2011

Dairy-Derived Bioactive Alpha-Linolenic Acid, Conjugated Linoleic Acid, and Calcium as Modulators of ST2 Stromal, MC3T3-L1 Adipocyte-like, and MC3T3-E1 Osteoblast-like Cell Metabolism

Youjin Kim

THE FLORIDA STATE UNIVERSITY

COLLEGE OF HUMAN SCIENCES

DAIRY-DERIVED BIOACTIVE ALPHA-LINOLENIC ACID, CONJUGATED LINOLEIC ACID, AND CALCIUM AS MODULATORS OF ST2 STROMAL, MC3T3-L1 ADIPOCYTE-LIKE, AND MC3T3-E1 OSTEOBLAST-LIKE CELL METABOLISM

By

YOUJIN KIM

A Dissertation submitted to the
Department of Nutrition, Food and Exercise Sciences
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Degree Awarded:
Fall Semester, 2011

Youjin Kim defended this dissertation on October 17, 2011.

The members of the supervisory committee were:

Jasminka Z. Ilich-Ernst
Professor Directing Dissertation

Robert Contreras
University Representative

Peggy Y. Hsieh
Committee Member

Michael Ormsbee
Committee Member

Maria Spicer
Committee Member

The Graduate School has verified and approved the above-named committee members, and certifies that the dissertation has been approved in accordance with university requirements.

This dissertation is dedicated to my husband, Dongwook Shin, and daughters, Chloe Shin and Raina Shin, as well as my parents in South Korea, for their constant support and for being a true inspiration.

ACKNOWLEDGEMENTS

I would like to give my deepest appreciation to my professor, Dr. Jasminka Ilich-Ernst for her constant enthusiasm, endless patience, and help. I am awed by her focus and dedication to academics, research, and helping students succeed and will never be able to thank her enough for all of time and support that she has put toward my pursuit of a doctoral degree.

I would like to express my heartfelt gratitude to my committee members, Drs. Peggy Hsieh, Maria Spicer, Michael Ormsbee, and Robert Contreras for their unwavering support through my doctoral program. Without their wisdom and guidance this dissertation would never have come to fruition.

I owe a special thanks to Dr. Bahram Arjmandi for his willingness to admit me to the program and for his constant encouragement.

I would also like to thank Dr. Owen Kelly for his endless help as I learned the intricate skills of cell culture and for sharing scientific thinking.

Ann Smith, David Parish, Joseph Carroll, and Tara Hartman gave generously of their assistance, guidance, and encouragement over the years.

I would like to express my appreciation to the Ilich-Ernst lab mates, Pei-Yang Liu, Hennie Shin, Jennifer Lemacks, and Yichih Chi, for making everyday life in the laboratory

enjoyable. They have helped me in their own and unique ways and I will never forget the great times I had with them all in the lab.

I am extremely thankful to my husband, Dongwook Shin, two daughters, Chloe Shin and Raina Shin, my sister and sister-in-law, Seung-Ah Kim and Jaeyoon Kim, and my parents, Yang-Suk Woo and Tae-Ki Kim. Words alone cannot describe the thanks I owe to them who guided me, inspired me, and encourage me throughout my entire life and especially during my doctoral program. Their unwavering faith and confidence has given me the energy to keep moving forward in academia.

"Thank my Lord and Savior Jesus Christ who has blessed me tremendously.

All praise and glory to you Lord!"

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: Milk and dairy foods are high in n-3 α-linolenic acid (ALA), conjugated linoleic acid (CLA), and calcium, all of which are regarded as health-beneficial by potentially promoting bone formation and decreasing adiposity. This study examined whether each of these components individually or in interaction with each other could regulate bone and adipose cells. The possible mechanisms underlying this regulation were examined as well.

Methods: Mouse ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cells were treated with: 1) ALA with LA:ALA=1-5:1; 2) individual/combinations of 20 μM *cis*-9,*trans*-11 (9,11) and *trans*-10,*cis*-12 (10,12) CLA isomers (80:10, 90:10, or 90:5%); 3) calcium phosphate (0.5-3.0 mM); or 4) combinations of ALA, CLAs, and calcium, with a slight modification, accordingly, during proliferation (8 days) and adipocytic and/or osteoblastic differentiation (6 days). Following the oil red O and alizarin red S staining, quantification of triglyceride accumulation and calcium deposition was performed. Secretion of eicosanoids and growth factors was determined from differentiation media.

Results: 1) ALA with LA:ALA=1-5:1 inhibited proliferation/differentiation of MC3T3-L1 but facilitated MC3T3-E1 cell differentiation, showing maximal osteoblastogenesis and minimal adipogenesis at LA:ALA=4:1. At this level, insulin-like growth factor-1 (IGF-1) and IGF binding protein-3 (IGFBP-3) production were lowest in MC3T3-L1 cells, implying that ALA may regulate adipocyte differentiation via IGF-1/IGFBP-3 signaling pathway. 2) Various combinations of 9,11/10,12-CLA mixtures had a tendency to inhibit MC3T3-L1 and MC3T3-E1 cell proliferation. During differentiation, the combination of 9,11- and 10,12-CLAs, exerted a promising outcome by further decreasing adipocytic and increasing osteoblastic differentiation. In both cell lines, most of CLA isomer mixtures resulted in insignificant increases in production

of prostaglandin E_2 (PGE_2). 3) The 1.5-2.5 mM calcium level was the best with regard to promoting ST2 and MC3T3-E1 and inhibiting MC3T3-L1 cell proliferation. 4) Combination of ALA, CLA isomers, and calcium generally decreased ST2 and MC3T3-E1 but not MC3T3-L1 cell proliferation. During differentiation, however, ALA (4:1)+CLA (90:10%)+calcium (2.0 mM) significantly attenuated lipid accumulation in MC3T3-L1 and increased calcium deposition in MC3T3-E1 cells.

Conclusions: These findings suggest that a LA:ALA=4:1 ratio may be beneficial for increasing osteoblastogenesis and decreasing adipogenesis, which may be partially mediated via IGF-1/IGFBP-3 signaling pathway. This favorable effect could be further enhanced when the LA:ALA=4:1 is incorporated with CLA (9,11:10,12=90:10%) and high calcium (2.0 mM), implying the possible benefit of this dietary regimen found in milk and dairy products in bone and body composition.

Keywords: α-Linolenic Acid, Conjugated Linoleic Acid, Calcium, Mesenchymal stem cell, Adipocyte, Osteoblast, Osteoporosis, Obesity

CHAPTER 1

INTRODUCTION

Osteoporosis and obesity are global health concerns, both of which can be influenced by dietary intake [Rosen and Bouxsein, 2006]. A growing body of recent evidence has suggested a close link between these abnormal conditions because osteoblasts and adipocytes share pluripotent mesenchymal stem cells (MSC) as their common origins in the bone microenvironment [Rosen and Bouxsein, 2006]. Studies in humans have also supported that excessive adiposity is inversely associated with bone mass and may be positively related to increased bone fracture [Goulding et al., 2001; Pollock et al., 2007; Weiler et al., 2000]. The pathophysiological connection between fat and bone is very complex and it is still uncertain whether fat infiltration causes bone loss or fat just fills the space where bone once existed [Cao, 2011]. However, it is regarded that MSC favor adipogenic differentiation by default and trans-differentiation of MSC to bone lineage can be triggered by various dietary, local, and systemic factors [Pittenger et al., 1999].

