterson et al. 2003b; Lin et al. 2004), fatty acid synthase (FAS) (Tsuboyama-Kasaoka et al. 2000; Kang et al. 2003; Peterson et al. 2003b; Lin et al. 2004) and stearoyl-CoA desaturase-1 (SCD-1) (in mammary tissue) (Peterson et al. 2003b; Lin et al. 2004), as well as the glycolytic/lipogenic enzyme glycerol dehydrogenase (Brown et al. 2003). Interestingly, Kang et al. (2004) reported an increase in SCD-2 expression in adipose tissue, with a decrease in 18:0/18:1. A recent study found that milk fat depression in dairy cattle resulted in increased levels of endogenous t10c12-CLA in milk fat which was correlated with a reduction in mRNA levels of ACC, FAS, LPL, and glycerol phosphate acyltransferase (Peterson et al. 2003a). Tsuboyama-Kasaoka et al. (2000) also reported a decrease in sterol regulatory binding protein-1 (SREBP-1) expression. The SREBPs represent a family of transcription factors that include SREBP-1a, SREBP-1c and SREBP-2. Collectively, SREBPs are involved in transcriptional activation of more than 30 genes

associated with cholesterol, fatty acid, TG and phospholipid synthesis (Horton et al. 2002). SREBP-1a can activate all of the SREBP-responsive genes, SREBP-1c is more specifically associated with fatty acid synthesis, as is SREBP-2 with cholesterol synthesis (Horton et al. 2002). Once cleaved by proteolysis, SREBP-1 releases a fragment that translocates to the nucleus and activates transcription (Peterson et al. 2003b). Treatment of a bovine mammary cell line (MAC-T) with t10c12-CLA did not yield a reduction in SREBP-1 mRNA concentration; however, there was a reduction of the SREBP-1 nuclear fragment, indicating that t10c12-CLA may reduce lipogenesis and lipogenic gene mRNA concentration by inhibiting the proteolytic cleavage of SREBP-1 (Peterson et al. 2003b).

Taken together, these data suggest that CLA imparts its de-lipidative activity through both metabolism and cell cycle control. Further research will be necessary to elucidate the basis for differences between species and confirm *in vitro* observations *in vivo*.

Liver:

Currently, mechanisms by which the liver becomes steatotic in response to CLA are unknown. Several studies have reported an increase in both liver fatty acid synthesis and oxidation in response to CLA supplementation (Belury et al. 1997; Moya-Camarena et al. 1999; Peters et al. 2001). Takahashi et al. (2003) confirmed these observations at both the gene and protein level in two lines of mice (ICR and C57BL/6J); however, the degree of increase differed between lines for some enzymes. For fatty acid synthesis, they reported an increase in activity and mRNA levels of ACC, FAS, ATP-citrate lyase and malic enzyme (Takahashi et al. 2003). Tsuboyama-Kasaoka et al. (2003) also reported an increase in ACC mRNA level, but not FAS. For fatty acid oxidation, Takahashi et al.

(2003) reported an increase in acivity of mitochondrial and peroxisomal palmitoyl-CoA oxidation, CPT, peroxisomal acyl CoA oxidase (ACO), 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, coupled with an increase in mRNA level of CPT I and II, trifunctional enzyme-α and -β, ACO and bifunctional enzyme. Several other experiments also reported that CLA causes an increase in CPT activity, with a greater effect being exerted by the t10c12-CLA isomer (Martin et al. 2000; Degrace et al. 2004). Degrace et al. (2004) recently described an increase in CPT I activity in mice supplemented with t10c12-CLA, along with an increased rate of carnitine-dependent palmitate oxidation. They also reported a 100% increase in expression of the CPT I liver isoform, a 200% increase in the CPT I muscle isoform, and almost doubled expression of CPT II compared to controls (Degrace et al. 2004). This was accompanied by an increase in ACO activity and gene expression (Degrace et al. 2004). Similar increases in ACO gene expression have been previously reported (Belury et al. 1997; Warren et al. 2003). Degrace et al. (2004) also observed an increase in mitochondrial and peroxisomal fatty acid oxidation capacity when measured *in vitro*, and suggested that hepatic steatosis is not a result of decreased oxidation (Degrace et al. 2004).

Since PPAR-α has been shown to regulate expression of enzymes associated with hepatic oxidation (Kersten et al. 1999; Peters et al. 2001), CLA may mediate its effects through this transcription factor. Indeed, both the c9t11- and t10c12-CLA isomers have been shown to be potent ligands of PPAR-α, and in addition to ACO, increase expression of cytochrome P450A1 (CYP4A1: ω-hydroxylation of fatty acids) and liver fatty acid binding protein, known gene targets of PPAR-α regulation (Moya-Camarena et al. 1999; Warren et al. 2003). However, a study conducted by Peters et al. (2001) in PPAR-α null

mice, showed that genes coding liver fatty acid oxidation and fatty acid binding were affected by CLA, and were therefore modulated independent of PPAR-α. Additionally, Warren et al. (2003) reported a decrease in PPAR-α expression by t10c12-CLA while still observing an increase in ACO (but not CYP4A1); however, PPAR-α expression was increased by c9t11-CLA. Therefore, it is probable that the effect of t10c12-CLA is not solely dependent on PPAR-α.

Effects of CLA on SFA:MUFA may be due to a decrease in SCD-1 expression that results from t10c12-CLA supplementation (Lee et al. 1998; Choi et al. 2000; Eder et al. 2002). However, a recent study using SCD-1 null mice, showed that t10c12-CLA exerts its de-lipidative effects independent of SCD-1 (Kang et al. 2004). Interestingly, SCD-1 null mice had a heavier liver mass, but displayed reduced hepatomegaly compared to wildtype (Kang et al. 2004). Supplementation with t10c12-CLA significantly reduced adipose tissue mass but did not significantly increase fat accumulation in liver or muscle, potentially confirming observations in earlier experiments that CLA increases energy expenditure (West et al. 1998; DeLany and West 2000; West et al. 2000; Kang et al. 2004). Use of knockout mice has proven to be a valuable tool in clarifying proposed CLA mechanisms. Evidence that CLA may be working either independently or dependently on SCD-1 or PPAR-α indicates that it may exert its effects through multiple mechanisms.

Statistical Analysis of Microarray and Quantitative Reverse Transcriptase PCR (QRT-PCR) Data:

Microarray analysis is a high throughput genomic technique that measures changes in expression of tens of thousands of genes simultaneously. Probes can be spotted onto

glass slides by several methods, one of the most common being an ink jet printer which sprays picoliter droplets of DNA under pressure (Gibson and Muse 2002). Treatment and control RNA is reverse transcribed into cDNA and labeled with either Cyanine-3 (Cy-3: green) or Cyanine-5 (Cy-5: red). Treatment and control cDNAs competitively hybridize to probes on the same array; therefore, samples whose gene is present in higher abundance will fluoresce more intensely. A ratio of the treatment:control fluorescence is indicative of a change in relative gene expression. However, the fluorescence intensities of each dye must be balanced to account for any bias that may occur as a result of differences in hybridization efficiency, heat and light sensitivity and scanner settings (Leung and Cavalieri 2003). Additionally, before ratios are measured, spots that are of too low an intensity (usually 2 standard deviations above background signal) are excluded (Leung and Cavalieri 2003). Background fluorescence is subtracted from the remaining spots to determine a more precise value (Gibson and Muse 2002). Once a ratio is obtained it is normalized by calculating the log base 2, thereby symmetrically distributing the data around zero (Gibson and Muse 2002). Ratios are then centered to produce a mean ratio of 1. The data may then be further normalized by utilizing the scatter plot smoother method, LOWESS, which is described in detail by Yang et al. (2002). Several software programs, many of which utilize proprietary error models, are now available that will further analyze data to determine statistical significance. Each software is fairly unique and may be more suitable for some projects than others; therefore it is recommended that the researcher study the market carefully before making a decision. It is also possible to utilize an analysis of variance (ANOVA) approach which works in two steps. First, after calculating the log base 2 of the fluorescence ratios, the dye and array effects are removed by normalizing the arrays

and dye channels within the arrays relative to one another, thereby normalizing expression levels relative to the sample mean (Gibson and Muse 2002). The treatments within a replicate are then compared to determine if the variability within an observation is less than the variability in other observations (Gibson and Muse 2002). Significance can also be determined using Student's t-test (Leung and Cavalieri 2003).

Often the relative gene expression from microarray experiments are validated using QRT-PCR. This method can also be used to determine absolute quantification of mRNA concentration. A method commonly used for relative quantification is the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen 2001). Briefly, $\Delta\Delta C_T$ is the difference between ΔC_T of the sample and ΔC_T of the calibrator (control). The difference between C_T of the target gene and housekeeping gene (determined to be unaffected by treatment or other variables in experiment) is ΔC_T, this subtraction step serves to normalize the data. The end result is a number that is representative of the gene expression relative to the calibrator. Error associated with the $2^{-\Delta\Delta C}{}_T$ method is asymmetric; however, it is possible to take the average of the range of error and determine significance using Student's t-test.

Conclusion:

The incidence of obesity has risen to epidemic proportions in the past two or three decades, and costs attributed to this disease are in the billions of dollars. With no sign of relent and a lack of medication that is both safe and effective, it has now become crucial for research spanning many areas of science to pursue a method that will attenuate this problem. No longer considered just a site for storage, adipose tissue is now recognized as an endocrine organ that secretes hormones (adipokines) such as leptin, adiponectin and

resistin. Implicated in roles associated with obesity and diabetes, adipokines display much potential as pharmacological targets against these diseases. Environment has been suggested to be a predominant factor in the obesity epidemic, and given the rapid onset of obesity, this is probably true. However, it is clear that obesity is a multi-factorial problem, and the role of genetics should not be underestimated. Use of animal models that closely mimic the human condition, such as the polygenic obese line of mice, M16, have been and will continue to be an indispensable tool for further elucidation of the physiological mechanisms associated with obesity.

A compound that has arisen from cancer research is CLA. In addition to its anti-carcinogenic qualities, CLA has beneficial effects against atherosclerosis, diabetes and obesity. Interestingly, these effects vary between different species, and as shown with diabetes, can be beneficial in one, and detrimental in another. Currently the precise reason for this is unknown. However, the de-lipidative effects of CLA have been replicated in several species and have thus been a topic of intense research. Despite this investigative flurry, the precise mechanisms by which CLA elicits its dramatic effects in adipose tissue and liver are still largely unknown. Whilst a reduction in feed intake, as reported in some experiments, may account for some decrease in adipose tissue mass, pair-feeding trials have established that it is not solely responsible for this effect. Indeed, given the dramatic effects of CLA, it is probable that diminution in energy intake may itself be a result of perturbations on metabolic homeostasis, rather than some organoleptic quality of the molecule.

In vivo and *in vitro* analyses of physiological modifications imparted by CLA on protein and gene expression, have confirmed that CLA exerts its de-lipidative effects by

modulating energy expenditure, apoptosis, fatty acid oxidation, lipolysis, stromal vascular cell differentiation, and lipogenesis (Figure 1.3). It is probable that this is accomplished, in part, through a signal transduction mechanism, as suggested by Brown et al. (2004). Use of functional genomic techniques, such as microarray analysis, to analyze effects of CLA on adipose tissue would make a tremendous contribution towards mechanistic elucidation. Towards this endeavor, we conducted an experiment analyzing the effects of t10c12-CLA on gene expression in the adipose tissue of the polygenic obese line of mice, M16. This is the first experiment that has employed a nutrigenomic approach to analyze gene expression changes during de-lipidation of adipose tissue, making the potentially novel results of this experiment imperative to the growing understanding of genetically induced obesity, as well as dietary and pharmacological methods for its treatment.

Chapter 1 Tables

Table 1.1a Comparison of reported anti-carcinogenic effects of CLA in the mouse, rat and human. +: beneficial effects; -:negative effect; ∅: no effect.

Species	Cancer	Ref
Mouse	+	(Ha et al. 1987; Ha et al. 1990; Ip et al. 1991; Liew et al. 1995; Visonneau et al. 1997; Cesano et al. 1998; Belury 2002b)
	∅	(Wong et al. 1997; Petrik et al. 2000)
Rat	+	(Ip et al. 1999; Ip et al. 2000; Ip et al. 2001; Park et al. 2001; Futakuchi et al. 2002; Park et al. 2004)
Human	+	(Palombo et al. 2002; Majumder et al. 2002; Oh et al. 2003; Ochoa et al. 2004; Cao et al. 2004)

Table 1.1b Comparison of reported anti-atherogenic effects of CLA in the mouse, rabbit, hamster and human. +: beneficial effects; -:negative effect; ∅: no effect.

Species	Atherosclerosis	Ref
Mouse	+	(Toomey et al. 2003)
	-	(Munday et al. 1999)
Rabbit	+	(Lee et al. 1994)
Hamster	+	(Nicolosi et al. 1997; Wilson et al. 2000; Gavino et al. 2000; Valeille et al. 2004)
Human	+	(Truitt et al. 1999)
	∅	(Benito et al. 2001a; Benito et al. 2001b)

Table 1.1c Comparison of reported anti-diabetogenic effects of CLA in the mouse, rat, and human. +: beneficial effects; -:negative effect; ∅: no effect.

Species	Diabetes	Ref
Mouse	-	(DeLany et al. 1999; Tsuboyama-Kasaoka et al. 2000; Clement et al. 2002; Roche et al. 2002)
Rat	+	(Houseknecht et al. 1998; Ryder et al. 2001; Henriksen et al. 2003; Nagao et al. 2003b; Taylor and Zahradka 2004)
Human	-	(Riserus et al. 2002; Terpstra 2004; Riserus et al. 2004b)

Table 1.1d Comparison of reported de-lipidative effects of CLA in the mouse, rat, pig and human. +: beneficial effects; -:negative effect; ∅: no effect.

Species	Obesity	Ref
Mouse	+	(Park et al. 1997); See Pariza et al. (2001) for comprehensive list of references.
Rat	+ (lean)	(Azain et al. 2000; Sisk et al. 2001; Yamasaki et al. 2003) (Akahoshi et al. 2004)
	- (obese)	(Szymczyk et al. 2000; Sisk et al. 2001)
Pig	+	(Dugan et al. 1997; Ostrowska et al. 1999; Thiel-Cooper et al. 2001; Wiegand et al. 2002; Ostrowska et al. 2003b)
Human	+*	(Blankson et al. 2000; Smedman and Vessby 2001; Thom et al. 2001; Riserus et al. 2004a)
	∅	(Larsen et al. 2003; Terpstra 2004; Malpuech-Brugere et al. 2004b)

* Note that CLA reduced body fat mass but had no effect on BMI.

Table 1.2 List of genes reported to be effected by the de-lipidative effects of CLA. Genes are classified by tissue and ascribed function.