Milk and dairy foods are nutrient-dense and provide essential macro- and micronutrients to the diet. In particular, polyunsaturated fatty acids (PUFA), mainly, n-6 linoleic acid (LA) and n-3 α-linolenic acid (ALA), account for ~7% of milk fatty acids with the ratio of ~2-5:1, which is very low compared with the ratio of 15-41:1 found in the typical Western diets [Haug et al., 2007; Simopoulos, 2006]. ALA, a metabolic precursor of long-chain n-3 PUFA, can be endogenously converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through a series of elongation and unsaturation processes. However, the conversion efficiency of ALA to EPA or DHA is very low; ~5% and <0.5%, respectively [Plourde and Cunnane, 2007]. According to recent reports, dietary intake of ALA was favorable to various bone properties in

postmenopausal women and osteoporotic animals [see review by Kim and Ilich, 2011]. Its bone-protective effect was more intensified when combined with estrogen therapy or in various pathological conditions [Cohen et al., 2005; Sacco et al., 2009a; 2009b]. The anti-adipogenic role of ALA was also documented that ALA-rich diets resulted in a significant reduction in body weight, visceral adiposity, and leptin in circulation [Begg et al., 2010; Chicco et al., 2009], whereas ALA-deficient diets led to a significant increase in hepatic *de novo* lipogenesis, total/epididymal fat mass, and adipocyte proliferation [Pouteau et al., 2010].

Conjugated linoleic acid (CLA), a mixture of positional and geometric isomers of LA, is mainly found in ruminant animals, milk, and dairy products [Hur and Park, 2007]. In nature, *cis*9, *trans*11- (9,11-CLA; 80-90%) and *trans*10, *cis*12-CLAs (10,12-CLA; 5-10%) are most predominant, biologically active; however, they may act differently in exerting many physiological functions [Hur and Park, 2007]. With respect to bone biology, the role of CLA is equivocal. In animals, although CLA supplementation had no effect on bone mineral content (BMC) and bone mineral mass, it increased or reduced bone formation [Burr et al., 2006; Kelly et al., 2003; Li et al., 1999; Watkins et al., 2003]. In ovariectomized (OVX) rats, CLA enhanced dietary calcium absorption, improved bone formation, and decreased bone resorption [Kelly and Cashman, 2004]. In addition, CLA reduced *ex vivo* prostaglandin E_2 (PGE_2) production, increased alkaline phosphatase (ALP*)* activity, but did not alter osteocalcin, urinary pyridinium crosslinks (Pyr), or insulin-like growth factor-1 (IGF-1) [Cusack et al., 2005; Kelly and Cashman, 2004; Li et al., 1999; Watkins et al, 2003]. The anti-adipogenic role of CLA, particularly 10,12-CLA, is also recognized *in vitro* where 10,12-CLA suppressed adipogenesis by reducing adipocytes' formation, differentiation, activity, and function, whereas 9,11-CLA induced

opposite outcomes [Brown et al., 2004; Evans et al., 2002]. Similar findings were observed in animals and humans [Navarro et al., 2006].

As mentioned above, milk contains some bioactive compounds, including ALA with ratios of n-6 LA to n-3 ALA of 2-5:1, CLA (especially 9,11- and 10,12-CLAs), and calcium, all of which are implicated to be beneficial to promote bone health and reduce adiposity. Mechanisms of how these bioactive compounds modulate bone and fat metabolism are not clearly known. In spite of it, it is largely understood that higher consumption of n-3 PUFA (or low dietary n-6:n-3 PUFA ratios) decreases the production of pro-inflammatory eicosanoids, such as PGE_2 and leukotriene B_4 (LTB_4), derived from n-6 arachidonic acid (AA). Hence, a dramatic increase of the n-6:n-3 PUFA ratio in a diet may accelerate the occurrence of many allergic and inflammatory diseases including osteoporosis and obesity [Haug et al**.,** 2007]. Similarly, CLA may also hinder the production of PGE_2 and LTB_4 by interfering AA synthesis (PGE_2 precursor) or gene expression/activity of Cox-2 (a key enzyme involved in PGE_2 synthesis) [Hur and Park, 2007]. The interplay between fat and bone can be mediated by various lipid signaling pathways. Some local growth factors, including IGF-1, IGF binding protein-3 (IGFBP-3), transforming growth factor- β (TGF-β), and bone morphogenetic protein-2 (BMP-2), are crucial for regulating a wide range of cellular processes by stimulating cellular growth, proliferation, and differentiation, implying a possible involvement of these factors in the regulation of bone and fat cell metabolism.

Aim of the Study

Based on findings of which individual ALA (with LA:ALA= 2-5:1), CLA, and calcium, are implicated to promote bone health and reduce adiposity, it is crucial to examine individual

and/or combined effects of these dairy components to gain better understanding about their roles in bone formation and adiposity. The objective of the study was, therefore, to elucidate individual and interactive effects of dairy components, including ALA with relatively low n-6:n-3 PUFA ratios (LA:ALA=1-5:1), CLA isomers (individual or a combination of 80-90% 9,11- and 5-10% 10,12-CLAs), and calcium (0.5-3.0 mM), on modulating cellular growth of MSCs, osteoblasts, and adipocytes, as well as to reveal the underlying mechanisms of the regulation of these cell developments.

Specific Aim 1. To investigate the effect of ALA with n-6:n-3 PUFA ratios=1-5:1 on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 1: It is hypothesized that n-6:n-3 PUFA ratios with a range of 1-5:1 will facilitate the commitment of MSC toward osteoblast lineage, decrease the growth (hyperplasia) of adipocytes, and increase the growth of osteoblasts, favoring bone development and inhibiting fat cell formation. According to recent findings, low n-6:n-3 PUFA ratios (or high n-3 PUFA) tend to reduce the risk for osteoporosis and obesity, mainly due to the benefit of n-3 PUFA in promoting overall bone integrity and at the same time, decreasing body weight/adipogenesis [Kim et al., 2006; Shirouchi et al., 2007]. To date, however, most of n-3 PUFA research has targeted long-chain n-3 EPA/DHA and thus, this study will be the first to investigate the direct role of ALA in the development of stromal cells, osteoblasts, and adipocytes.

S**pecific Aim 2.** To examine the effect of CLA (9,11- and 10,12-CLAs) on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 2: It is hypothesized that individual and/or a combination of CLA isomers (80-90% 9,11- and 5-10% 10,12-CLAs; the ratio of 9,11- and 10,12-CLAs=100:0, 0:100, 80:10, 90:10, and 90:5) will trigger the trans-differentiation of MSC toward osteoblasts, attenuate the growth

(proliferation/differentiation) of adipocytes, and facilitate the growth of osteoblasts by modulating lipid signaling pathways which may involve local regulatory factors (some eicosanoids and growth factors). The proposed CLA isomer mixtures are mimicking CLA blends found in nature (in contrast to the synthetic type of CLA consisting of 40:40% 9,11- and 10,12-CLAs, commonly used for CLA supplements). This hypothesis is based on evidence supporting the bone-protective and anti-adipogenic property of CLA, in spite of existing controversies between different species, CLA isomer types, and various CLA concentrations utilized in different studies [Brown et al., 2004; Burr et al., 2006; Kloss et al., 2005; Watkins et al., 2003]. The anti-adipogenic effect of CLA would also be beneficial to promote bone mass by attenuating adipogenesis in bone marrow [Hur and Park, 2007].

Specific Aim 3. To investigate the effect of different levels of calcium on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 3: It is hypothesized that higher levels of calcium (in form of calcium phosphate found in milk) favor the commitment of MSCs' lineage to osteoblasts, increase osteoblast formation (osteoblastogenesis), and decrease adipocyte formation (adipogenesis). It is widely recognized that calcium promotes bone formation [Insel et al., 2004]. In the meantime, a growing body of evidence has supported that calcium is effective in reducing adiposity (body weight/fat) by directly altering intracellular calcium concentrations, which may modulate a subsequent lipid metabolic process [Zemel et al., 2002].

Specific Aim 4. To examine the possible interaction among ALA, CLA, and calcium on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 4: It is hypothesized that the incorporation of major milk components, such as ALA with n-6:n-3 PUFA ratios=1-5:1, CLA, and calcium, may further promote the potential of each

component's capability to increase osteoblastogenesis and inhibit adipogenesis. While the health benefits of these molecules are promising, it is unknown whether these bioactive milk compounds can truly interact together and exert additional benefits beyond their individual effects. From this perspective, the study will provide a new insight into the effect of milk components on bone and fat cells and show the possible interactions among them.