Gene	Eff.	Tissue	Species	Literature
Lipoprotein lipase	↓	Adipose	Mouse	(Park et al. 1997; Park et al. 1999; Lin et al. 2001; Kang et al. 2003; Xu et al. 2003)
Fatty acid binding protein	↑	Liver	Rat	(Moya-Camarena et al. 1999)
aP2	↓	Adipose	Mouse	(Kang et al. 2003; Granlund et al. 2003)
			Human	(Brown et al. 2003; Granlund et al. 2003)
	↑		Rat	(Houseknecht et al. 1998)
Acyl-CoA binding protein	↓	Adipose	Human	(Brown et al. 2003)
Glycerol-3-phosphate acyltransferase	↓	Mammary	Bovine	(Peterson et al. 2003a)
Perilipin	↓	Adipose	Human	(Brown et al. 2003)
Glucose transporter 4	↓	Adipose	Mouse	(Takahashi et al. 2002)
			Human	(Brown et al. 2003)
ATP-citrate lyase	↑	Liver	Mouse	(Takahashi et al. 2003)
Glycerol-3-phosphate dehydrogenase	↓	Adipose	Human	(Brown et al. 2003)

Table 1.2 (continued)

Acetyl CoA carboxylase	↓	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003)
			Human	(Brown et al. 2003)
		Mammary	Mouse	(Lin et al. 2004)
			Bovine	(Peterson et al. 2003b)
	↑	Liver	Mouse	(Takahashi et al. 2003)
	↔			(Lin et al. 2004)
Fatty acid synthase	↓	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000; Kang et al. 2003)
		Mammary		(Lin et al. 2004)
			Bovine	(Peterson et al. 2003b)
	↑	Liver	Mouse	(Takahashi et al. 2003)
	↔			(Lin et al. 2004)
Malic enzyme	↑	Liver	Mouse	(Takahashi et al. 2003)
Stearoyl-CoA Desaturase 1	↓	Adipose	Mouse	(Choi et al. 2000)
		Mammary		(Lin et al. 2004)
			Bovine	(Peterson et al. 2003b)
		Liver	Mouse	(Lee et al. 1998)
	↔			(Lin et al. 2004)
Stearoyl-CoA Desaturase 2	↑	Adipose	Mouse	(Kang et al. 2003)
Cytochrome P450	↑	Liver	Mouse	(Warren et al. 2003)
	↔		Rat	(Moya-Camarena et al. 1999)
Hormone sensitive lipase	↓	Adipose	Human	(Brown et al. 2003)
Peroxisomal acyl-CoA oxidase	↑	Adipose	Mouse	(Belury et al. 1997; Warren et al. 2003; Takahashi et al. 2003; Degrace et al. 2004)
Peroxisomal bifunctional enzyme	↑	Liver	Mouse	(Takahashi et al. 2003)
Trifunctional enzyme-α	↑	Liver	Mouse	(Takahashi et al. 2003)
Trifunctional enzyme-β	↑	Liver	Mouse	(Takahashi et al. 2003)
Carnitine palmitoyl-transferase I (liver)	↑	Liver	Mouse	(Takahashi et al. 2003; Degrace et al. 2004)
Carnitine palmitoyl-transferase I (muscle)	↑	Liver	Mouse	(Degrace et al. 2004)
Carnitine palmitoyl-transferase II	↑	Liver	Mouse	(Takahashi et al. 2003)

Table 1.2 (continued)

Uncoupling protein-1	↓	Adipose (Brown)	Mouse	(Takahashi et al. 2002; Ealey et al. 2002)
	↔			(West et al. 2000)
			Rat	(Ryder et al. 2001; Ealey et al. 2002)
Uncoupling protein-2	↑	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000; West et al. 2000; Ryder et al. 2001; Takahashi et al. 2002; Ealey et al. 2002; Kang et al. 2004)
		Mammary		(Ealey et al. 2002)
	↔	Adipose	Rat	(Ealey et al. 2002)
Uncoupling protein-3	↓	Adipose	Mouse	(Takahashi et al. 2002; Ealey et al. 2002)
	↔		Rat	(Ealey et al. 2002)
Leptin	↑	Adipose	Mouse	(Takahashi et al. 2002; Warren et al. 2003)
			Rat	(Nagao et al. 2003a)
	↓		Human	(Brown et al. 2003)
Adiponectin	↓	Adipose	Mouse	(Warren et al. 2003)
Interleukin-6	↑	Adipose	Human	(Brown et al. 2004)
Interleukin-8	↑	Adipose	Human	(Brown et al. 2004)
Peroxisome proliferator activated receptor-α	↓	Liver	Mouse	(Warren et al. 2003)
Peroxisome proliferator activated receptor-γ	↓	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000; Evans et al. 2000b; Takahashi et al. 2002; Kang et al. 2003; Granlund et al. 2003; Kang et al. 2004)
			Human	(Brown et al. 2003; Granlund et al. 2003; Brown et al. 2004)
Sterol Regulatory Element Binding Protien 1	↓	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000)
CAAT/enhancer binding protein-α	↓	Adipose	Human	(Brown et al. 2003)
Tumor Necrosis Factor-α	↑	Adipose	Mouse	(Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003)

Chapter 1 Figures

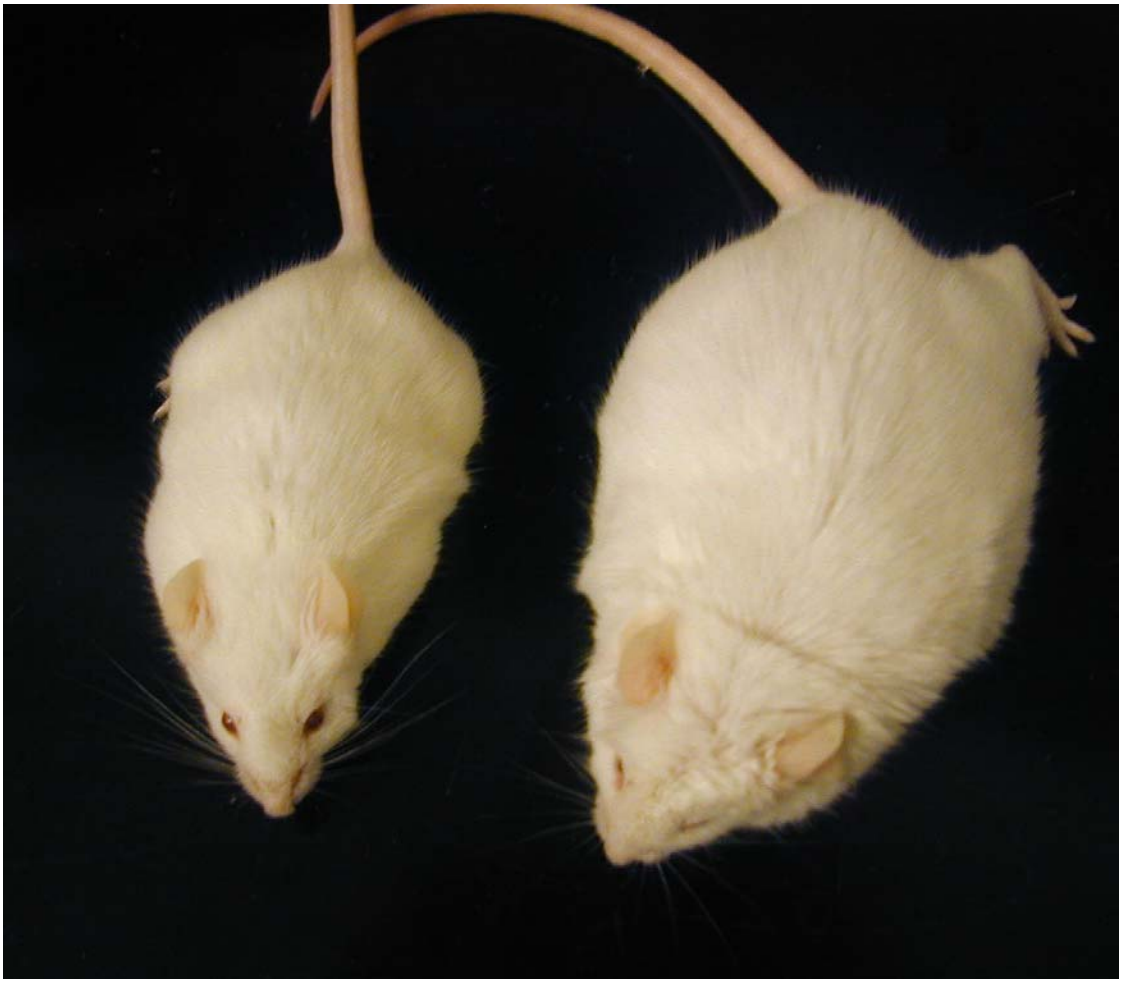

Figure 1.1 Comparison of the non-obese ICR (left) and the polygenic obese M16 (right) line of mice.

Figure 1.2 Examples of currently marketed products that contain CLA.

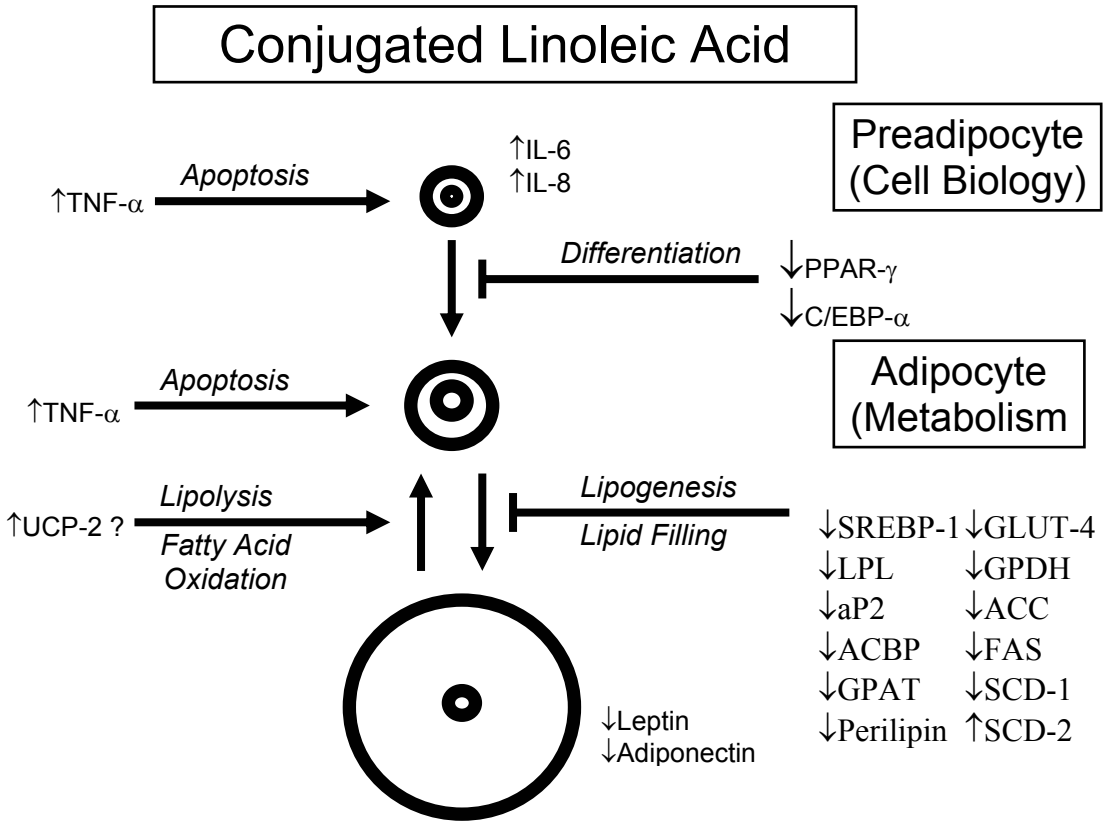

Figure 1.3 Proposed model of the de-lipidative effects of CLA; suggests that CLA imparts its effects by increasing apoptosis, fatty acid oxidation, and lipolysis, as well as decreasing stromal vascular cell differentiation, and lipogenesis. Genes affected by CLA are annotated adjacent to their respective function.

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CHAPTER II

MASTER OF SCIENCE DEGREE PROJECT

FUNCTIONAL GENOMIC CHARACTERIZATION OF THE DE-LIPIDATIVE EFFECTS OF *trans* 10, *cis*12-CONJUGATED LINOLEIC ACID (t10c12-CLA) IN A POLYGENIC OBESE LINE OF MICE

Introduction:

Over the past twenty years the incidence of obesity in society has reached epidemic proportions. According to the 1999-2000 National Health and Nutrition Examination Survey (NHANES), approximately 64% of U.S. adults ≥ 20 years old are overweight or obese (www.cdc.gov/nchs/products/pubs/pubd/hestats/obese/obse99.htm) (Hill et al. 2003). In 2003 Sturm reported that extreme obesity (BMI ≥ 40) quadrupled between 1986 and 2000 from 1 in 200 adults to 1 in 50. Additionally, within the same time period, there was an increase by a factor of 5 of Americans with a BMI ≥ 50, from 1 in 2000 to 1 in 400 (Sturm 2003). Internationally, the World Health Organization reported that from 1995 to 2000 the number of obese adults throughout the world increased from approximately 200 million to over 300 million adults; 115 million were from developing countries (www.who.int/nut/obs.htm). In 2003, $75 billion was spent on medical treatment ascribed to obesity, half of which was paid for by Medicare and Medicaid (Finkelstein et al. 2004).

The rapid ascent in the prevalence of obesity has attracted much attention on the de-lipidative effects of conjugated linoleic acid (CLA). Found naturally in ruminant products (such as beef and cheese) the CLA family consists of several conjugated and stereoisomeric

variations of linoleic acid (*cis, cis*-$\Delta^{9,12}$-octadecadienic acid) (Chin et al. 1992). Arising from anaerobic bacterial biohydrogenation of linoleic acid and α-linolenic acid obtained from plant material (Kepler et al. 1966; Pariza et al. 2001; Martin and Valeille 2002), the predominant (80-90%) natural form is the *cis* 9, *trans* 11-CLA (c9t11-CLA) isomer, also called rumenic acid (Parodi 1977; Chin et al. 1992; Ma et al. 1999; Martin and Valeille 2002). Experiments conducted using a synthetic mixture of c9t11- and t10c12-CLA (usually a 1:1 ratio) have shown that CLA has beneficial effects against cancer (Belury 2002b), diabetes (Taylor and Zahradka 2004), atherosclerosis (McLeod et al. 2004) and obesity (Wang and Jones 2004). It should be noted, however, that effects of CLA differ between and among species.

The de-lipidative effects of CLA were first observed in the ICR line of mice which displayed a 60% decrease in body fat after about four to five weeks of feeding (Park et al. 1997). These effects on body composition have been replicated in different lines of mice and have shown similar results (Pariza et al. 2001). The t10c12-CLA isomer is predominantly responsible for the de-lipidative activity observed with mixed (c9t11/t10c12) CLA treatment (de Deckere et al. 1999; Park et al. 1999b; Pariza et al. 2001; Hargrave et al. 2002; Clement et al. 2002; Evans et al. 2002b). Consequently, our work focused solely on t10c12-CLA's effects on adiposity.

Several groups have shown that CLA may impart its de-lipidative effects by increasing energy expenditure (West et al. 1998; DeLany and West 2000; West et al. 2000; Ohnuki et al. 2001), apoptosis (Tsuboyama-Kasaoka et al. 2000; Miner et al. 2001; Hargrave et al. 2002; Tsuboyama-Kasaoka et al. 2003), fatty acid oxidation (Park et al. 1997; Evans et al. 2002b), and lipolysis (Park et al. 1997; Brown et al. 2003), as well as,

decreasing stromal vascular cell (pre-adipocyte) differentiation (Satory and Smith 1999; Brodie et al. 1999; Evans et al. 2000a; Brown and McIntosh 2003), and lipogenesis (Brown et al. 2001; Evans et al. 2002b; Brown et al. 2004). Gene expression studies using quantitative real time reverse transcriptase PCR (QRT-PCR) or Northern blot analysis have confirmed these observations at the gene expression level (Table 2.1a, b).

Based on *in vivo* (West et al. 1994; Park et al. 1997; Park et al. 1999b; Tsuboyama-Kasaoka et al. 2000; Miner et al. 2001; Takahashi et al. 2002; Hargrave et al. 2002; Ealey et al. 2002; Warren et al. 2003; Xu et al. 2003; Lin et al. 2004) and *in vitro* (Satory and Smith 1999; Brodie et al. 1999; Park et al. 1999b; Evans et al. 2000a; Evans et al. 2000b; Lin et al. 2001; Evans et al. 2002b; Kang et al. 2003; Granlund et al. 2003) observations made in murine white adipose tissue with regard to proposed mechanisms of CLA and the reduced-fat phenotype, we propose a broad list of genes that may be affected by t10c12-CLA supplementation (Table 2.2), recognizing that not all the genes listed are highly expressed in white adipose tissue. To our knowledge, this is the first experiment to utilize functional genomic techniques to analyze the broad effects of CLA in mice. To more closely resemble the major human obesity condition we utilized the polygenic obese line of mice M16 (Eisen and Leatherwood 1978a; West et al. 1994; Warden et al. 1995; Rocha et al. 2004). The M16 line was selected over more than 27 generations for rapid post-weaning gain from an outbred ICR albino population and exhibits positive correlated responses in body weight and percent body fat (Hanrahan et al. 1973; Hanrahan and Eisen 1973; Eisen 1975; Eisen and Leatherwood 1978b). Thus, we conducted a study probing for changes in gene expression elicited by dietary t10c12-CLA in this polygenic obese mouse model using microarray analysis.