Significance

Research elucidating the link between obesity and osteoporosis is a new area of investigation. To date, the assessment of osteoporosis and obesity connection has been largely based on cross-sectional data with limited ability to reveal the exact relationship between these pathophysiological conditions. Hence, there is a need for further research *in vivo* and *in vitro.* To our knowledge, this is the first study investigating the interaction among bioactive milk components, including ALA with low LA:ALA ratios (1-5:1), CLA, and calcium, in modulating bone formation and adiposity. Along with the basic findings that osteoblasts and adipocytes share a common progenitor cell (MSC) as their common origin, there is growing evidence supporting an inverse association between bone marrow adiposity and bone mass. Therefore, it is crucial to identify factors capable of stimulating MSC's lineage commitment from adipocytes to osteoblasts, or directly enhancing osteoblast differentiation and inhibiting adipocyte differentiation, all of which will ultimately contribute to increased bone mass. This *in vitro* study will, therefore, form a basis for further research elucidating the potential health benefits of dairy products for osteoporosis and obesity. Also, the results might provide useful strategies to manipulate milk composition leading to ways of using milk as a possible delivery system for

nutraceuticals with known benefits to human health and to combat clinical pathological conditions including obesity or osteoporosis.

CHAPTER 2

LITERATURE REVIEW

Link between Osteoporosis and Obesity

Osteoporosis and obesity are global health concerns, both of which can be influenced by dietary intake [Rosen and Bouxsein, 2006]. Osteoporosis is typically defined as the progressive loss of bone mass and deterioration of bone tissue, leading to skeletal weakness and increased susceptibility to bone fracture [Huth et al., 2006]. At present, almost 44 million US adults over age 50 years are diagnosed with either osteoporosis or osteopenia (lesser bone mass), putting them at the higher risk of bone fracture [Cashman, 2007]. At the same time, a dramatic increase of obesity, especially during the past 20 years in the US, has been directly related to the development of numerous chronic diseases such as cardiovascular disease, type II diabetes, and a certain types of cancer. According to the recent report (2007-2008), more than one-third of children and over two-thirds of adults in the US are estimated as overweight or obese [Flegal et al., 2010].

Osteoporosis and obesity were once thought to be mutually exclusive. However, a line of recent evidence has suggested a close link between these abnormal conditions because osteoblasts and adipocytes share pluripotent mesenchymal stem cells (MSC) as their common origins in the bone microenvironment [Rosen and Bouxsein, 2006]. Studies in humans have also supported that excessive adiposity was inversely associated with bone mass and may positively related to increased bone fracture [Goulding et al., 2001; Pollock et al., 2007; Weiler et al., 2000]. The pathophysiological connection between fat and bone is very complex and it is still uncertain whether fat infiltration causes bone loss or fat just fills the space where bone once existed [Cao,

2011]. However, it is regarded that MSC favors adipogenic differentiation by default and trans-differentiation of MSC to bone lineage can be triggered by various dietary, local, and systemic factors [Pittenger et al., 1999]. In fact, physiological conditions such as aging, estrogen depletion, sedentary lifestyle, and exposure to microgravity encouraged the commitment of MSC to the adipocyte lineage [Cartwright et al., 2007; Foo et al., 2007; Goodpaster and Brwon, 2005; Meyers et al., 2005]. Conditions promoting bone loss also increased fat accrual within bone marrow and osteoporotic and/or osteopenic bones typically had facilitated adipogenesis [Duque, 2008; Baksh, 2004]. Therefore, maintaining osteogenic environment in bone marrow is critical to maintain/promote overall bone health.

Bioactive Compounds in Milk

Milk is a nutrient-dense food that provides essential macro- and micronutrients to the diet. Aside from the nutritious value, milk also contains a variety of potent bioactive substances including certain proteins (α-lactalbumin, β-lactoglobulin), phosphopeptides (caseinophosphopeptides), non-digestible oligosaccharides (lactose, lactulose), organic acids (fatty acids), cytokines, growth factors, and hormones [Ebringer et al., 2008]. On average, total lipid content in whole milk is about 33 g/L [Jensen and Newburg, 1995]. Although saturated- (SFA; ~65%) and monounsaturated fatty acids (MUFA; ~28%) are predominant, PUFA, mainly LA and ALA, accounts for ~7% of milk fatty acids (varied in breeds and living environments) with LA:ALA=~2-5:1, which is very low compared with the ratio of 15-41:1 found in the typical Western diets [Haug et al., 2007; Simopoulos, 2006]. With this reason, despite the concern about milk having a potency capable of increasing body weight/fat due to the high content of total fat and SFA, milk is regarded as health beneficial by its relatively high PUFA (LA and ALA)

content and low n-6:n-3 PUFA ratios (~2-5:1). Because milk fat content is readily manipulated by changing the type/amount of fatty acids in ruminant diets, incorporating fish oil to the ruminant diet to increase n-3 EPA and/or DHA amounts in milk became popular [Dhiman et al., 2005; Jenkins and McGuire, 2006]. The detailed composition of whole milk is described in Table 1.

Table 1. Nutrient composition of whole milk (liquid and dry)

Milk component	Content	
	Whole, liquid	Whole, dry
Water, %	88.32	2.47
Protein, %	3.22	26.32
Carbohydrate, %	4.52	38.42
Fat, %	3.25	26.71
Saturated fatty acids (% of total fat)	(64.9)	(66.1)
Monounsaturated fatty acids (% of total fat)	(28.3)	(31.3)
Polyunsaturated fatty acids (% of total fat)	(6.8)	(2.6)
Linoleic acid [% of total PUFA]	[54.3]	-
Alpha-linolenic acid [% of total PUFA]	[33.9]	-

Source. USDA National Nutrient Database for Standard Reference

Polyunsaturated Fatty Acids (PUFA)

1. Dietary Ratios of n-6:n-3 PUFA

The modern Western diet has changed enormously over the last several decades, compared with the diet people evolved with, notably in a dramatic increase in saturated-, trans-, and n-6 fatty acid consumptions. In particular, the increase of n-6 PUFA is mainly due to the indiscriminate recommendation of this fatty acid to substitute for SFA, in order to lower serum cholesterol levels. Consequently, the n-6:n-3 PUFA ratio in a typical Western diet has increased from estimated 1-2:1 (in hunter-gatherer societies) to 15-41:1 presently [Simopoulos, 2000; 2002]. It is largely understood that increasing the consumption of n-6 PUFA encourages the

synthesis of pro-inflammatory PGE_2 and LTB_4, derived from AA, whereas higher intake of n-3 PUFA facilitates the production of anti-inflammatory PGE_3 and LTB_5, converted from EPA. Hence, a dramatic increase of n-6:n-3 PUFA ratios can accelerate the occurrence of many allergic/inflammatory diseases including osteoporosis and obesity [Simopoulos, 2006]. The optimal n-6:n-3 PUFA ratio in a diet is still under debating because it can vary in different physiological/pathological conditions [Cook, 1996; de Longeril et al., 1994]. According to the dietary guidelines of Health Canada and US dietary reference intakes (DRIs), it is recommended to decrease n-6:n-3 PUFA ratios as low as 4:1 and ~5-10:1, respectively, for healthy adults [Kris-Etherton and Hill, 2008].

2. α-Linolenic Acid (ALA)

ALA (18:3n-3), the parent fatty acid of n-3 PUFA family, is abundant in some plant seeds (flax, canola, rape, chia, perilla), green leafy vegetables (purslane, cauliflower, spinach), and walnuts. ALA can be endogenously converted into EPA (20:5n-3) and DHA (22:6n-3) through a series of elongation and unsaturation processes. However, the conversion efficiency of ALA to EPA or DHA is very low as only ~5% and <0.5%, respectively, indicating that ALA can be distinct from longer n-3 EPA and DHA in regulating various physiological conditions [Plourde and Cunnane, 2007]. Long-chain n-3 EPA and DHA are mostly found in fatty fish (herring, sardines, salmon, tuna), shellfish, some algae, and fungi [Harris, 2004; Weber et al., 1986]. According to the recent DRIs (2010), the acceptable macronutrient distribution range (AMDR) for ALA is 0.6-1.2% of daily energy intakes and adequate intakes (AIs) for ALA are 1.6 g/d for men and 1.1 g/d for women, respectively. To meet the recommendation of n-3 PUFA, the American Dietetic Association and Dietitians of Canada encourage the consumption of 2 servings of fatty fish (~8 oz cooked or ~10.5 oz raw) per week to provide 500 mg/d of

EPA+DHA in healthy people. For individuals suffering from coronary heart disease or those who attempt to lower plasma triglyceride levels, over 1.0 g/d of EPA+DHA is recommended under physician's care. The general 'safe' level for EPA+DHA is set at 3.0 g/d by the US Food and Drug Administration [Kris-Etherton et al., 2002; Kris-Etherton and Hill, 2008].

Role of ALA in Bone. Flaxseeds and flaxseed oil are common sources of ALA in nature and thus, most of ALA studies have been performed by using these food sources. According to recent data, although supplementation of whole flaxseeds did not lead to marked improvement of osteoporotic bones in humans and animals, when combined with estrogen therapy, they offered an extra benefit to bone in animal models [Kim and Ilich, 2011]. Similar results were found in studies using flaxseed oil where its bone-protective effect was more intensified when combined with estrogen therapy or in various pathological conditions (kidney disease, obesity with insulin resistance) [Cohen et al., 2005; Sacco et al., 2009a; 2009b]. Based on these available data, it is assumed that supplementation of ALA in the form of flaxseeds/flaxseed oil may contribute to some improvement in osteoporotic bone properties in postmenopausal women and osteoporotic animals.

Of note, compared with ALA, there are numerous studies examining the effect of long-chain n-3 EPA and DHA on bone. In animal studies, diets rich in fish oil (EPA+DHA) prevented low-calcium induced bone deteriorations, potentiated the bone-protective effect of estrogen, and maintained BMD in ovariectomized (OVX) rodents [Sakaguchi et al., 1994; Schlemmer et al., 1999; Sun et al., 2003]. However, they did not influence BMD and bone mineral content (BMC) in obese, insulin-resistant rats [Mollard et al., 2005]. On the contrary, diets deficient in fish oil resulted in greater bone loss in OVX rats and exhibited reductions in urinary calcium excretion and overall bone integrity in normal rats [Sun et al., 2003; Shen et al., 2007; Watkins et al.,

2005]. In humans, fish oil improved intestinal calcium absorption, increased plasma levels of bone formation markers (osteocalcin, procollagen), and helped maintain or increase bone mass (lumbar vertebrae, femur) in osteoporotic patients [Kruger et al., 1998; Van Papendrop, 1995]. Dietary DHA showed a positive correlation with bone mineral accrual and BMD in young men [Högström et al., 2007] but taking the Efacal® consisting of primrose oil (rich in n-6 γ-linoleic acid), fish oil, and calcium did not affect total BMD in women [Bassey et al., 2000].

Bone formation and resorption procedures are tightly controlled by numerous factors, mainly systemic hormones and local factors, produced by bone. The primary mechanism by which n-3 PUFA may modulate bone biology is through altering cell membrane composition by replacing n-6 AA with n-3 PUFA, which possibly decreases the production of PGE_2 and LTB_4 [Bhattacharya et al., 2007]. It is well established that a high concentration of PGE_2 is a potent inducer of bone resorption that is partially mediated by bone-derived IGFs [Raisz et al., 2005]. Likewise, 4-series of LTs (LTB_4, LTC_4, LTD_4, LTE_4) generated by AA may be also involved in bone metabolism by facilitating bone resorption and osteoclastogenesis [Watkins et al., 2001]. In addition, n-3 PUFA appears to lower the production of pro-inflammatory cytokines (tumor necrosis factor α (TNF-α), interleukine-1β (IL-1β), IL-6) and increase the synthesis of anti-inflammatory cytokines (IL-10) [Bhattacharya et al., 2007]. Pro-inflammatory cytokines may negatively influence bone mass by stimulating osteoclastic activity and osteoclastogenesis, thereby leading to increased bone resorption [Nanes, 2003; Ragab et al., 2002; Roodman, 1996].

Role of ALA in Adiposity. The anti-adipogenic role of ALA has also been documented. In animals, ALA-rich diets resulted in reductions in body weight, visceral adiposity, and leptin in circulation [Begg et al., 2010; Chicco et al., 2009], whereas ALA-deficient diets led to increases in hepatic *de novo* lipogenesis, total/epididymal fat mass, and adipocyte proliferation in

subcutaneous adipose tissue [Pouteau et al., 2010]. In spite of it, the anti-adipogenic effect of ALA was reported as less effective than EPA and DHA [Flachs et al., 2005; Ruzickova et al., 2005].

Regarding the anti-adipogenic property of EPA/DHA, Japanese and Eskimo people who consume fish more often than other populations were found to have lower incidence of obesity-induced diseases (i.e., cardiovascular disease), which first revealed the possible anti-adipogenic property of fish (high in EPA+DHA) [Dyerberg et al., 1978; Kagawa et al., 1982]. Later on, similar results were reported *in vitro* and *in vivo* studies. In cultured pre-adipocytes, DHA facilitated apoptosis and inhibited differentiation of the cells [Kim et al., 2006]. In rodents, EPA+DHA lowered adipose tissue mass [Gaiva et al., 2001; Minami et al., 2002; Shirouchi et al., 2007] and exhibited a further anti-adipogenic effect (preferentially in the abdomen) over ALA [Flachs et al., 2005; Ruzickova et al., 2005]. In obese females, an energy-restricted diet high in EPA+DHA resulted in significant weight loss, possibly being attributed to DHA [Kunesova et al., 2006]. In young, overweight men, incorporation of fish and/or fish oil as part of an energy-restricted diet resulted in greater weight loss, although it was not observed in young, overweight women [Thorsdottir et al., 2007].

Despite the existing controversies, n-3 PUFA may attenuate energy intake, which partly explains the anti-obesogenic mechanism of n-3 PUFA [Li et al., 2008; Perez-Matute et al., 2007]. It is known that n-3 PUFA decreases white fat mass at specific fat depots (retroperitoneal and epididymal fat pads) and enhances thermogenesis in brown adipose tissue (BAT), which is partially mediated by altering gene expression of uncoupling proteins (UCPs) [Oudart et al., 1997; Takahashi and Ide, 2000]. In addition, n-3 PUFA may participate in the regulation of lipid metabolism by lowering lipid uptake by adipocytes via suppressing lipoprotein lipase activity

[Raclot et al., 1997], increasing lipid catabolism via enhancing fatty acid oxidation [Guo et al., 2007], reducing lipid synthesis via inhibiting activities of stearoyl-CoA desaturase and fatty acid synthase [Kim et al., 2003], and possibly increasing intracellular lipolysis [Raclot et al., 1997]. n-3 PUFA may also control the status of adipocytes by regulating pre-adipocyte differentiation and/or apoptosis [Kim et al., 2006; Perez-Matute et al., 2007].

3. Conjugated Linoleic Acid (CLA)

CLA is a collective term used to describe a mixture of positional and geometric isomers of LA (*cis-9, cis-12* octadecadienoic acid; 18:2n-6) [Kepler et al., 1966]. In nature, CLA is formed as a result of biohydrogenation by LA isomerase produced by luminal microbes (*Butyrivibrio fibrisolvens*) and therefore, is mainly found in ruminant animals (beef, lamb), milk, and dairy products (Table 2) [Hur and Park, 2007].