Materials and Methods:

Diet Composition:

Purified diets (Harlan Teklad, Madison, WI) were formulated with either 1% t10c12-CLA or linoleic acid as a treatment control (Table 2.3). Diet fatty acid content was analyzed and confirmed in our laboratory (data not shown). The t10c12-CLA was kindly donated by the BASF corporation (Ludwigshafen, Germany), and linoleic acid (LA) was purchased from Nu-Chek-Prep (Elysian, MN). Purity of these samples was analyzed in our laboratory, confirming that LA and t10c12-CLA were 99% and 92% pure, respectively. Inert red and blue dyes were incorporated into the pellets to ensure mice were administered their respective diet at all times. Pelleted diets were separated into equal portions, vacuum sealed and stored at 4°C until feeding.

Mouse Selection and Treatment:

Animal protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University. To ensure that mice had attained ≥ 95% of their mature body mass, dietary treatment began when they reached 9 weeks of age (Eisen et al. 1978a). The experiment was conducted with a total of 185 male mice and was split into two replicates (50 mice/line/replicate, except the obese line in replicate two, which only contained 35 mice; Figure 2.1). Each line contained a t10c12-CLA and an LA fed group. Mice were individually housed in 10.5”x6.5” polypropylene cages in a windowless humidity-controlled room maintained at 21°C, and provided a 12 hour day/night cycle. Water was available at all times and mice were fed *ad libitum*. The LA diet was fed to all animals during the first seven days of acclimation to their new environment. Food was

presented in cubes hung above the cage to reduce waste caused by spilling. Feed intake and body weight of individual mice were recorded on days two, five, eight, eleven and fourteen of the trial. Mice were euthanized by CO_2-asphyxiation, and epididymal, mesenteric, and scapular brown adipose tissues, as well as liver tissue samples were collected from mice chosen at random on days zero, five, and fourteen of the study. Dissected tissue samples were weighed, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Fatty Acid Analysis:

Lipids were extracted from epididymal, mesenteric and brown adipose tissues taken from obese and non-obese animals at day 14, and fatty acids were quantified using a procedure previously reported by Averette-Gatlin *et al.* (2002). Briefly, lipids were saponified with 3.75 M NaOH dissolved in a 1:1 (vol/vol) methanol, distilled water mixture. Samples were then methylated in a 1.7:1 (vol/vol) methyl alcohol and 6.0 N hydrochloric acid mixture (Averette Gatlin et al. 2002). Fatty acid methyl esters were quantified by gas chromatography (HP 5890), using a 100-meter capillary column and flame ionization detection.

RNA Isolation:

Total RNA was isolated from adipose and liver tissue using both Tri Reagent (Sigma, Saint Louis MO) and the Qiagen RNeasy Mini kit (Qiagen, Valencia CA). Tissue was homogenized and chloroform extracted using the manufacturer's protocol for Tri Reagent (derived from (Chomczynski and Sacchi 1987)), incorporating the following modifications.

1) A 10 min incubation step at room temperature after homogenization, followed by centrifugation at 13,000 X g (4°C) for 20 min after which the clear upper layer was discarded.

2) Following addition of chloroform, samples were allowed to incubate at room temperature for 30 min followed by centrifugation at 13,000 X g (4°C) for 20 min. The resulting clear upper layer was transferred to a new tube.

3) Approximately one volume (usually 500μL) of 70% EtOH was added to the reserved supernatant, mixed, and immediately transferred to a Qiagen RNeasy column.

The RNA was then purified following the Qiagen RNeasy Mini kit manufacturer's protocol (**www1.qiagen.com/literature/handbooks/INT/rnalit.aspx#rnymicro**), incorporating modifications that have been previously reported (Vidal 2001). Total RNA concentration was measured by spectrophotometrically analyzing a 1:20 solution of RNA diluted in Tris-DEPC water at 260nm. Integrity was verified electrophoretically using 3μg of RNA on a 1% native agarose gel. Fluorescence was visualized after staining with ethidium bromide.

RNA Labeling and Hybridization:

Total RNA was labeled by direct reverse transcriptional incorporation of cyanine 3 (Cy3) and cyanine 5 (Cy5) labeled dCTP using an oligo dT primer. The Agilent Fluorescent Direct Label Kit protocol was followed with exception to a modification in the starting amount (10 μg of starting material was used, rather than 20 μg) (www.agilent.com). Hybridizations were performed for 16 h in a rotating hybridization oven using the Agilent 60-mer oligo microarray processing protocol (www.agilent.com). Slides were then washed

using a 6X SSC, 0.005% Triton X-102 wash solution for 10 min at room temperature, and then a second wash using a solution of 0.1X SSC and 0.005% Triton X-102 for 5 min. Slides were dried under a nitrogen stream and scanned with an Agilent G2565BA Microarray Scanner (www.agilent.com).

Microarray Design:

Functional genomic analysis was conducted on RNA samples isolated from epididymal adipose and liver tissue extracted on day 5 and day 14 (day 14 only for liver tissue). Because line X treatment interactions were not detected (P>0.05) in growth and fat depot weights, only tissues from obese mice (M16) were screened. A total of 106 samples were pooled into four groups (per treatment per day) totaling 24 samples and yielding a total of 12 microarray slides (4 slides per tissue*day) (Figure 2.2). Therefore, each observation was replicated on four arrays with a fluor reversal applied to two of the four replicate slides (Figure 2.2). Agilent Mouse Oligo microarray slides (Agilent Technologies, Palo Alto, CA) containing >20,000 probes, designed in collaboration with the NIEHS, Toxicogenomics Research Consortium and Paradigm Genetics (Research Triangle Park, NC) were used for the analysis. 60-mer oligonucleotide probes were oriented in the sense (5'-3') direction and spotted using Agilent's SurePrint fabrication technology which utilizes an industrial-scale inkjet printer that synthesizes oligonucleotide probes *in situ* on glass wafers, which are then scribed onto barcoded 1"x3" glass slides. A complete probe list can be found at **http://dir.niehs.nih.gov/microarray/chips.htm.**

Microarray Data Collection:

Gene expression data were obtained using Agilent G2567AA Feature Extraction software (see above), using defaults for all parameters, except the ratio terms, which were

changed according to the Agilent protocol to fit the direct labeling procedure. Files and images, including error values and P-values, were exported from the Agilent Feature Extraction software and loaded into Rosetta Resolver® (version 3.2, build 3.2.2.0.33) (Rosetta Biosoftware, Kirkland, WA). Four arrays for each sample pair, including fluor reversals, were combined into ratio experiments in Rosetta Resolver® as described in US patent # 6,351,712 (Stoughton and Dai 2002). Intensity plots were generated for each ratio experiment, and genes specifically output by this software were considered "signature genes" if the P-value was less than 0.01. P-values reflecting both microarray slide and biological variation were calculated using the Rosetta Resolver® Error Model with Agilent error terms (Stoughton and Dai 2002). In compliance with the "minimum information about microarray experiments" (MIAME) (Brazma et al. 2001) our raw data has been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and have been assigned the following GEO accession numbers: Epididymal adipose tissue: GSM27145, GSM27154, GSM27155, GSM27158, GSM27162, GSM27164, GSM27166, GSM27168 and GSE1580; Liver tissue: GSM27354, GSM27355, GSM27356, GSM27357 and GSE1594.

Real Time Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR):

To validate microarray results, two genes of interest, caveolin-1 (cav-1) (NM_007616) and caspase-3 (casp-3) (NM_009810), were further analyzed using QRT-PCR. These genes were chosen because they represented spots of high (cav-1) and low (casp-3) intensity on the microarray and are associated with metabolism (cav-1) and apoptosis (casp-3); β-actin (X03672) was chosen as a normalization standard housekeeping gene. A 1ug aliquot of total RNA was reverse transcribed into cDNA using an oligo-dT

primer (Roche Applied Science, Indianapolis IN) and the Qiagen Omniscript Reverse Transcriptase kit (Qiagen, Valencia CA) according to the manufacturer's protocol. RNase inhibitor (Roche Applied Science, Indianapolis IN) was included in the reaction. Each treatment*day (eg. CLA*day 5) was replicated using at least 14 animals for each gene. Primers were designed to span introns to prevent amplification of possible genomic DNA contamination. DNASTAR PrimerSelect software (DNASTAR, Madison WI) was used to design the following primers: NM_007616 (cav-1); forward: 5'-ACGCGCACACCAAGGAG-3' reverse: 5'-CAAAGTAAATGCCCCAGATGAG-3'; NM_009810 (casp-3); forward: 5'-AATGGGCCTGTTGAACTGAAAAAG-3' reverse: 5'-CCTGTTAACGCGAGTGAGAATGTG-3'; X03672 (β-actin) forward: 5'-CGGCCAGGTCATCACTATTG-3' reverse: 5'-GCTAGGAGCCAGAGCAGTAATC-3'. Quantitative PCR was carried out with 2μL of cDNA in a 20μL total reaction using Qiagen's Quantitect SYBR Green PCR kit (Qiagen, Valencia CA), following the manufacturer's protocol. Negative controls were included on each 96 well plate. Fluorescence measurements were recorded in real time using the OPTICON by MJ Research (Waltham, MA). Melting curve analysis was used to verify primer quality (no primer dimers or non-specific amplification). Amplification was verified electrophoretically using 2μg of cDNA on a 2% MetaPhor® Agarose (Cabrex BioScience, Rockland ME) gel.

Statistical Analysis:

Phenotypic Analysis:

This experiment followed a two by two factorial, with two lines of mice (obese (M16) and non-obese (ICR)) fed either a treatment or control diet (t10c12-CLA vs. LA,

respectively) (Figure 2.1). Male mice were allocated to each treatment group over two replicates using a completely randomized design. Time effects on body weight and feed intake were analyzed using repeated measures ANOVA. Differences between lines and treatments were calculated using the SAS general linear model procedure (SAS Institute, Cary, NC). Fixed effects included line (M16 and ICR) ($P<0.0001$), treatment (t10c12-CLA and LA) ($P<0.001$), interval (time points that body weight and feed intake measurements were recorded) ($P<0.0001$) and replicate (2 total replicates) ($P<0.0001$ for body weight and $P>0.05$ for feed intake and tissue weight). Values were represented using least square means (LSM); P-value indicated significance of the LSM difference between treatments for each observation.

Microarray Data Analysis:

A population of controls was present on each slide and used to calculate the population statistics of the intensities of each spot and background region using a 99% level of confidence. Normalization was conducted using Agilent G2567AA Feature Extraction Software (version 6.1.1), a detailed description of the algorithms that were utilized in the software can be found at www.chem.agilent.com/scripts/literaturePDF.asp?iWHID=37629. Briefly, after the background signal had been subtracted from spots on the array, the software used a "rank consistency filter" method, which identified spots that had trends consistent between the red and green channels and tended to fall in the center of the data. These "normalization features" then became equivalent to housekeeping genes. Normalization was then accomplished in two steps. First, geometric mean signal intensity of the normalization features was set to 1000 to calculate the linear dye normalization factor. This was a global approach because it assumed that intensity is independent of dye

bias. The second method, LOWESS dye normalization, assumed the opposite and was, therefore, a local approach. Each normalization feature was assigned a LOWESS normalization factor that was determined by fitting a locally weighted regression curve to the normalization features. Following this step, the central tendency of the log ratio of all intensity ranges was to fall along zero. The linear dye normalization factor was then multiplied to the LOWESS normalization factor to yield a "dye normalization factor." The dye normalized signal (processed signal) for each spot was then calculated by multiplying the background subtracted signal of the respective spot by the dye normalization factor. Processed signals were then used to calculate the log ratio for each gene represented on the array, as well as the P-value and error associated with a given ratio. These numbers from each replicate were then combined and assigned P-values using Rosetta Resolver® software.

Differentially expressed genes identified by Rosetta Resolver® were screened against our hypothesized genes of interest (Table 2.2). An initial analysis for genes not included in our hypotheses was conducted using Ingenuity Pathway software (Mountain View, CA). Analysis of the datasets (day 5 and day 14 epididymal "signature genes") was conducted by increasing stringency with each iteration (2-fold change, $P<0.001$). A description of the software can be accessed at www.ingenuity.com/products/product_overview.html. Briefly, data were arranged to include an identifier (gene name) and a rank cutoff (fold change or P-value). Input was compared to a database of biological networks to determine potential interactions within genes. Additionally, "signature gene" lists from both timepoints were compared with each other to determine genes common to both datasets. These datasets were also compared to

the GeneSpring® (Silicon Genetics, Redwood CA) database to identify genes associated with apoptosis, metabolism and transcription factors.

Real Time Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR) Analysis:

Relative quantification was calculated using the $2^{-\Delta\Delta C}{}_T$ method described by Livak and Schmittgen (2001). The value C_T, represents the cycle number at which the fluorescence of the sample exceeds the threshold level (threshold determined by multiplying the standard deviation of the baseline by 10). Briefly, $\Delta\Delta C_T$ is the difference between ΔC_T of the sample and ΔC_T of the calibrator (control). The difference between C_T of the target gene and housekeeping gene is ΔC_T. The end result is an estimate that is representative of the gene expression relative to the calibrator. Error associated with the $2^{-\Delta\Delta C}{}_T$ method has a tendency to be asymmetric; therefore, we approximated a standard error by dividing the range of error by 2. The resulting number was used in a one-sided t-test to determine statistical significance at the $P<0.05$ level.

Results:

Body Composition Changes Associated With t10c12-CLA:

Time-dependent changes in body weight of t10c12-CLA-fed mice and LA-fed mice were similar in obese and non-obese lines (Line x treatment interaction: $P>0.7$) (Figure 2.3). Differences in body weights of t10c12-CLA vs. LA-fed mice were detected after day five and increased progressively to day fourteen (obese LA: 61.23 ± 1.24; obese CLA: 52.79 ± 1.23; non-obese LA: 40.46 ± 1.16; non-obese CLA: 36.13 ± 1.17; $P<0.01$; Figure 2.3).

Differences in feed intake between t10c12-CLA and LA-fed mice (Figure 2.4) were observed after the second day (obese: 1.61±0.24g; $P<0.0001$, non-obese: 0.70±0.22g; $P<0.05$) and persisted throughout the trial. However, differences decreased as the trial progressed and by day fourteen were 1.19±0.22g ($P<0.0001$) and 0.48±0.21g ($P<0.05$) for obese and non-obese lines, respectively (Figure 2.4). To test if the decreased feed intake could account for the reduced fat pad mass, we conducted a covariance analysis of feed intake on body and tissue weights. We found that there was still a significant difference between the LA and t10c12-CLA-fed groups, indicating that feed intake could not completely account for the loss in fat pad mass or difference in body weight. The effect of t10c12-CLA on the differences between tissue weights was similar in both lines of mice (line x treatment: $P>0.05$) (Figure 2.5) and increased progressively over the duration of the trial. Epididymal and mesenteric adipose tissues changed similarly, progressively decreasing in mice supplemented with t10c12-CLA. By day fourteen there was 30% less epididymal adipose ($P<0.0001$) (Figure 2.5) and 27% less mesenteric adipose tissue ($P<0.0001$) in t10c12-CLA-fed mice. By day five there was 54% ($P<0.0001$) less brown adipose in t10c12-CLA-fed mice, and by day fourteen the difference increased marginally to 58% ($P<0.0001$). There was about a 33% ($P<0.0001$) increase in liver weight by day 14 (Figure 2.5), with 61% more fat present in the liver of t10c12-CLA-fed mice versus LA-fed mice ($P<0.001$; data not shown).

Fatty Acid Composition of Tissues:

The t10c12-CLA isomer was only present in the adipose depots of mice fed the t10c12-CLA supplemented diet (Table 2.4a, b, c). The ratio of 16:0/16:1 increased in all three fat pads of mice fed t10c12-CLA ($P \leq 0.0011$); however, no detectable change was

observed between treatments in 18:0/18:1 ($P>0.05$) except for a decrease in brown adipose tissue ($P<0.05$). 14:0 decreased in all three fat pads of t10c12-CLA-fed mice ($P<0.0001$); however both 18:2, *cis*-9, *cis*-12 and 20:1, *cis*-11 + 18:3, *cis*-9, *cis*-12, *cis*-15 decreased in epididymal and mesenteric adipose tissues ($P\leq0.0007$), but increased in brown adipose tissue ($P<0.0001$) of mice supplemented with t10c12-CLA.

Microarray and Pathway Analysis:

Output from Rosetta Resolver® (Rosetta Biosoftware, Kirkland, WA) resulted in 1,030 (4.9%) (Figure 2.6a), 1,229 (5.9%) (Figure 2.6b) (see GEO accession number GSE1580; http://www.ncbi.nlm.nih.gov/geo/ for day 5 and 14 epididymal adipose tissue gene lists) and 1,394 (6.7%) (see GEO accession number GSE1594; http://www.ncbi.nlm.nih.gov/geo/ for day 14 liver tissue gene list) genes differentially expressed ($P<0.01$) in day five and day fourteen epididymal adipose tissue, as well as day fourteen liver tissue, respectively. Within these, 29 (0.14%) (Figure 2.6a), 125 (0.60%) (Figure 2.6b) and 80 (0.38%) (data not shown) genes were expressed ≥ two fold in epididymal adipose (day 5 & 14) and liver tissue (day 14), respectively.

Comparisons of our "signature gene" datasets (all genes $P<0.01$ output from Rosetta Resolver®) for the epididymal fat pad with the GeneSpring database resulted in 15 genes matching GeneSpring's apoptosis database at day 5 and 14, respectively (Figure 2.7a); 49 genes for day 5 and 75 genes for day 14 matched GeneSpring's metabolism database (Figure 2.7b); as well as 45 genes for day 5 and 38 genes for day 14 matched genes coding for transcription factors in the GeneSpring database (Figure 2.7c). Even though both day 5 and day 14 epididymal fat pads both shared 15 genes with the GeneSpring apoptosis database, only 2 of the 15 were shared between timepoints (Table 2.6a); 6 genes were

shared between timepoints in the metabolism comparison (Table 2.5b) and 5 in the transcription factor comparison (Table 2.5c).

Ingenuity pathway analysis (Mountain View, CA) of day 14 epididymal adipose tissue (Figure 2.8) yielded several genes of interest, including the peroxisome proliferator activated receptor-γ (PPAR-γ), which has been previously reported in the literature to decrease in expression (Tsuboyama-Kasaoka et al. 2000; Evans et al. 2000b; Takahashi et al. 2002; Kang et al. 2003; Granlund et al. 2003)). Potentially novel CLA-gene interactions that resulted from this analysis included caveolin-1 (cav-1) (proposed to function in free fatty acid and triglyceride transport and storage) and caspase-3 (casp-3) (part of the apoptotic pathway). Cav-1 was also present in the day 5 pathway analysis, implicating it as a potentially early responder to CLA treatment (data not shown).

Genes identified by our analyses were compared with our *a priori* hypotheses and are shown in Table 2.6. Overall, anti-apoptotic and lipid storage/transport genes had a tendency to be down-regulated while genes that were pro-apoptotic and involved in fatty acid oxidation tended to be up-regulated. Whilst we did not observe a change in leptin mRNA concentration, the adipokines adiponectin and resistin were down-regulated. In addition to a reduction in PPAR-γ expression, we observed a decrease in CCAAT/enhancer-binding protein α (C/EBPα) and the retinoic acid receptor (RXR). We also observed a dramatic reduction (~7-fold) in mRNA concentration of the adipocyte marker adipsin. Results from our microarray analysis confirm observations that have been previously reported with glucose transporter-4 (GLUT-4) (Takahashi et al. 2002; Brown et al. 2003), perilipin (Brown et al. 2003), adiponectin (Warren et al. 2003) and uncoupling protein-2 (UCP-2) (Tsuboyama-Kasaoka et al. 2000; West et al. 2000; Ryder et al. 2001; Takahashi

et al. 2002; Ealey et al. 2002). Additionally, we propose potentially novel CLA-gene interactions in adipose tissue with caveolin-1 (cav-1), phosphofructokinase-2 (PFK-2), pyruvate dehydrogenase (PDH), diacylglycerol acyltransferase (DGAT), adipsin, resisitin, B-cell lymphoma 2 (Bcl-2), caspase-3 (casp-3), and cytochrome-c (cyt-c) (see Table 2.6 for a complete list).

The relative increase (day 5 & 14) in cav-1 and no change (day 5) or decrease (day 14) in casp-3 mRNA concentration was confirmed using QRT-PCR (Table 2.7). Cav-1 at day 5 was downregulated 2.00 fold ($P<0.001$), and no significant difference was detected for casp-3, in congruence with the array data. We also found that cav-1 was downregulated at day 14 ~1.55 fold ($P<0.05$) and that casp-3 was upregulated ~1.41 fold ($P<0.05$) (Table 2.7). Except for cav-1 at day 14, which was slightly higher than the array data (~0.16 fold difference), the QRT-PCR results were very similar to the results from the array.

Discussion:

Phenotypic Effects:

Effects of CLA on fat pad reduction in mice have been well established. However, the mechanism of action still remains unclear. Several studies have shown that the t10c12-CLA isomer is solely responsible for reduction of fat pad weight (de Deckere et al. 1999; Park et al. 1999b; Pariza et al. 2001; Hargrave et al. 2002; Clement et al. 2002; Evans et al. 2002b), and as a result, this study focused solely on this isomer. In agreement with previously reported data (West et al. 1998; Park et al. 1999a; DeLany and West 2000; Pariza et al. 2001; Terpstra et al. 2002; Hargrave et al. 2002; Tsuboyama-Kasaoka et al. 2003), we observed reduced body weight gain in both lines of mice fed t10c12-CLA

(Figure 2.4). Feed intake was also reduced in the t10c12-CLA supplemented group ($P<0.0001$), an issue that is a source of controversy in the literature, with some groups reporting little to no effect (Ostrowska et al. 1999; DeLany et al. 1999; DeLany and West 2000; Azain et al. 2000; West et al. 2000; Sisk et al. 2001; Thiel-Cooper et al. 2001; Terpstra et al. 2002; Wiegand et al. 2002; Yamasaki et al. 2003), while others have reported a reduction (Dugan et al. 1997; West et al. 1998; Szymczyk et al. 2000; Miner et al. 2001; Ohnuki et al. 2001; Ryder et al. 2001; Hargrave et al. 2002; Ostrowska et al. 2003b). However, studies conducted with a pair-fed group of mice and rats on a CLA supplemented diet confirmed a significant decrease in fat pad mass compared to a control group with the same energy intake (Ryder et al. 2001; Ntambi et al. 2002; Hargrave et al. 2002). Additionally, after conducting covariance analysis of feed intake on body and tissue weights we showed that there was still a significant difference between the LA and t10c12-CLA-fed groups, indicating that feed intake could not completely account for the loss in fat pad mass or difference in body weight. It is possible that CLA has either an averse organoleptic quality or alters metabolism in such a way as to impart a reduction in feed intake.

Our observations of adipose tissue reduction and increased hepatic lipid further confirms previous reports in the literature (Belury and Kempa-Steczko 1997; West et al. 1998; Park et al. 1999a; DeLany and West 2000; Tsuboyama-Kasaoka et al. 2000; Terpstra et al. 2002; Clement et al. 2002; Warren et al. 2003; Degrace et al. 2003; Kelley et al. 2004). Interestingly, t10c12-CLA had the same effect on adipose tissue and body weight in both lines of mice (line x treatment: $P>0.05$), indicating that t10c12-CLA effects are

independent of genetic strain. Hargrave *et al.* (2002) reported a similar observation in mice selected for high or low energy expenditure.

Isomers and metabolites of CLA have been shown to incorporate into phospholipid and neutral lipid fractions of different tissues (Belury 2002a). Consequently, in agreement with previously reported observations (Xu et al. 2003; Kang et al. 2004), presence of t10c12-CLA in adipose tissue was evident only in mice that had consumed the t10c12-CLA supplemented diet (Table 2.4a, b & c). Studies analyzing effects of CLA on fatty acid composition in different tissues of several species have shown that it significantly alters fatty acid profiles (Sebedio et al. 2001; Banni et al. 2001; Xu et al. 2003; Ostrowska et al. 2003a; Kang et al. 2004; Kelley et al. 2004). Excluding 16:0 and 16:1, we did not observe a significant difference in a majority of the fatty acid levels between treatments in each adipose depot (Table 2.4a, b & c). In mice, lipogenesis occurs predominantly in liver tissue, which may partially explain the minor differences we observed. Also, net lipogenesis in these mice near maintenance is less likely than in growing and lactating animals, and this could impact effects on fatty acid profiles.

Several groups have reported a shift in the ratio of palmitate:palmitoleate (16:0/16:1) and stearate:oleate (18:0/18:1) with t10c12-CLA supplementation (Lee et al. 1998; Choi et al. 2000; Sebedio et al. 2001; Eder et al. 2002; Evans et al. 2002a; Brown et al. 2003; Xu et al. 2003; Kang et al. 2004). This result may be due to a reduction in stearoyl-CoA desaturase-1 (SCD-1) activity (Lee et al. 1998; Choi et al. 2000; Eder et al. 2002). Our results show an increase in the ratio of 16:0/16:1, but we were unable to detect a significant increase in 18:0/18:1. Since SCD-1 is predominantly expressed in liver tissue, our observations may be due to secondary effects of CLA interactions with the liver. A

study conducted by Xu et al. (2003), analyzing the early effects of CLA (4 days), did not detect any changes in the ratios of 16:0/16:1 and 18:0/18:1 in adipose tissue, further indicating that this modulation may be due to downstream effects. A recent experiment using SCD-1 null mice, showed that t10c12-CLA exerts its de-lipidative effects independent of SCD-1 (Kang et al. 2004); however, in the same study control mice (SCD1 +/+) fed t10c12-CLA had an increase in the ratio of 16:0/16:1, and a decrease in ratio of 18:0/18:1. The authors suggest that the decrease in 18:0/18:1 is due to an increase in SCD-2 gene expression (predominantly expressed in brain and adipose tissue); they also report that t10c12-CLA had no effect on SCD-1 gene expression in adipose tissue. Whilst we did not detect a significant decrease in 18:0/18:1 in epididymal and mesenteric adipose tissues, results from our microarray analysis confirm these observations. We detected ~1.3 fold induction in SCD-2 ($P<0.05$) (Table 2.6) expression in t10c12-CLA-fed mice, with no significant change in SCD-1 expression between treatment groups. The duration of the Kang et al. (2004) study was four weeks whilst our experiment only lasted two, which may explain why we had not yet seen a significant reduction in 18:0/18:1. Given these results, it seems probable that CLA is exerting its de-lipidative effects through multiple mechanisms.

Gene Expression Effects:

In order to further explore mechanisms associated with t10c12-CLA supplementation, we conducted a functional genomic analysis on epididymal adipose tissue extracted on day 5 and day 14 of the trial from the obese line of mice. Following models previously proposed for potential CLA mechanisms (Pariza et al. 2001; Brown and McIntosh 2003), we hypothesized that genes associated with lipid metabolism and apoptosis would be modulated by t10c12-CLA. Specifically, genes associated with

lipogenesis (anabolic) and anti-apoptotis would be down-regulated, and those involved with fatty acid oxidation, lypolysis (catabolic) and pro-apoptosis would be up-regulated. Several studies have analyzed the expression of genes associated with these functions and are summarized in Tables 2.1a, b. The majority of microarray data confirmed our hypotheses and other results previously reported. Additionally, our analyses presented potentially novel genes that are modulated by t10c12-CLA supplementation.

A powerful aspect of this experiment has been the degree of biological replication. Wary of the large degree of error that can occur with microarray experiments and the difficulty in experimental replication, we used no less than four arrays that were hybridized with RNA samples that had been pooled from a total of 70 mice (34 from day 5 and 36 from day 14) (Figure 2.2) to test each observation. Unlike traditional microarray experiments that use two arrays hybridized with identical samples (switching dyes between arrays) to correct for dye bias, we applied a dye switch on two of our four replicate arrays (Figure 2.2). This approach ensured that for each pair of chips, the treatment and control (pooled from no less than four animals per sample) were labeled with a different dye (Cy-3 or Cy-5), but by not utilizing identical samples we maximized our biological replication, thereby increasing the statistical power of our test. Furthermore, we validated our results using QRT-PCR to confirm the change in mRNA concentration of cav-1 and casp-3, genes that were representative of either high or low intensity on the array, and are involved in metabolism or apoptosis, respectively.

The gene that appeared to be most highly expressed in our analysis was UCP-1 (~12 fold) (Table 2.6); however, this is a gene that is predominantly expressed in brown adipose tissue, and the high increase in expression may be due to large changes in minor

concentrations, rendering it biologically unimportant. However, a study conducted by Tsukiyama-Kohara et al. (2001) showed that Eif4ebp1-/- mice had smaller white fat pads and increased metabolic rate, with no change in feed intake. Additionally, the white adipose tissue adopted a multilocular appearance similar to brown adipocytes and expressed UCP-1 (~6-fold) (Tsukiyama-Kohara et al. 2001). Eukaryotic translation initiation factor 4E binding protein 1(Eif4ebp1) is a gene that codes for eIF4E-binding protein-1 (4EBP1), part of a family of 4EBPs that bind to eIF4E, thereby preventing its association with eIF4F and preventing translation of a subset of genes, including the PPAR-γ co-activator-1 (PGC1) (Tsukiyama-Kohara et al. 2001). We did not detect a significant change in PGC1, but we observed a reduction in Eif4ebp1 mRNA concentration (~1.5 fold reduction) (Table 2.6). Furthermore, we observed a decrease in phosphatidylinositol 3-kinase (PI3K) expression at day 5 (~1.1 fold) (Table 2.6). The PI3K signaling pathway is a mechanism by which extracellular stimuli are able to alter eIF4F activity (Gingras et al. 1999). The increase in UCP-1 coupled with a decrease in Eif4ebp1 expression, as well as the similar phenotypic response that we observed in our t10c12-CLA-fed mice, may indicate that CLA is working in part through an eIF4-dependent mechanism. Recently, Brown et al. (2004) showed that one of the ways that t10c12-CLA reduced triglceride content in human adipocytes was through activation of the mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) signaling pathway. This was coupled with hypersecretion of interleukin-6 (IL-6) and IL-8, and they proposed that this induction occurred through the autocrine/paracrine actions of these adipokines (Brown et al. 2004). Whilst we did not detect a change in IL-8 message, we found that IL-6 was up-regulated (~1.7 fold) in day 14 epididymal adipose tissue (Table 2.6). Collectively, this

may suggest that t10c12-CLA is imparting its effects through signal transduction mechanisms.

Our data yielded a significant increase in enzymes that catalyze the initial steps of fatty acid biosynthesis, such as acetyl-CoA carboxylase (ACC) at day 14 and malic enzyme at day 5 and 14 (Table 2.6). We also failed to detect a significant change in fatty acid synthase (FAS). These data are in conflict with previously reported observations (Tsuboyama-Kasaoka et al. 2000; Brown et al. 2003; Peterson et al. 2003b; Kang et al. 2004; Lin et al. 2004) and our hypothesis that t10c12-CLA works by decreasing lipogenesis. However, because lipogenesis in the mouse primarily occurs in the liver with very little occurring in adipose tissue, it is probable that the de-lipidative effect of t10c12-CLA in the mouse may be working through mechanisms other than lipogenesis. Indeed, we show that t10c12-CLA has a negative effect on glucose transport (GLUT-4) in adipose tissue and transport of triglycerides and fatty acids for storage in lipid droplets (cav-1) (Table 2.6).