Table 2. Conjugated linoleic acid (CLA) content in various foods

Food	Amount (mg/g fat)	Food	Amount (mg/g fat)
Dairy Products		Meat and Fish	
Homogenized milk	5.5	Lamb	5.8
2% milk	4.1	Ground beef	4.3
Condensed milk	7.0	Veal	2.7
Cultured buttermilk	5.4	Ground turkey	2.6
Butter	4.7	Chicken	0.9
Butter fat	6.1	Pork	0.6
Sour cream	4.6	Egg yolk	0.6
Ice cream	3.6	Salmon	0.3
Plain yogurt	4.8		
Frozen yogurt	2.8	Vegetable Oil	
Medium cheddar	4.1	Safflower oil	0.7
American processed	2.0	Sunflower oil	0.4

Source. Adapted from MacDonald, 2000

Although over 25 different CLA isomers have been identified in nature, 9,11- (80-90%) and 10,12-CLAs (5-10%) are most predominant and biologically active; however, they may act differently in exerting many physiological functions [Hur and Park, 2007]. Currently, synthetic type of CLA is also available but it contains approximately equal amounts (~40% each) of 9,11- and 10,12-CLA isomers [Pariza et al., 2001]. Figure 1 represents the structure of LA, 9,11-CLA, and 10,12-CLA.

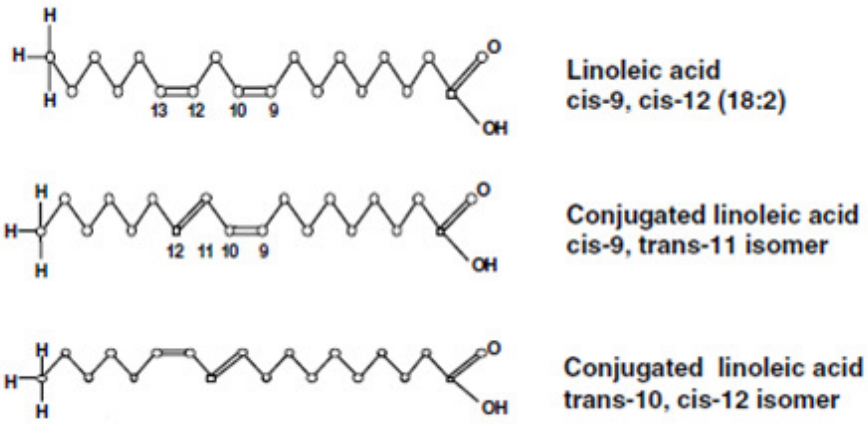


Figure 1. Structure of LA and its major CLA derivatives, 9,11- and 10,12-CLA isomers [modified from Wahle et al., 2004]

To date, it has been reported that CLA exerts a variety of different biological functions: anti-carcinogenic, anti-diabetic, anti-adipogenic, anti-inflammatory, and anti-atherosclerotic activities [Wahle et al., 2004]. However, the biological properties of 9,11- and 10,12-CLAs seem to be distinct and they may act independently or synergistically each other, which possibly results in an antagonistic or additive net effect in regulating many physiological functions. It is also evident that functions of CLA isomers vary in different strains and species of animals [Pariza et al., 2003]. Table 3 represents the major biological functions of 9,11- and 10,12-CLA isomers.

Table 3. Biological effects of 9,11- and 10,12-CLA isomers

Biological effect	9,11-CLA		10,12-CLA
Anti-cancer	+	>	+
Anti-Adiposity	-		+
Anti-atherosclerosis	+		+
Anti-diabetes	+ (?)		+
Anti-inflammation	+	>	+

Source. Adapted from Churruca et al., 2009
Symbols. +, positive effect; -, no effect; ?, no general consensus; >, greater effect

***Role of CLA in Bone*.** Studies investigating the role of CLA in bone biology are limited and the findings are equivocal. It is probably because the net effect of CLA could be affected by types of CLA isomers mixed, type/amount of other fatty acids making up the experimental diet, and variations of tested animals in their age and sex [Watkins et al., 2003]. In animals, although CLA supplementation had no effect on BMC and bone mineral mass, it increased or reduced bone formation [Burr et al., 2006; Kelly et al., 2003; Li et al., 1999; Watkins et al., 2003]. In OVX rats, CLA enhanced dietary calcium absorption, improved bone formation, and decreased bone resorption [Kelly et al., 2004]. In addition, CLA reduced *ex vivo* PGE_2 production, increased ALP activity, but did not alter osteocalcin, urinary Pyr, or IGF-1 [Cusack et al., 2005; Kelly and Cashman, 2004; Li et al., 1999; Watkins et al., 2003]. In most human studies, CLA consumption failed to show a positive link to bone mass [Doyle et al., 2005; Gaullier et al., 2004; Kreider et al., 2002], except one study showing a significant correlation between >63 mg/d CLA consumption and increased forearm BMD in postmenopausal women [Brownbill et al., 2005]. When examining bone markers, CLA supplementation did not lead to any significant changes in bone formation and bone resorption markers, as well as serum/urinary calcium levels [Doyle et al., 2005; Hur and Park, 2007]. Unlike these mixed results in animals and humans, findings in

cultured cells are quite consistent. Evidently, CLA increased the production of core binding factor α-1 (Cbfa1; osteoblast-specific transcription factor), leading to elevated osteoblast differentiation [Watkins et al., 2003]. In another study, 9,11-CLA, but not 10,12-CLA, significantly enhanced bone mineralization accompanied by an increase in ALP activity [Platt et al., 2007].

In respect to the possible mechanism of CLA in modulating bone formation, first, CLA may decrease the production of some pro-inflammatory eicosanoids such as PGE_2, PGF_2, and LTB_4 [Lee, 2008], of which PGE_2 is the most potent pro-inflammatory agent capable of causing bone resorption. There are several ways explaining how CLA lowers PGE_2 synthesis: 1) LA is the parent molecule of both CLA and AA. CLA can compete with LA in their synthesis, which may hinder AA synthesis and a subsequent PGE_2 production. 2) CLA may suppress the gene expression/activity of Cox-2 (a key enzyme involved in PGE_2 synthesis), which, in turn, can influence some hormones (parathyroid hormone (PTH), IGF-1) and PGE_2-dependent bone resorption by stimulating osteoclast activity [Hur and Park, 2007; Lee, 2008]. 3) CLA can inhibit the synthesis of pro-inflammatory cytokines (TNF-α, IL-1, -2, -4, -6, -8, -10, interferon-γ (IFN-γ)), which can contribute to decreased PGE_2 production [Hur and Park, 2007]. Second, CLA can stimulate osteoblast differentiation by facilitating Cbfa1 and inhibiting peroxisome proliferator-activated receptor-γ (PPAR-γ) gene expression and production. PPAR-γ, highly expressed in adipose tissues and macrophages, is essential in adipocyte differentiation and inflammation. CLA acts as a natural inhibitory ligand of PPAR-γ in adipose tissue, mainly through nuclear factor-kappa-B (NFκB) (a transcription factor related to cell signaling pathways) [Brown et al., 2004; Granlund et al., 2005; Kang et al., 2003], in spite of data showing an opposite result in muscles and macrophages [Meadus et al., 2002; Yu et al., 2002]. Third, CLA can reduce the

production of leptin (adipose tissue-derived hormone), which is known to be unfavorable to bone formation, hence possibly contributing to the protection of bone loss [Mallamaci et al., 2005]. However, the role of CLA in leptin regulation is still controversial, making it difficult to define the involvement of leptin in the regulation of CLA in bone [Corino et al., 2002].

Role of CLA in Adiposity. At present, it is generally agreed that 10,12-CLA, rather than 9,11-CLA, has an anti-adipogenic activity [Evans et al., 2001; 2002]. Evidently, although 10,12-CLA attenuated differentiation and lipid droplet accumulation, 9,11-CLA brought about opposite outcomes in white and brown adipocytes *in vitro* [Brown and McInstosh, 2003; Rodriguez et al., 2002]. Similar results were observed in animals [Martin and Valeille, 2002; Wang and Jones, 2004] but the results are quite inconsistent in humans [Navarro et al., 2006; Salas-Salvado et al., 2006]. In some human studies, CLA supplementation induced a moderate body fat reduction but overall, this anti-adipogenic effect of CLA was not as prominent as the effect shown in animals. Contradictive results were also reported in other human studies [Navarro et al., 2006; Salas-Salvado et al., 2006]. The discrepant results found in humans may be due to the following reasons: 1) In most human studies, a synthetic type of CLA (containing approximately equal amounts of 9,11- and 10,12-CLAs) has been used, which can compromise (negate or boost) the individual functions of CLA isomers. 2) Despite the threshold of CLA as 3-6 g/d to elicit a significant fat reduction, a range of various 10,12-CLA concentrations has been used in different human studies [Churruca et al., 2009].