Studies conducted on 3T3-L1 adipocytes and human adipocytes have confirmed a reduction in the size of the lipid droplet upon CLA supplementation (Choi et al. 2000; Kang et al. 2003; Brown et al. 2004). Additionally, negative effects of t10c12-CLA on perilipin (protein associated with intracellular lipid droplets) would add credence to the suggestion that the morphology of the lipid droplet is being compromised. Our analysis showed a decrease in cav-1 mRNA expression (Table 2.6). Adipose tissue has a high abundance of caveolae, accounting for ~30% of the surface area of an adipocyte (Razani et al. 2002). Razani et al. (2002) showed that cav-1 null mice were resistant to diet induced obesity, despite being hyperphagic. Mice were unable to convert lipoprotein triglycerides to lipid

droplet form for storage (Razani et al. 2002). Additionally, there may be a reciprocal relationship between cav-1 and the MEK/ERK pathway (Krajewska and Maslowska 2004). Therefore, if t10c12-CLA stimulates MEK/ERK signaling as proposed by Brown et al. (2004), then this may be the mechanism by which cav-1 expression is reduced. This observation may indicate that t10c12-CLA reduces adipose tissue, at least partially, through a caveolin-dependent mechanism, and adds credence to the proposal that it is working through the MEK/ERK signaling pathway. Additionally, we observed a decrease in phosphoenolpyruvate carboxykinse (PEPCK) mRNA concentration (Table 2.6). Presence of PEPCK may not be expected in adipose tissue, as it is predominantly expressed in the liver and kidney. However, an experiment conducted by Olswang et al. (2002) on mice, mutated to abolish expression of PEPCK in white adipose tissue, showed they had reduced triglyceride deposition.

Collectively, it appears that the de-lipidative effects of t10c12-CLA in mice is occurring through reduced fatty acid and triglyceride translocation and storage, as well as decreased glucose availability, rather than affecting endogenous lipogenesis. Furthermore, it has been proposed that CLA reduces triglyceride content by increasing fatty acid oxidation (Park et al. 1997; Evans et al. 2002b). This is further evident by an increase in carnitine palmitoyl transferase (CPT) activity (Park et al. 1997; Rahman et al. 2001); a mitochondrial membrane bound protein essential for shuttling long chain fatty acids into the mitochondria where they undergo β-oxidation. Our results confirm these observations on a genetic level (Table 2.6). We observed an increase in both liver and muscle isoforms of CPT-1, as well as an increase in acyl-carnitine translocase. Currently, the effects of CLA on lipolysis are conflicting. Several *in vitro* studies have reported an increase in lipolysis in

response to CLA (Park et al. 1997; Brown et al. 2003); however, our data are in agreement with a study that reported no effect *in vivo* in mice (Xu et al. 2003). Our results would therefore suggest that fatty acid oxidation, not lipolysis is indeed one mechanism by which t10c12-CLA imparts its de-lipidative effects.

Among genes associated with cell biology, we observed an increase in expression of tumor necrosis factor-α (TNF-α) ($P<0.05$), and a decrease in expression of the peroxisome proliferator activated receptor-γ (PPAR-γ), adipsin, resistin and adiponectin ($P<0.01$; Table 2.6). The reduction in adiponectin confirms previously reported observations (Warren et al. 2003). This result is intriguing because it has been shown that adiponectin levels are negatively correlated with BMI (Hu et al. 1996; Arita et al. 1999; Hotta et al. 2000; Weyer et al. 2001), therefore it may be expected that adiponectin levels would increase with weight loss. However, adiponectin mRNA is expressed predominantly in mature adipocytes and that differentiation dramatically increases its expression (Hu et al. 1996). Negative effects of t10c12-CLA on pre-adipocyte differentiation are well established (Houseknecht et al. 1998; Satory and Smith 1999; Brodie et al. 1999; Tsuboyama-Kasaoka et al. 2000; Evans et al. 2000a; Evans et al. 2000b; Takahashi et al. 2002; Belury et al. 2002; Kang et al. 2003; Brown et al. 2003; Granlund et al. 2003; Kang et al. 2004; Brown et al. 2004), and are represented in this study by a reduction in PPAR-γ, RXR and C/EBP-α mRNA concentration (Table 2.6); therefore, a reduction in adiponectin expression may be a result of decreased differentiation. Additionally, adiponectin may increase insulin sensitivity (Hu et al. 1996; Arita et al. 1999; Hotta et al. 2000; Yamauchi et al. 2001; Weyer et al. 2001; Fruebis et al. 2001); however, the opposite has been reported in mice fed t10c12-CLA (DeLany et al. 1999; Tsuboyama-Kasaoka et al. 2000; Clement et al. 2002;

Roche et al. 2002), and this may be due to decreased adiponectin expression. It should be noted that the precise mechanism of action of adiponectin currently remains unclear.

Several groups have reported an increase in apoptotic activity in adipose tissue upon t10c12-CLA supplementation (Tsuboyama-Kasaoka et al. 2000; Evans et al. 2000a; Miner et al. 2001; Hargrave et al. 2002; Tsuboyama-Kasaoka et al. 2003); however, studies analyzing this potential interaction on a genetic level have been sparse. Our results ($P<0.05$) confirm a report by Tsuboyama-Kasaoka et al. (2003), that CLA increases TNF-α expression (Table 2.6). TNF-α is a cytokine that induces lipolysis, adipocyte de-differentiation, as well as apoptosis of pre- and mature adipocytes (Prins et al. 1997). A study using colon SW480 tumor cells showed that t10c12-CLA increased casp-3 activity, reduced bcl-2 expression and increased cytosolic cytochorome c (cyt c) (Miller et al. 2002). We show that t10c12-CLA works similarly in adipose tissue, by increasing casp 3 and cyt c expression, as well as a decreasing bcl-2 expression (Table 2.6). Therefore we confirm on a genetic level, the increase in apoptosis reported by Miner et al. (2001) and Hargrave et al. (2002), based on internucleosomal DNA degradation measurements. It therefore seems probable that t10c12-CLA is additionally imparting its de-lipidative effects through an apoptotic mechanism.

In summary, we suggest that at the genetic level, t10c12-CLA is imparting its anti-adipogenic effects by inhibiting pre-adipocyte differentiation, increasing fatty acid oxidation and apoptosis, as well as preventing storage and translocation of fatty acids and triglycerides into lipid droplets (Figure 2.9). Additionally, it seems highly probable that these effects arise through t10c12-CLA induction of signal transduction mechanisms. We

also propose that little if any effect is being contributed by induction of lipolysis or inhibition of de novo lipogenesis in murine white adipose tissue.

This experiment is unique in that to our knowledge, this is the first study utilizing functional genomic techniques to characterize gene expression during CLA induced degradation of body fat. In addition to genes previously reported, this experiment has identified potentially novel gene candidates that are involved in the mechanisms associated with lipid metabolism and apoptosis that are affected by dietary intake of t10c12-CLA. The long-range goal of this research is to better understand the mechanisms associated with obesity and identify genes that may be targeted for pharmacologic development. We believe that the power of the nutrigenomic approach, coupled with the potent effects of CLA presented herein, provide valuable insight towards achieving this goal.

Chapter 2 Tables

Table 2.1a Previously reported literature regarding gene expression effects of CLA on adipocyte *lipid metabolism* in white adipose and liver tissue (arrows pertain to adipose tissue, unless stated otherwise).

Gene Name	***Effect***	***Ref.***
Anabolic:		
Lipoprotein lipase	↓	(Park et al. 1997; Park et al. 1999b; Lin et al. 2001; Kang et al. 2003; Xu et al. 2003)
Fatty acid binding protein (liver)	↑:Liver	(Moya-Camarena et al. 1999)
aP2	↓↑	(Houseknecht et al. 1998; Kang et al. 2003; Brown et al. 2003; Granlund et al. 2003)
Acyl-CoA binding protein	↓	(Brown et al. 2003)
Glycerol-3-phosphate acyltransferase	↓:Mammary	(Peterson et al. 2003a)
Perilipin	↓	(Brown et al. 2003)
Glucose transporter 4	↓	(Takahashi et al. 2002; Brown et al. 2003)
ATP-citrate lyase	↑:Liver	(Takahashi et al. 2003)
Glycerol-3-phosphate dehydrogenase	↓	(Brown et al. 2003)
Acetyl CoA carboxylase	↓ ↑:Liver ↓:Mammary	(Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003; Brown et al. 2003; Takahashi et al. 2003; Peterson et al. 2003b; Lin et al. 2004)
Fatty acid synthase	↓ ↑:Liver ↓:Mammary	(Tsuboyama-Kasaoka et al. 2000; Kang et al. 2003; Takahashi et al. 2003; Peterson et al. 2003b; Lin et al. 2004)
Malic enzyme	↑:Liver	(Takahashi et al. 2003)
Stearoyl-CoA Desaturase 1	↓ ↓:Liver ↓:Mammary	(Lee et al. 1998; Choi et al. 2000; Eder et al. 2002; Peterson et al. 2003b; Lin et al. 2004)
Stearoyl-CoA Desaturase 2	↑	(Kang et al. 2003)
Catabolic:		
Cytochrome P450	↑↔:Liver	(Moya-Camarena et al. 1999; Warren et al. 2003)
Hormone sensitive lipase	↓	(Brown et al. 2003)

Table 2.1a (continued)

Peroxisomal acyl-CoA oxidase	↑	(Belury et al. 1997; Warren et al. 2003; Takahashi et al. 2003; Degrace et al. 2004)
Peroxisomal bifunctional enzyme	↑:Liver	(Takahashi et al. 2003)
Trifunctional enzyme-α	↑:Liver	(Takahashi et al. 2003)
Trifunctional enzyme-β	↑:Liver	(Takahashi et al. 2003)
Carnitine palmitoyl transferase I (liver)	↑:Liver	(Takahashi et al. 2003; Degrace et al. 2004)
Carnitine palmitoyl transferase I (muscle)	↑:Liver	(Degrace et al. 2004)
Carnitine palmitoyl transferase II	↑:Liver	(Takahashi et al. 2003)
Uncoupling protein-1	↔,↓	(West et al. 2000; Takahashi et al. 2002; Ealey et al. 2002)
Uncoupling protein-2	↑	(Tsuboyama-Kasaoka et al. 2000; West et al. 2000; Ryder et al. 2001; Takahashi et al. 2002; Ealey et al. 2002; Kang et al. 2004)
Uncoupling protein-3	↓	(Takahashi et al. 2002; Ealey et al. 2002)

Table 2.1b Previously reported literature regarding gene expression effects of CLA on adipocyte *cell biology* in white adipose and liver tissue (arrows pertain to adipose tissue, unless stated otherwise).

Gene name	***Effect***	***Ref.***
Adipokines:		
Leptin	↓↑	(Takahashi et al. 2002; Warren et al. 2003; Brown et al. 2003; Nagao et al. 2003)
Adiponectin	↓	(Warren et al. 2003)
Interleukin-6	↑	(Brown et al. 2004)
Interleukin-8	↑	(Brown et al. 2004)
Tanscription factors:		
Peroxisome proliferator activated receptor-α	↓:Liver	(Warren et al. 2003)
Peroxisome proliferator activated receptor-γ	↓	(Tsuboyama-Kasaoka et al. 2000; Evans et al. 2000b; Takahashi et al. 2002; Kang et al. 2003; Brown et al. 2003; Granlund et al. 2003; Kang et al. 2004; Brown et al. 2004)

Table 2.1b (continued)

Sterol Regulatory Element Binding Protien 1	↓	(Tsuboyama-Kasaoka et al. 2000)
CAAT/enhancer binding protein-α	↓	(Brown et al. 2003)
Pro-apoptosis:		
Tumor Necrosis Factor-α	↑	(Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003)

*Please note that since this study has focused on murine white adipose tissue, no genes effected by CLA in cancer cells are presented in this table.

Table 2.2 *A priori* hypotheses on the effects of t10c12-CLA supplementation on gene expression in murine white adipose tissue, specifically associated with lipid metabolism and cell biology.

LIPID METABOLISM		***LIPID METABOLISM***		***CELL BIOLOGY***		***CELL BIOLOGY***	
Anabolic		***Catabolic***		***Pro-apoptosis***		***Adipokines***	
Lipoprotein lipase	↓	Hormone sensitive lipase	↑	TNF-a	↑	Leptin	↓
Fatty acid binding protein 4	**?**	Cytochrome P450	↑	Fas	↑	Adiponectin	↓
aP2	↓	Phospholipase A2	↑	c-jun NH2-terminal kinase	↑	Adipsin	↓
Acyl CoA synthetase 1-5	**?**	Acyl-CoA oxidase	↑	p53	↑	Resistin	↓
Acyl-CoA binding protein	↓	Peroxisomal bifunctional enzyme	↑	p21 WAF1/CIP1	↑	IL-6	↓
Glycerol-3-phosphate acyltransferase	↓	Catalase	↑	Bax	↑	IL-8	↓
Monoacylglycerol acyltransferase	↓	CPT IA (liver)	↑	Cytochrome -c	↑	***Txn factors***	
DAG-acyltransferase	↓	CPT IB (muscle)	↑	Apaf-1	↑	PPAR-a	↑
Perilipin	↓	CPT II	↑	Caspase 3	↑	STAT 1	↑
Glucose transporter 4	↓	Acyl-carnitine translocase	↑	Bad	↑	STAT 3	↓
Hexokinase	↓	Acyl-CoA dehydrogenase	↑	Bcl-Xs	↑	STAT 5	↓

Table 2.2 (continued)

Phospho-fructokinase	↓	Enoyl-CoA hydratase	↑	Hrk	↑	PPAR-g	↓
Pyruvate dehydrogenase	↓	Hydroxyacyl-CoA dehydrogenase	↑	***Anti-apoptosis***		SREBP 1c	↓
ATP-citrate lyase	↓	Ketoacyl-CoA thiolase	↑	c-Myc	↓	SREBP 2	↓
Acetyl CoA carboxylase	↓	HMG CoA synthase	↑	Cyclin D1	↓	C/EBP-a	↓
Fatty acid synthase	↓	HMGl CoA lyase	↑	Cyclin E	↓		
Malic enzyme	↓	UCP-1	↑	Retinoblastoma protein	↓		
Isocitrate dehydrogenase 2	↓	UCP-2	↑	Bcl-2	↓		
Stearoyl-CoA Desaturase 1	↓						

Table 2.3 Control (linoleic acid) and treatment (t10c12-CLA) diet composition.

Ingredients*	**LA (g/Kg)**	**CLA(g/Kg)**
Corn Starch	346.3	346.3
Casein. "Vitamin-free"	200	200
Sucrose	150	150
Maltodextrin	130	130
Soybean Oil	***60***	***60***
Cellulose	50	50
Mineral Mix, AIN-93G-MX	35	35
Vitamin Mix, AIN-93-VX	10	10
Linoleic Acid	***10***	***-***
t10c12-Conjugated Linoleic Acid	***-***	***10***
CaHPO4	3	3
DL-Methionine	3	3
Choline Bitartrate	2.5	2.5
Diet Marker	0.15	0.15
Tert-Butylhydroquinone (antioxidant)	0.014	0.014

Table 2.4a Fatty acid profile of *epididymal* adipose tissue of obese and non-obese mice fed either t10c12-CLA or LA.*

Fatty Acid g/100g fatty acids	Obese		Non-obese		Pooled SEM	P-values Mouse Line	Treatment	Line X Treatment
	LA	CLA	LA	CLA				
14:0	1.61	1.30	2.32	1.13	0.15	0.0820	<0.0001	0.0071
16:0	18.93	20.13	19.73	19.80	0.54	0.6662	0.2474	0.2986
16:1, *cis*-9	6.32	4.66	5.62	2.95	0.31	0.0006	<0.0001	0.1141
18:0	1.98	2.00	2.38	3.44	0.15	<0.0001	0.0013	0.0018
18:1, *trans*-9	0.60	0.51	0.25	0.70	0.24	0.5262	0.1428	0.0288
18:1, *cis*-9	27.65	31.16	32.40	28.99	1.84	0.0011	<0.0001	0.0212
18:2, *cis*-9, *cis*-12	29.07	27.06	38.26	28.14	1.13	0.0001	<0.0001	0.0013
18:2, *trans*-10, *cis*-12	n.d.	0.59	n.d.	0.87	0.71	0.0150	-	-
20:1, *cis*-11 + 18:3, *cis*-9, *cis*-12, *cis*-15	2.34	1.65	3.09	1.98	0.13	0.0005	<0.0001	0.1365
Ratio								
16:0/16:1	3.10	4.40	3.57	6.78	0.22	<0.0001	<0.0001	0.0002
18:0/18:1	0.06	0.06	0.10	0.10	0.01	<0.0001	0.8775	0.3031

* Values represent least square means (LSM); P-value indicates significance of the LSM difference between treatments for each fatty acid

Table 2.4b Fatty acid profile of *mesenteric* adipose tissue of obese and non-obese mice fed either t10c12-CLA or LA.*

Fatty Acid g/100g fatty acids	Obese		Non-obese		Pooled SEM	P-values Mouse Line	Treatment	Line X Treatment
	LA	CLA	LA	CLA				
14:0	1.36	1.01	1.64	0.90	0.09	0.3403	<0.0001	0.0319
16:0	19.33	19.80	22.22	19.23	0.65	0.0862	0.0636	0.0129
16:1, *cis*-9	6.26	4.43	6.87	3.32	0.71	0.6036	<0.0001	0.0811
18:0	2.83	2.71	3.04	3.51	0.27	0.0747	0.5274	0.2960
18:1, *trans*-9	0.53	0.70	0.30	0.97	0.15	0.8861	0.0087	0.0986
18:1, *cis*-9	31.10	33.53	24.05	30.68	1.71	0.0073	0.0131	0.2301
18:2, *cis*-9, *cis*-12	27.93	25.94	32.95	27.23	1.02	0.0044	0.0007	0.0789
18:2, *trans*-10, *cis*-12	n.d.	0.44	n.d.	0.84	0.12	0.0292	-	-
20:1, *cis*-11 + 18:3, *cis*-9, *cis*-12, *cis*-15	2.30	1.69	1.91	1.32	0.14	0.0135	0.0003	0.9280
Ratio								
16:0/16:1	3.17	4.56	3.62	6.39	0.57	0.0554	0.0011	0.2336
18:0/18:1	0.09	0.08	0.14	0.12	0.02	0.0473	0.3961	0.7281

* Values represent least square means (LSM); P-value indicates significance of the LSM difference between treatments for each fatty acid

Table 2.4c Fatty acid profile of *brown* adipose tissue of obese and non-obese mice fed either t10c12-CLA or LA.*

Fatty Acid	Obese		Non-obese			P-values		
g/100g fatty acids	LA	CLA	LA	CLA	Pooled SEM	Mouse Line	Treatment	Line X Treatment
14:0	2.91	1.23	3.56	0.83	0.09	0.2182	<0.0001	<0.0001
16:0	25.94	18.88	30.06	17.92	0.78	0.0526	<0.0001	0.0030
16:1, *cis*-9	7.80	2.32	7.63	2.56	0.72	0.9638	<0.0001	0.7770
18:0	4.14	3.45	4.69	3.76	0.31	0.1730	0.0140	0.7150
18:1, *trans*-9	0.37	1.11	0.08	1.42	0.16	0.9841	<0.0001	0.0640
18:1, *cis*-9	30.82	28.38	27.37	29.65	1.05	0.3083	0.9413	0.0330
18:2, *cis*-9, *cis*-12	21.79	30.94	22.57	31.32	1.48	0.6985	<0.0001	0.8918
18:2, *trans*-10, *cis*-12	n.d.	1.86	n.d.	2.34	0.14	0.0163	-	-
20:1, *cis*-11 + 18:3, *cis*-9, *cis*-12, *cis*-15	1.43	2.19	1.12	2.46	0.14	0.8763	<0.0001	0.0489
Ratio								
16:0/16:1	3.76	8.55	4.23	9.19	0.83	0.5135	<0.0001	0.9167
18:0/18:1	0.13	0.12	0.18	0.12	0.01	0.1018	0.0137	0.1696

* Values represent least square means (LSM); P-value indicates significance of the LSM difference between treatments for each fatty acid

Table 2.5a List of genes that are shared amongst the day 5 and 14 epididymal adipose "signature gene" datasets (P<0.01) and the GeneSpring database of genes associated with *apoptosis*

Gene Description	Accession #
Mus musculus TRB-3 (TRB-3), mRNA	BC012955
Mus musculus Bcl-2-related ovarian killer protein (Bok), mRNA	NM_016778

Table 2.5b List of genes that are shared amongst the day 5 and 14 epididymal adipose "signature gene" datasets (P<0.01) and the GeneSpring database of genes associated with *metabolism*

Gene Description	Accession #
Mus musculus galactosidase, alpha (Gla), mRNA	NM_013463
Mus musculus low density lipoprotein receptor-related protein 1 (Lrp1), mRNA	NM_008512
Mus musculus malic enzyme, supernatant (Mod1), mRNA	NM_008615
Mus musculus ATP-binding cassette, sub-family D (ALD), member 2 (Abcd2), mRNA	NM_011994

Table 2.5b (continued)

Mus musculus, Similar to carbonyl reductase 3, clone MGC:41226 IMAGE:1313642, mRNA, complete cds	BC028763
Mus musculus acetyl-Coenzyme A synthetase 1 (AMP forming) (Acas1), mRNA	NM_019811

Table 2.5c List of genes that are shared amongst the day 5 and 14 epididymal adipose "signature gene" datasets (P<0.01) and the GeneSpring database of genes associated with *transcription factors*

Gene Description	**Accession #**
Mus musculus SRY-box containing gene 6 (Sox6), mRNA	NM_011445
Mus musculus, clone MGC:38361 IMAGE:5344900, mRNA, complete cds	BC027771
Mus musculus Notch gene homolog 4, (Drosophila) (Notch4), mRNA	NM_010929
Mus musculus synuclein, alpha interacting protein (synphilin) (Sncaip), mRNA	NM_026408
Mus musculus SRY-box containing gene 7 (Sox7), mRNA	NM_011446

Table 2.6 Comparison of results from our microarray analyses with our *a priori* hypotheses of the effects of t10c12-CLA on genes associated with lipid metabolism and cell biology. All genes reported are statistically significant at the P<0.01 level unless stated otherwise; no significant change is represented by a hyphen (-).

Gene Name	***Accession #***	***Hypothesis***	***Observed Results (Fold Change; CLA:LA)***	
			d5	***d14***
Lipid Metabolism:				
Glycerol-3-phosphate acyltransferase	NM_008149	↓	-	1.59
Diacylglycerol acyltransferase	NM_026713	↓	-	-1.24
Perilipin	AK031445	↓	-	-1.48
Caveolin	NM_007616	n/a***	-1.96	-1.71
Eukaryotic translation initiation factor 4E binding protein 1	NM_007918	n/a***	-1.77	-1.40
Phosphatidylinositol 3-kinase	NM_008839	n/a***	-1.33	-
Glucose transporter 4	NM_009204	↓	-	-2.33
Phosphofructokinase 2	NM_133232	↓	-	-2.24

Table 2.6 (continued)

Phosphoenolpyruvate carboxykinase	NM_011044	n/a***	-	-1.52
Pyruvate dehydrogenase	NM_008810	↓	-1.49	-
Acetyl CoA carboxylase	BC022940	↓	-	1.44
Malic enzyme	NM_008615	↓	1.30	1.79
Isocitrate dehydrogenase 2	NM_008322	↓	-1.79	-2.41
Phospholipase A2	NM_012044	↑	1.54	3.13
Carnitine palmitoyl transferase I (liver)	AF017175	↑	-	1.54
Carnitine palmitoyl transferase I (muscle)	NM_009948	↑	-	1.18
Acyl-carnitine translocase	NM_020520	↑	-	1.33
Uncoupling protein-1	NM_009463	↑	-	12.85
Uncoupling protein-2	NM_011671	↑	1.32	1.49
Stearoyl-CoA Desaturase 2*	NM_009128	n/a***	-	1.33
Adipokines:				
Adiponectin	NM_009605	↓	-	-3.68
Adipsin	NM_013459	↓	-	-7.79
Resistin	BB609634	↓	-	-2.81
Interleukin-6	NM_031168	n/a***	-	1.76
Transcription Factors:				
Peroxisome proliferator activated receptor-γ	NM_011146	↓	-	-1.70
Retenoic acid receptor	NM_009107	n/a***	-	-1.29
CAAT/enhancer binding protein-α	NM_007679	↓	-1.65	-
Apoptosis:				
Tumor Necrosis Factor-α*	NM_013693	↑	1.11	1.36**
Cytochrome-c	AK077566	↑	-	1.81
Caspase 3	NM_009810	↑	-	1.38
Bcl-2	NM_016778	↓	-1.58	-1.44

*P<0.05

**P=0.07

***Represents genes of interest not included in our list of hypotheses (Table 2.2)

Table 2.7 Comparison of microarray and QRT-PCR results. Statistical significance was determined using Rosetta Resolver® software for microarray data and a one-sided t-test for QRT-PCR. Relative expression for QRT-PCR analysis was determined using the $2^{-\Delta\Delta C}{}_t$ method (Livak and Schmittgen 2001).

Gene	Microarray Results (Fold Change)				QRT-PCR Results (Fold Change)			
	Day 5	P-value	Day 14	P-value	Day 5	P-value	Day 14	P-value
Cav-1	-1.96	<0.01	-1.71	<0.01	-2.00	<0.001	-1.49	<0.05
Casp-3	-1.01	>0.05	1.38	<0.01	1.10	>0.05	1.41	<0.05

Chapter 2 Figures

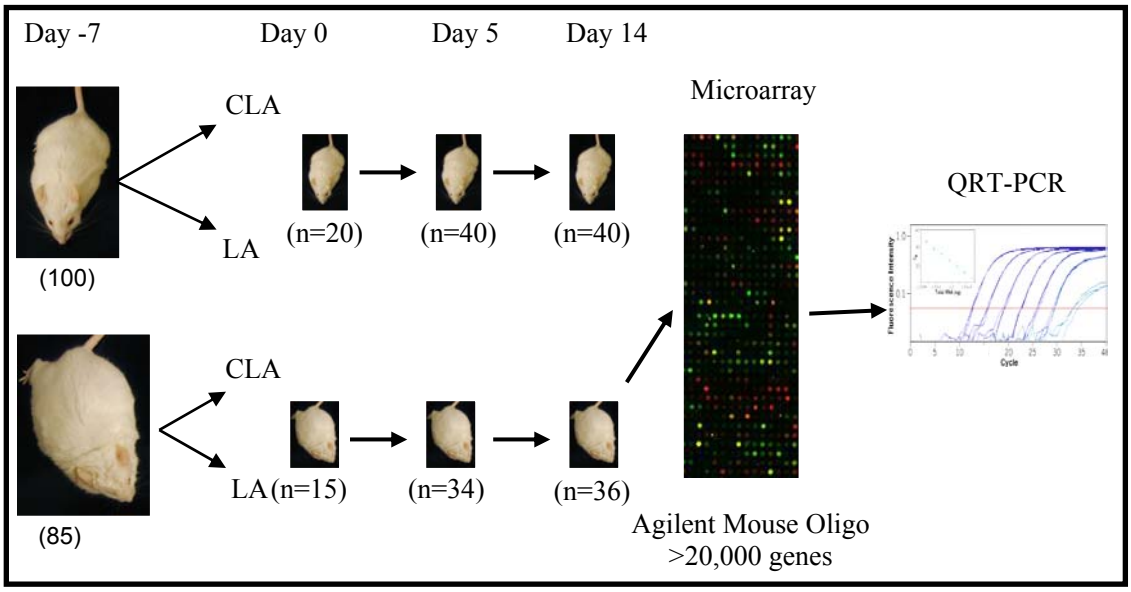

Figure 2.1 Experimental scheme outlining the random allocation of mice into treatment groups over the course of the fourteen day trial. Tissue was collected on day 0, 5 and 14 of the trial from non-obese(ICR) and obese(M16) mice. Epididymal adipose tissue take from obese mice on day 5 and 14 was subjected to microarray analysis, and select genes were further verified using QRT-PCR.

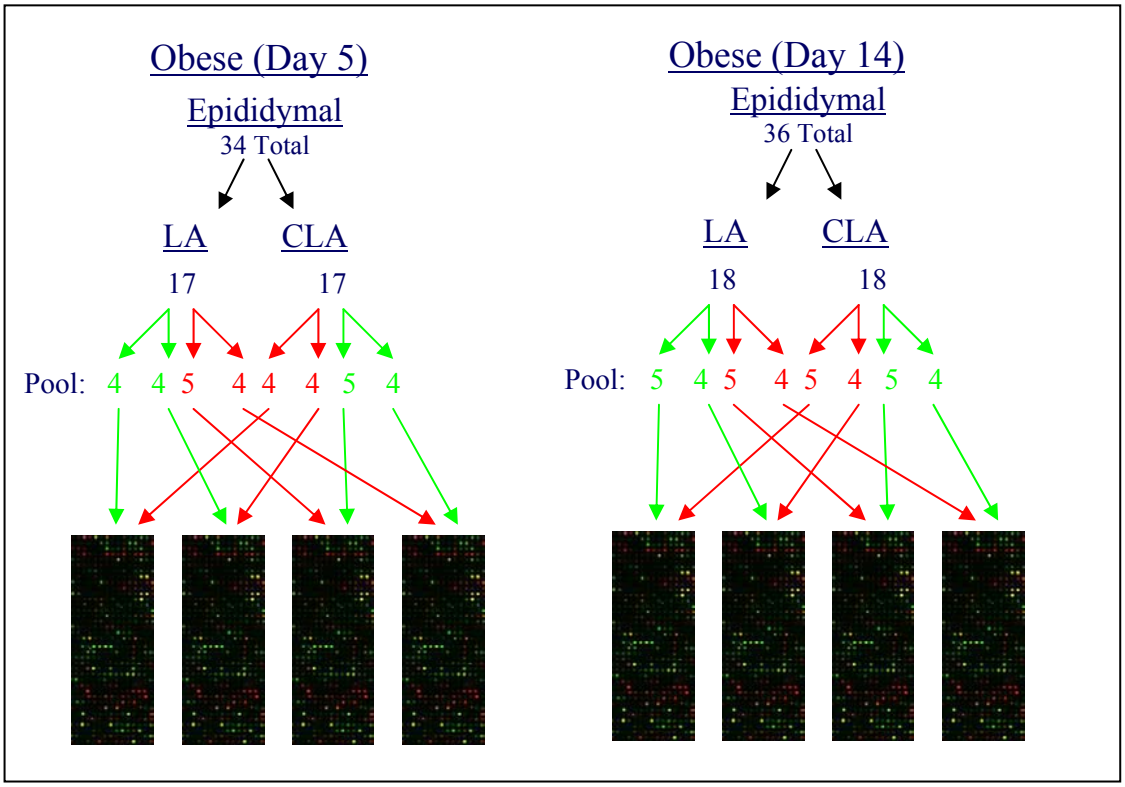

Figure 2.2 Experimental strategy diagramming RNA isolation and analysis from epididymal adipose tissue (day 5 and day 14). Samples were pooled and labeled with Cy-3 (green) or Cy-5 (red) before hybridization to Agilent microarray slides. Numbers indicate the number of animals or RNA samples.