In general, the anti-adipogenic property of 10,12-CLA could be attributed to its ability to stimulate adipocyte apoptosis, reduce adipocyte size, decrease intracellular triglyceride content, or increase fatty acid oxidation [Kloss et al., 2005; Larsen et al., 2003; Mirand et al., 2004]. Furthermore, 10,12-CLA may suppress some transcription factors capable of enhancing

adiposity, such as PPAR-γ and CCAAT/enhancer binding protein-α (C/EBPα), which can direct a corresponding reduction in adipocyte differentiation [Brown and McIntosh, 2003; Kang et al., 2003]. The 10,12-CLA may also attenuate the release of pro-inflammatory cytokines, particularly TNF-α, which can subsequently reduce the production of pro-inflammatory eicosanoids, including PGE_2 and LTB_4 [Platt and El-Sohemy, 2009]. Lastly, 10,12-CLA may stimulate the gene expression of UCPs in brown/white adipose and liver tissues, which can induce a higher metabolic rate and resultant lesser adiposity [Salas-Salvado et al., 2006].

Milk and Calcium

Role of Milk/Calcium in Bone. The calcium content in milk is about 1 g/L. Milk products not only supply more than half of the US dietary calcium but also provide other bone minerals (phosphorous, magnesium) and vitamin D. To date, commercial milk is often fortified with vitamin D that is believed to synergistically interact with calcium, offering an extra bone protection beyond calcium alone. The benefit of calcium in bone is widely recognized and reviewed elsewhere [Cashman, 2002; Insel et al., 2004]. Along with calcium, non-digestible oligosaccharides (lactose, lactulose), basic whey protein fractions (α-lactalbumin, β-lactoglobulin, lactoferrin), and caseinophosphopeptides are also present in milk, all of which are implicated as bone-protective, possibly by increasing calcium absorption, stimulating bone formation and mineralization, or lowering bone resorption [Cashman, 2003; Cashman and Flynn, 1999; Uenishi et al., 2007]. Since 1975, a substantial number of epidemiological and clinical studies have consistently indicated that consuming adequate amount of calcium or calcium-rich foods help attain optimal peak bone mass by age 30 or earlier, slow age-induced bone loss, and lower the risk of osteoporotic fractures in later life [Ryan-Harshman and Aldoori, 2005]. The

significance of calcium intake is clearly stated in the latest Dietary Guidelines for Americans (2010) and the Surgeon General's Report on Bone Health and Osteoporosis (2004), in which 3 cups of milk a day is currently recommended [Huth et al., 2006].

Role of Milk/Calcium in Adiposity. To date, an inverse relationship between milk/calcium consumption and body adiposity has almost univocally confirmed in a number of population-based epidemiological studies, such as the CARDIA study (Coronary Artery Risk Development in Young Adults, 1985-2001) [Rosell et al., 2004], the HERITAGE (Health, Risk factors, exercise Training and Genetics) Family Study (1992-2004) [Loos et al., 2004], and the large Portuguese Health Interview Survey (1998-1999) [Marques-Vidal et al., 2006]. Similar results were also observed in some case-control studies [Carruth and Skinner, 2001; Dixon et al., 2005; Drapeau et al., 2004; Elwood et al., 2005], except one study showing a somewhat opposite result [Berkey et al., 2005]. Evidently, dairy consumption showed an inverse correlation with body fat and BMI, as well as a positive association with lean body mass in children [Carruth and Skinner, 2001; Dixon et al., 2005], adolescents [Berkey et al., 2005], and adults [Elwood et al., 2005]. However, results in intervention trials were not conclusive. Dairy-rich, energy-restricted diets were effective in reducing BMI and waist circumferences in a short-term (12-24 weeks) but not in a long-term (48-52 weeks) in obese adults [Harvey-Berino et al., 2005; Thompson et al., 2005; Zemel et al., 2004; 2005]. An inverse correlation between calcium intake and adiposity was also found in male adolescents [Goldberg et al., 2009], obese girls [dos Santos et al., 2008], postmenopausal women [Heiss et al., 2008], young adult white males [Brooks et al., 2006], and in young, healthy women [Eagan et al., 2006]. On the contrary, some reports have demonstrated no effect of calcium intake on body weight/fat mass changes in young, healthy women [Gunther et al., 2005] and overweight/obese adults [Yanovski et al., 2009]. Meanwhile, emerging evidence

has indicated that the milk type of calcium may induce greater fat loss than the equivalent amount of calcium supplement [Summerbell et al., 1998; Zemel and Miller, 2004], implying that milk products may have some bioactive components capable of reducing body weight/fat mass by interacting with calcium independently or synergistically.

Regarding the possible anti-adipogenic mechanism of calcium, ingested calcium can directly regulate the intracellular calcium concentration, which is closely related to the regulation of lipid metabolism. Briefly, lower calcium intake stimulates the secretion of both PTH and 1,25-dihydroxy-vitamin D (1,25-$(OH)_2D$), which leads to the elevated calcium level in bloodstream, thereby facilitating calcium influx into adipocytes. The higher level of intracellular calcium in adipocytes will stimulate both transcription and activity of fatty acid synthase, leading to increased *de novo* lipogenesis, reduced lipolysis, and expanded triglyceride storage in adipocytes. In contrast, higher calcium intake attenuates hormonal responses of PTH and 1,25-$(OH)_2D$, thereby leading to the up-regulation of lipolysis and the down-regulation of lipogenesis (Figure 2) [Fujita and Palmieri, 2000; Zemel, 2002]. As an alternative mechanism, dietary calcium can bind to fatty acids in the intestinal tract that hinders overall fat absorption [Parikh and Yanovski, 2003].

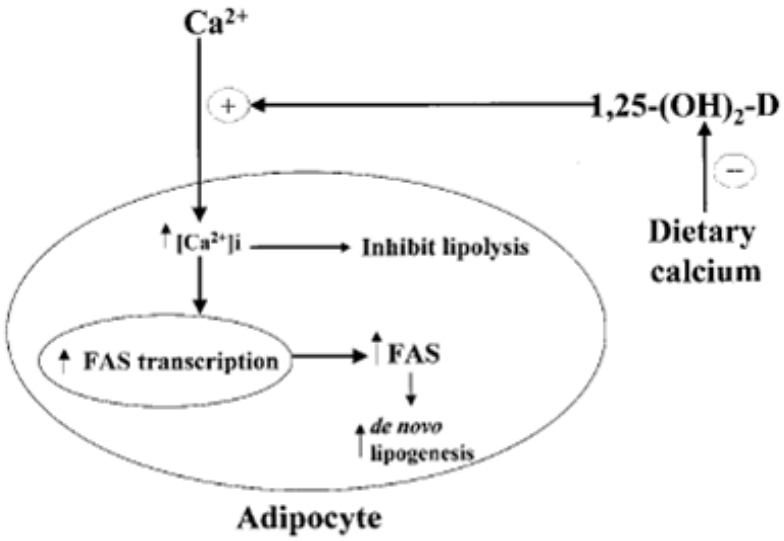


Figure 2. Role of dietary calcium in lipid metabolism [Zemel, 2002].

CHAPTER 3

EXPERIMENTAL DESIGNS AND METHODS

Reagents and Chemicals

Triglycerides of ALA, EPA, and DHA, as well as free fatty acids of 9,11- and 10,12-CLAs were purchased from Nu-check Prep, Inc. (Elysian, MN). Fetal bovine serum and heat-inactivated new-born calf serum were obtained from SAFC Biosciences (Lenexa, KS) and Sigma-Aldrich (St. Louis, MO), respectively. The 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (CalBiochem, San Diego, CA), and insulin (MP Biomedicals, Solon, OH) were added to adipogenic media. The β-glycerophosphate (CalBiochem, San Diego, CA) and L(+)-ascorbic acid (EMD Chemicals Inc., Gibbstown, NJ) were added to osteogenic media. Oil red O and alizarin red S powders were purchased from Alfa Aesar (Ward Hill, MA) and used for cell staining. All other chemicals used were reagent grade or better.

Cell Culture

The ST2 mouse bone marrow-derived stromal cell line was purchased from Riken BioResource Center (Ibaraki, Japan) and maintained in RPMI Medium1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. The MC3T3-L1 mouse adipocyte-like cell line was obtained from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated new-born calf serum. The MC3T3-E1 mouse osteoblast-like cell line was purchased from ATCC (Manassas, VA) and maintained in Minimum Essential Medium-α (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. All culture media were added with 1%

penicillin(100 U/ml)/streptomycin (100 μg/ml) (Gibco, Grand Island, NY) and changed with fresh growth media every 2-3 days. Cells were incubated at 37°C with a humidified atmosphere of 95% air and 5% CO_2. Cells used for experiments were from passages 7-13. When reached 70-80% confluence, cells were subcultured using 0.25% trypsin with 0.02% EDTA (Mediatech, Inc., Herndon, VA). All experiments were repeated in triplicate.

Experimental Procedures

For ~10 days (~3 passages), ST2, MC3T3-L1, and MC3T3-E1 cells were plated in petri-dishes (CellStar®; Greiner Bio-one, Monroe, NC) at a density of 1.5-2.5x10^5 cells/cm^2 and cultured in individual growth media supplemented with 2.5 μg/ml of n-3 PUFA mixture (ALA:EPA:DHA=1:3:6) [Welch et al., 2006] dissolved in dimethylsulfoxide (DMSO; CalBiochem, San Diego, CA) as vehicle (< 1 μl/ml that had no effect on cell growth). It was performed to provide growth media with the n-6:n-3 PUFA ratio of 20:1, reflecting the current n-6:n-3 PUFA ratio found in typical Western diets. Throughout experiment 1-4, cells were pre-treated with n-6:n-3 PUFA=20:1, then transferred to either 96-well or 6-well plates accordingly for further experiments. Overall procedures of experiment 1-4 are represented in Table 4.

Experiment 1.The effect of ALA with n-6:n-3 PUFA ratios (1-5:1) on ST2, MC3T3-L1, and MC3T3-E1 cell metabolism: Briefly, to measure proliferation, all cell lines were seeded in separate 96-well plates (at a density of 1x10^3 cells/well) and grown in respective culture media supplemented with different concentrations of purified ALA to make the ratio of n-6 LA (sourced from cell culture sera) to n-3 ALA as 1-5:1 in growth media (for 8 days). Purified ALA was dissolved in DMSO as vehicle. ALA concentrations used for developing LA:ALA=1-5:1 are as follows: For ST2 and MC3T3-E1 cells, LA:ALA=1:1 (54:54 μg/ml by adding 200 μM ALA),

2:1 (54:27 μg/ml by adding 100 μM ALA), 3:1 (54:18 μg/ml by adding 66.7 μM ALA), 4:1 (54:13.5 μg/ml by adding 50 μM ALA), and 5:1 (54:10.8 μg/ml by adding 40 μM ALA). For MC3T3-L1 cells, however, LA:ALA=1:1 (9.8:9.8 μg/ml by adding 35 μM ALA), 2:1 (9.8:4.9 μg/ml by adding 17.5 μM ALA), 3:1 (9.8:3.26 μg/ml by adding 11.6 μM ALA), 4:1 (9.8:2.45 μg/ml by adding 8.75 μM ALA), and 5:1 (9.8:1.96 μg/ml by adding 7 μM ALA). In this study, different amounts of ALA were added to develop equivalent LA:ALA ratios for all cell lines, because fat-free serum was unavailable and fetal bovine and heat-inactivated new-born calf sera contain different levels of LAs as 54 μg/ml and 9.8 μg/ml, respectively. To facilitate differentiation, cells were seeded in separate 6-well plates (at a density of $1x10^5$ cells/well) and allowed to reach 100% confluence. On day 2 post confluence (day 0), cells were exposed to either adipogenic (ST2, MC3T3-L1) or osteogenic media (ST2, MC3T3-El), both of which were supplemented with various concentrations of ALA (for LA:ALA = 1-5:1) for additional 6 days. For adipogenic differentiation, cells were cultured in adipogenic media (standard growth media containing 0.5 mM IBMX+1 μM dexamethasone+1 μg/ml insulin for day 0-2, 1 μg/ml insulin for day 2-4, and normal growth media for day 4-6). For osteogenic differentiation, cells were cultured in osteogenic media (standard growth media containing 5 mM β-glycerophosphate+100 μg/ml L(+)-ascorbic acid for day 0-6). On day 7, cell culture supernatants were collected and stored at -80°C until being used for protein assays. At the same time, cells were prepared for appropriate staining. The general differentiation procedure was identical throughout experiment 1-4.

Experiment 2. The effect of CLA (9,11- and 10,12-CLAs) on ST2, MC3T3-L1, and MC3T3-E1 cell metabolism: To measure proliferation, cells seeded in 96-well plates (at a density of $1x10^3$ cells/well) were maintained in individual culture media supplemented with various combinations

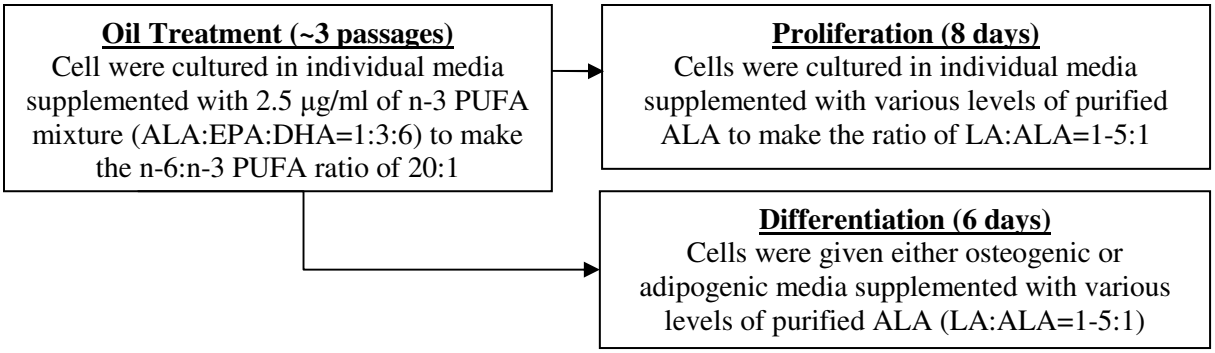
of 20 µM purified 9,11- and 10,12-CLA isomers dissolved in DMSO (for 8 days). The 9,11- (80-90%) and 10,12-CLA (5-10%) mixtures used are as follows: 100:0%, 0:100%, 80:10%, 90:10%, or 90:5%, reflecting possible 9,11- and 10,12-CLA blends found in milk. During differentiation, cells maintained in either adipogenic or osteogenic condition, accordingly, were also treated with the same CLA isomer mixtures for 6 days.

Experiment 3. The effect of different levels of calcium on ST2, MC3T3-L1, and MC3T3-E1 cell metabolism: To measure proliferation, cells seeded in 96-well plates (at a density of $1x10^3$ cells/well) were maintained in individual culture media (for 2 days) which were, then, switched to calcium-free Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY) supplemented with appropriate serum and various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mM) of calcium phosphate (monobasic; Mallinckrodt Baker, Inc., Phillipsburg, NJ), which reflect possible calcium levels found in milk (for 6 days).