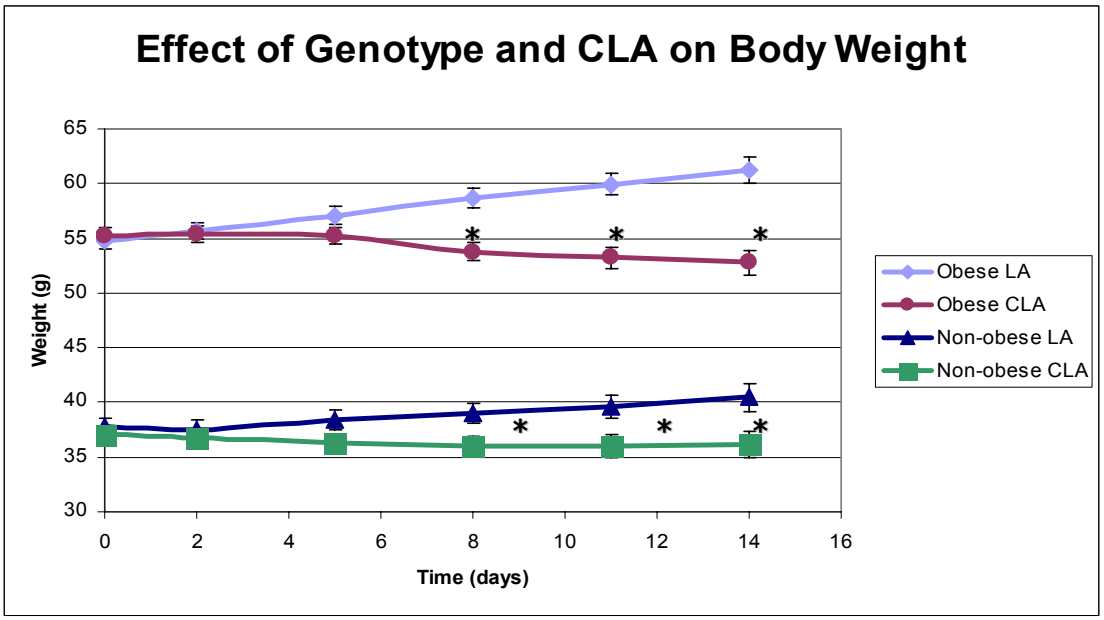

Figure 2.3 Body weights of obese and non-obese mice fed either t10c12-CLA or LA. Values represent least square means +/- SEM. Line main effect: P<0.0001, treatment main effect: P<0.0006, line by treatment interaction: P>0.7
*Differs from LA control at similar time and within genetic line

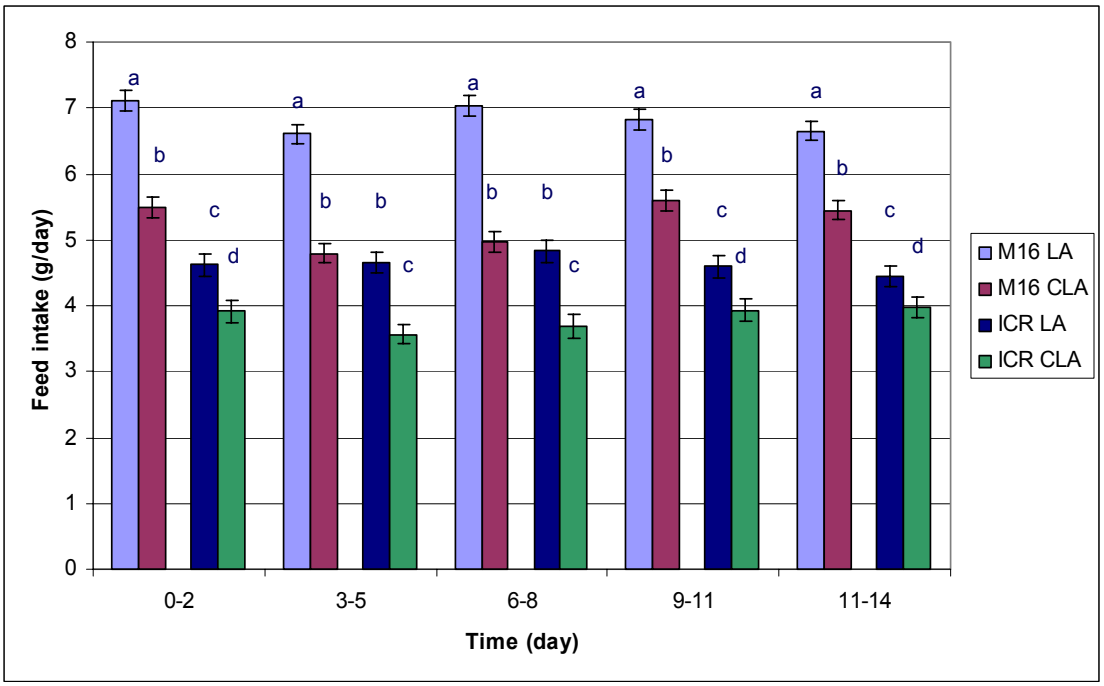

Figure 2.4 Feed intake values of obese and non-obese mice fed either CLA or LA. Values represent least square means +/- SEM. Line effect: P<0.0001, treatment main effect: P<0.0001, line by treatment interaction: P=0.002; a, b, c, d: significance within interval, bars lacking a common letter are different P<0.0001.

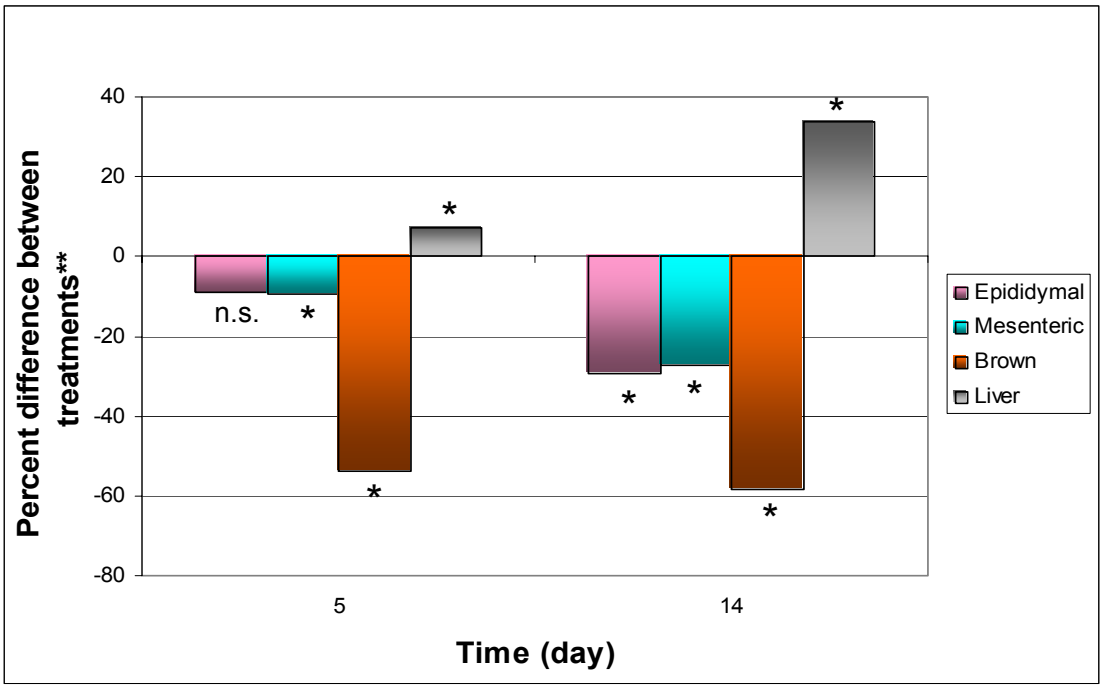

Figure 2.5 Tissue weight differences (epididymal, mesenteric, brown adipose and liver tissue) between t10c12-CLA and LA supplemented mice, expressed as a percentage of body weight.*

*CLA effect $p<0.0001$

**100(CLA-LA)/LA

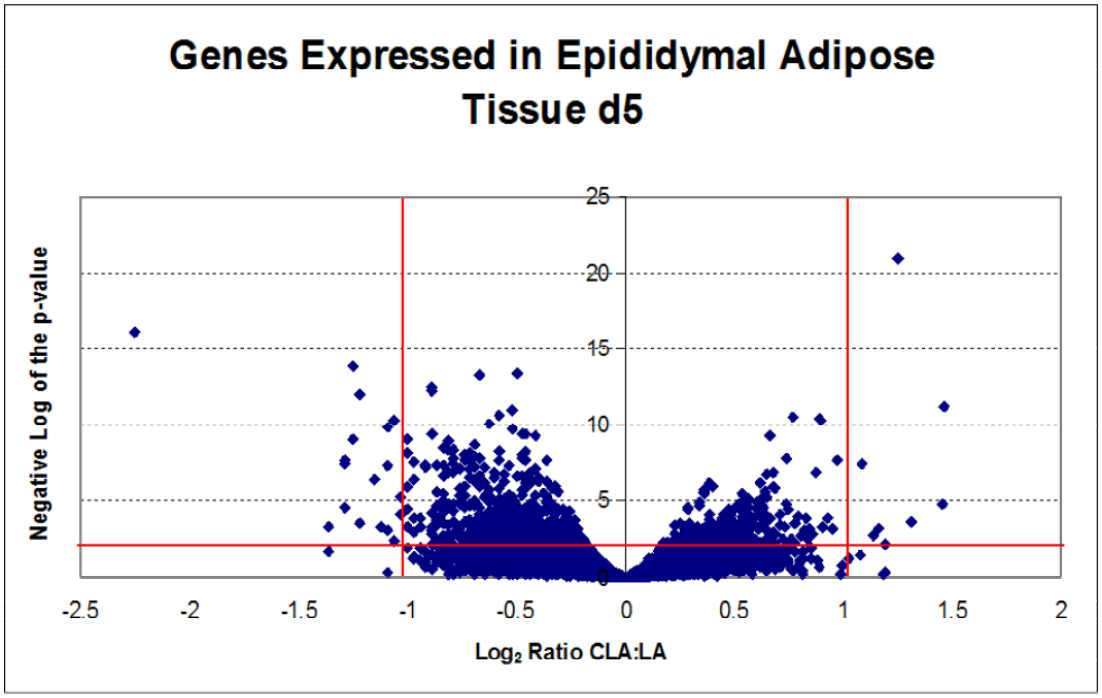

Figure 2.6a Relative expression of genes represented on the microarray for the day 5 epididymal fat pad analysis. The horizontal line represents a P<0.01 cutoff, therefore genes above this line are considered to be significantly effected by t10c12-CLA at the P<0.01 level and totaled 1,030 genes. Vertical lines represent ≥2-fold cutoff, therefore genes exceeding these lines were considered differentially expressed ≥2-fold by t10c12-CLA treatment and totaled 29 genes.

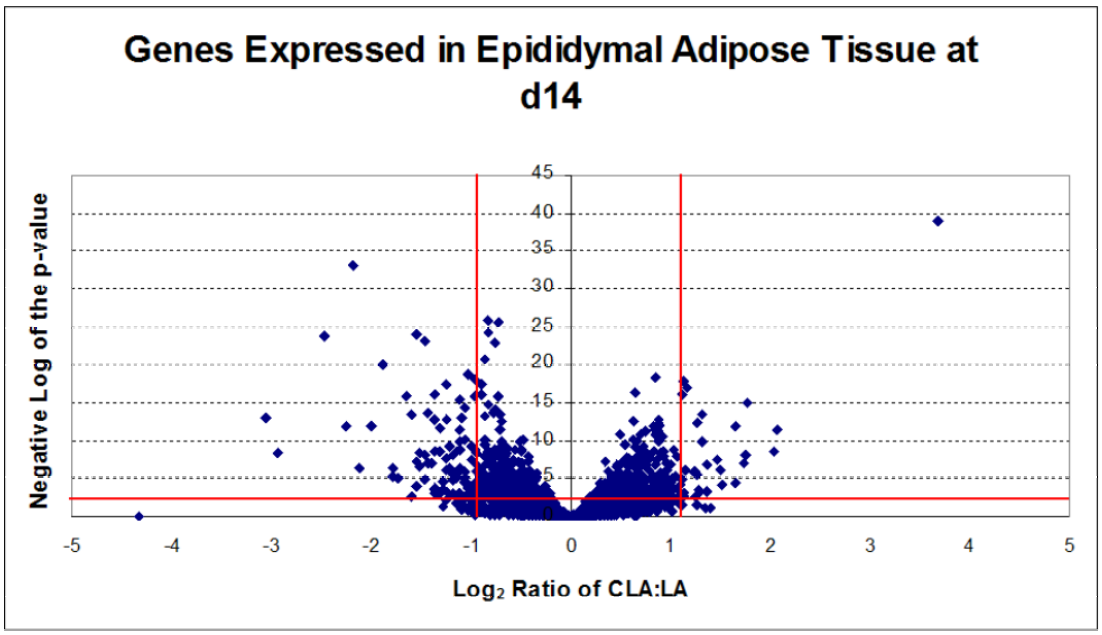

Figure 2.6b Relative expression of genes represented on the microarray for the day 14 epididymal fat pad analysis. The horizontal line represents a P<0.01 cutoff, therefore genes above this line are considered to be significantly effected by t10c12-CLA at the P<0.01 level and totaled 1,229 genes. Vertical lines represent ≥2-fold cutoff, therefore genes exceeding these lines were considered differentially expressed ≥2-fold by t10c12-CLA treatment and totaled 125 genes.

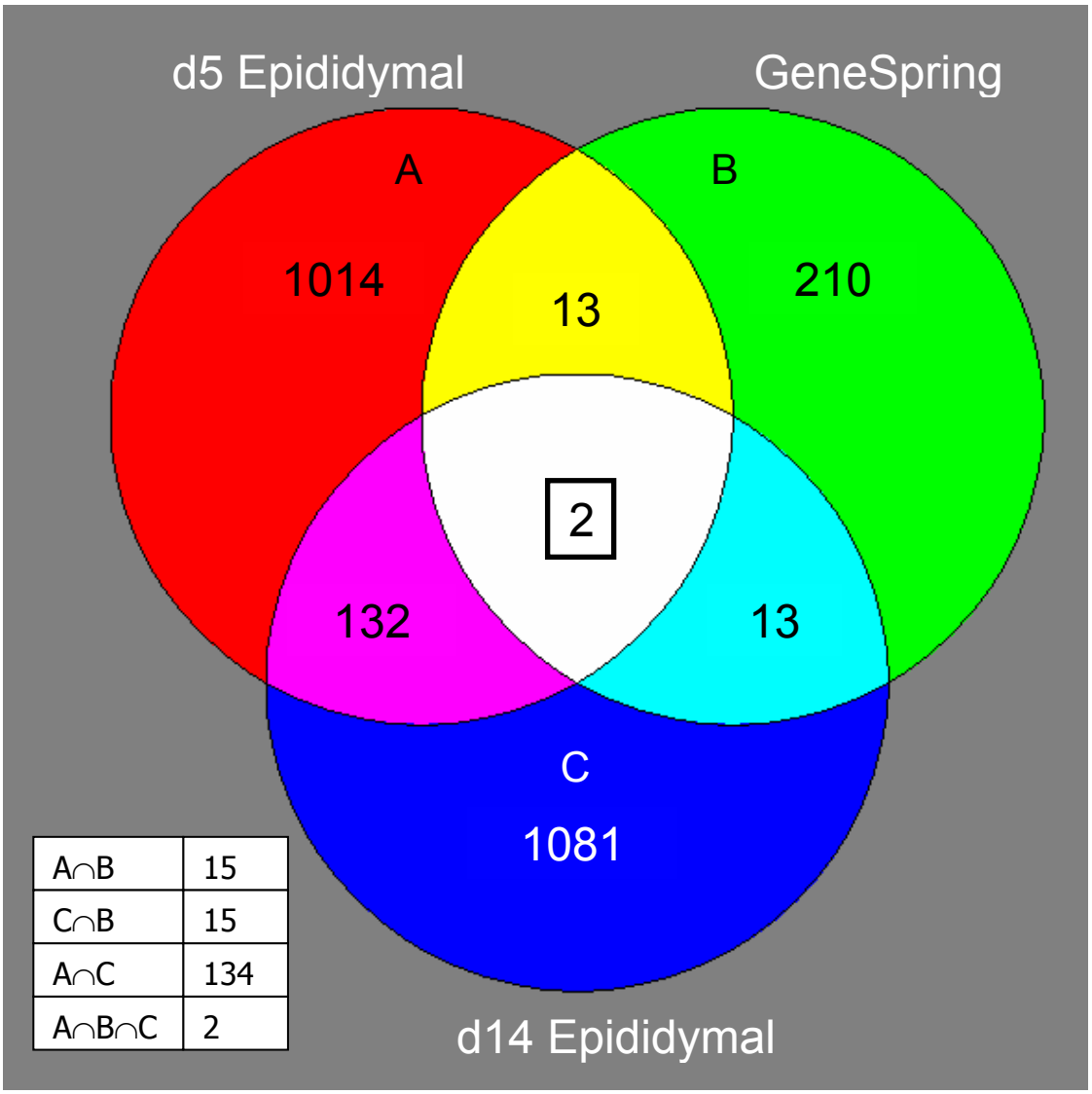

Figure 2.7a Comparison of day 5 and 14 epididymal fat pad "signature gene" datasets (P<0.01) with each other and the GeneSpring database of genes associated with *apoptosis*

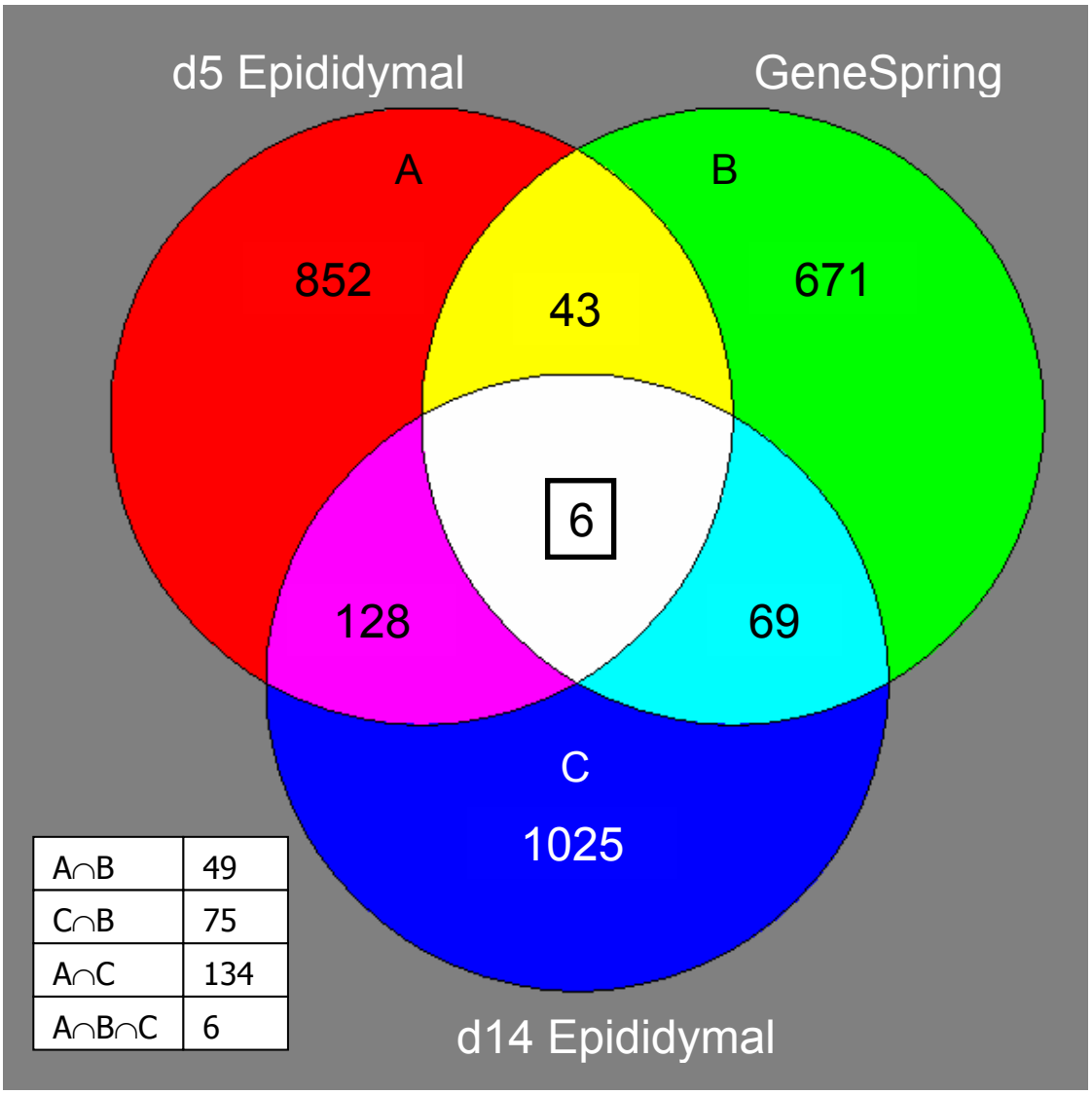

Figure 2.7b Comparison of day 5 and 14 fat pad "signature gene" datasets (P<0.01) with each other and the GeneSpring database of genes associated with *metabolism*

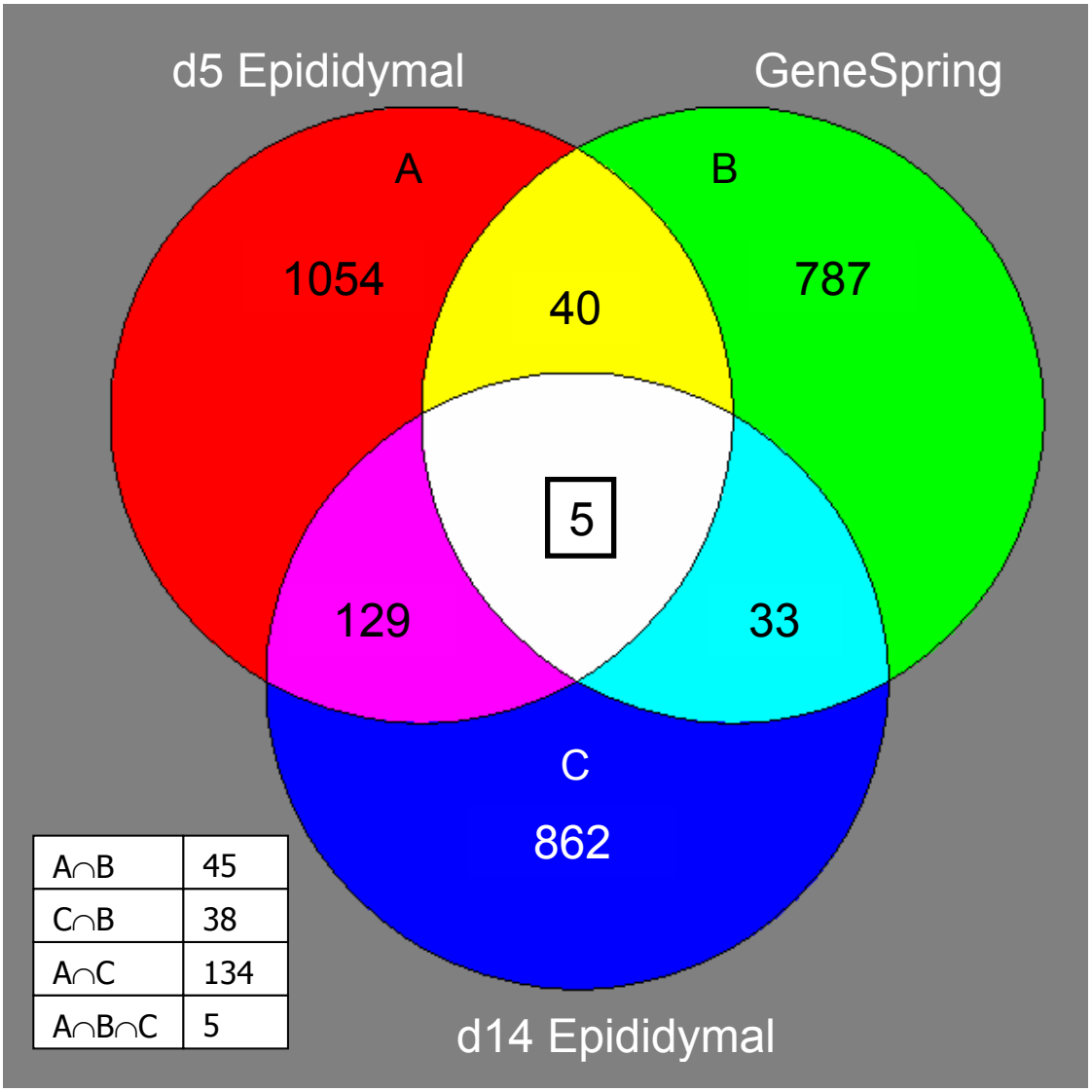

Figure 2.7c Comparison of day 5 and 14 epididymal fat pad "signature gene" datasets (P<0.01) with each other and the GeneSpring database of genes associated with *transcription factors*

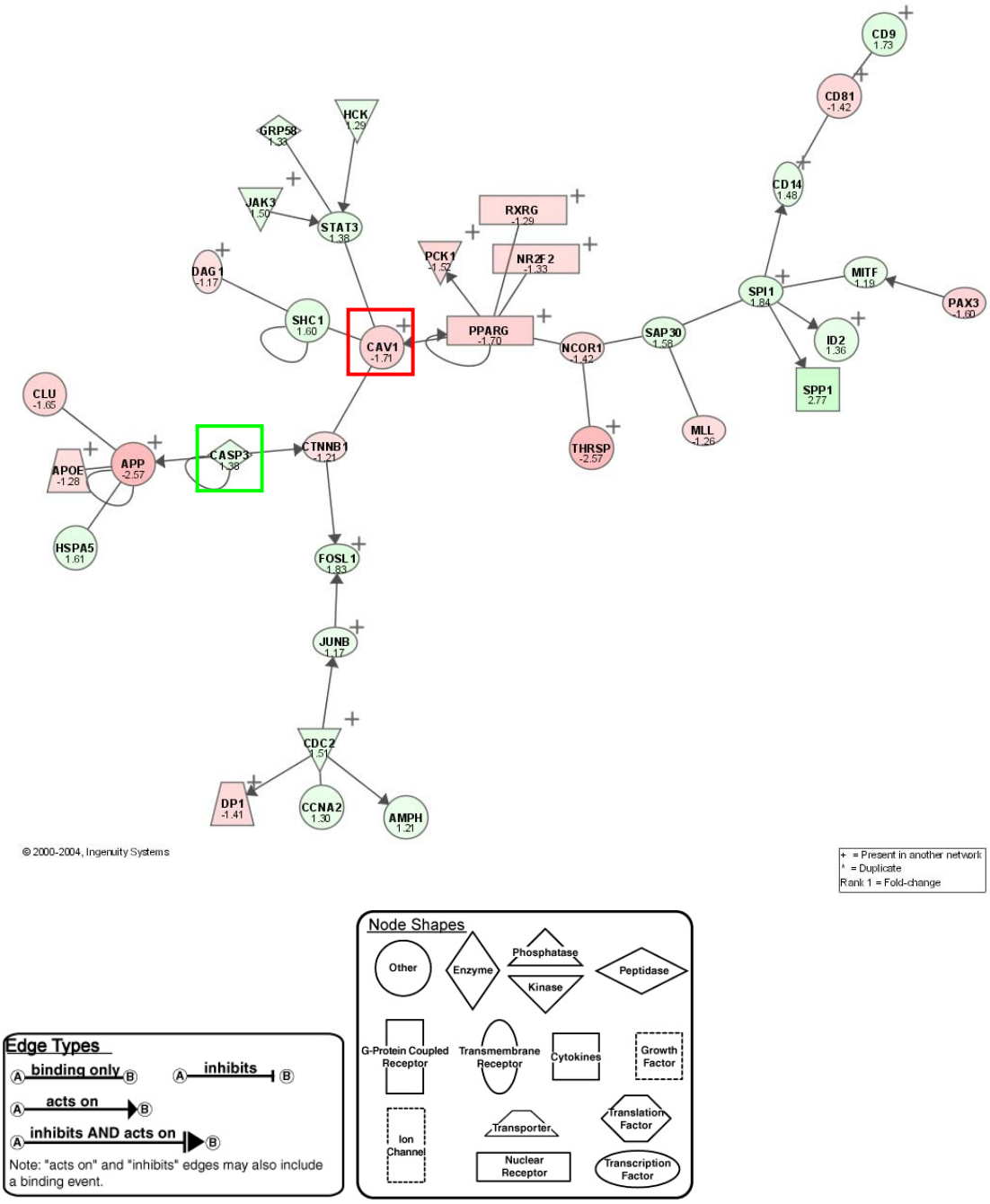

Figure 2.8 Ingenuity pathway analysis on the day 14 epididymal adipose"signature gene" dataset (P<0.01). Figure depicts networks created between gene interactions within the dataset; determined by mining databases of previously reported literature

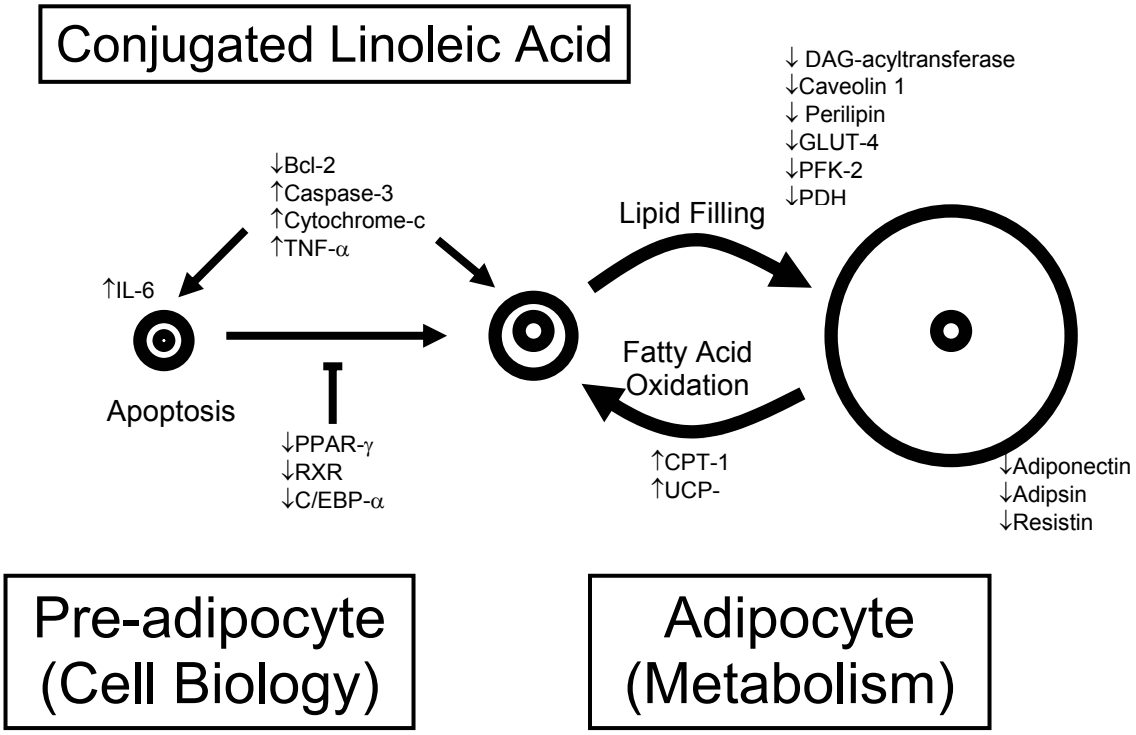

Figure 2.9 Schematic representation of the proposed CLA mechanism in murine white adipose tissue with a subset of our observations annotated adjacent to their respective function.

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