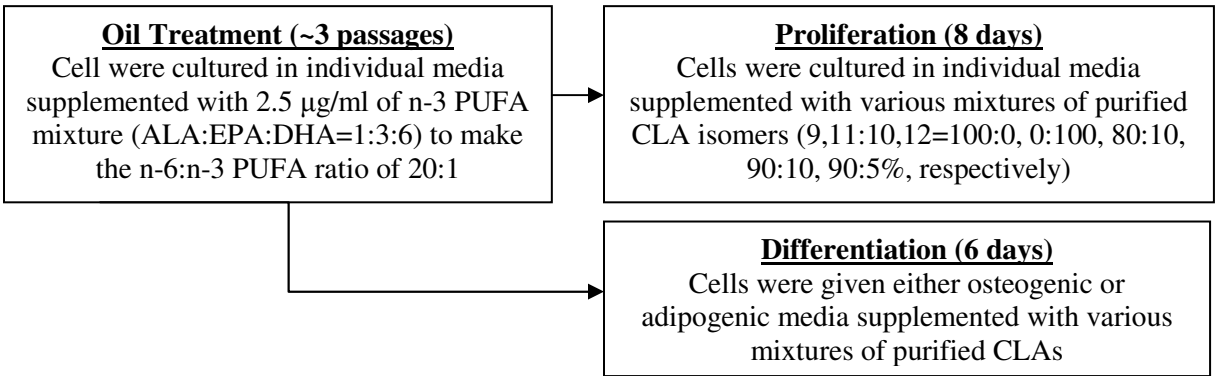
Experiment 4. The possible interaction among ALA, CLA, and calcium on ST2, MC3T3-L1, and MC3T3-E1 cell metabolism: To measure proliferation, cells seeded in 96-well plates (at a density of $1x10^3$ cells/well) were maintained in individual culture media (for 2 days) which were, then, switched to calcium-free Dulbecco's Modified Eagle's Medium supplemented with appropriate serum and various combinations of ALA (LA:ALA = 4:1), CLA (9,11:10,12 = 80:10%, 90:10%, or 90:5%), and calcium (0.5 or 2.0 mM) for 6 days. These treatments were chosen from the experiment 1-3 results showing the best in promoting osteoblastogenesis and inhibiting adipogenesis. During differentiation, cells exposed to adipogenic or osteogenic conditions, accordingly, were treated with similar ALA, CLA, and calcium combinations for 6 days.

Table 4. Experimental Procedures

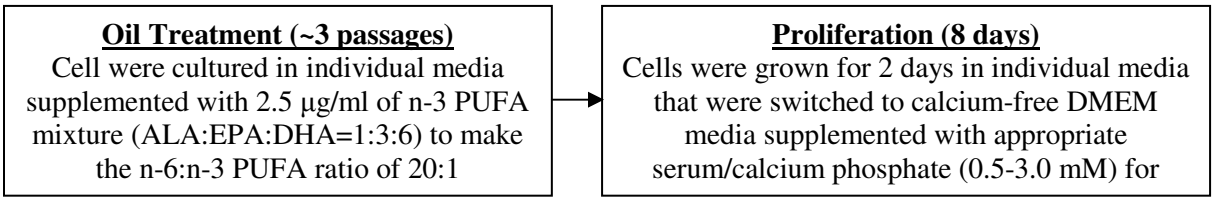
Experiment 1.



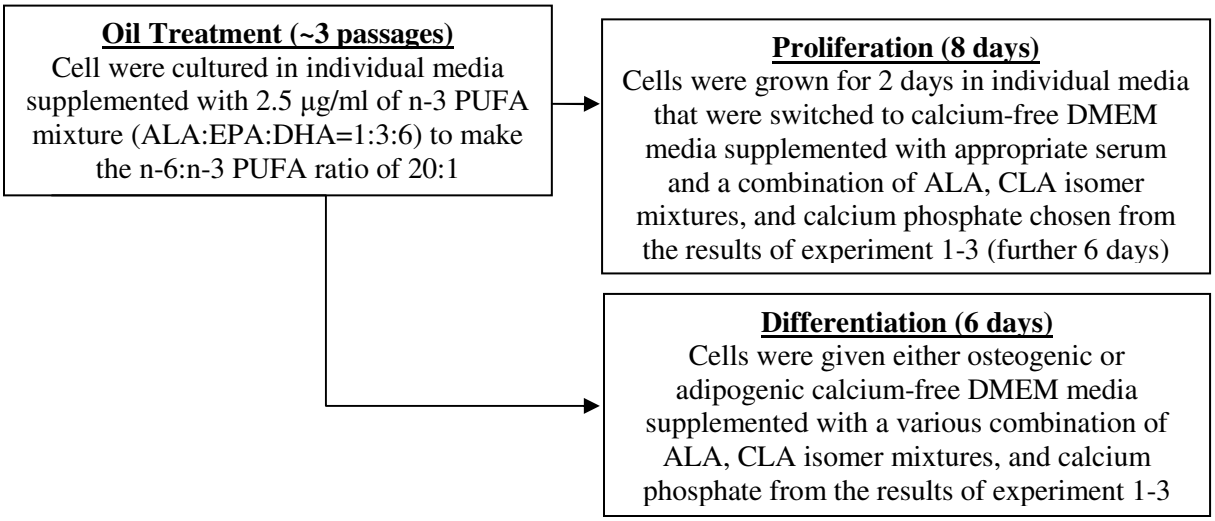
Experiment 2.



Experiment 3.



Experiment 4.



Cell Proliferation Assay

Assay based on Resazurin sodium salt (Sigma-Aldrich Corp., St. Louis, MO) was used for measuring cell proliferation. After 8 days of treatment of each test compound, cells cultured in a 96-well plate was incubated for 2 hr in 100 µl of respective fresh media (no phenol red) containing 10 µl of Resazurin sodium salt solution (1 mg Resazurin sodium salt in 1 ml PBS). Fluorescence was detected using a microplate reader (SpectraMax M5; Molecular Devices, Inc., Sunnyvale, CA) at a wavelength of 590 nm.

Oil Red O Staining

Lipid droplet accumulation was measured in ST2 and MC3T3-L1 cells grown under adipogenic condition. First, oil red O stock solution was prepared by mixing 700 mg of oil red O powder in 200 ml of 100% isopropanol. It was stirred overnight, paper-filtered (Whatman, grade No.1), and stored at 4^{o}C. On staining, oil red O working solution was prepared by adding 6 parts of oil red O stock to 4 parts of ddH_2O. Cells in 6-well plates were rinsed with PBS and fixed with 10% formaldehyde in PBS for 1 hr. After fixation, cells were rinsed with 60% isopropanol, air-dried, and stained with oil red O working solution (1 ml/well) for ~1 hr. Cells were then washed with ddH_2O and eluted with 100% isopropanol to quantify relative triglyceride content in cells.

Alizarin Red S Staining

Calcium deposition was measured in ST2 and MC3T3-E1 cells grown under osteogenic condition. Alizarin red S solution was freshly prepared by adding 100 µl of 28% *(w/v)* ammonia solution to 1% *(w/v)* alizarin red S solution (1 g of alizarin red S in 100 ml ddH_2O; pH 6.36-6.40).

Cells in 6-well plates were rinsed with PBS and fixed with 10% formaldehyde in PBS for 15 min. After fixation, cells were rinsed five times with ddH_2O, air-dried, and stained with alizarin red S solution (1 ml/well) for 20 min. Cells were then washed with ddH_2O and eluted with 10% (*v/v*) acetic acid to quantify relative calcium deposits in cells.

Quantification of Lipid Droplet Accumulation

Oil red O stain was eluted by incubating dyed cells in 100% isopropanol for 10 min with gentle shaking. After transferring oil red O dye and isopropanol mixtures to 96-well plates, absorbance was measured using a microplate reader (Powerwave; Bio-Tek instruments, Inc., Winooski, VT) at a wavelength of 500 nm.

Quantification of Calcium Deposits

Alizarin red S stained cells were washed with ddH_2O (4 x 5 min with gentle shaking) and incubated in 10% (*v/v*) acetic acid (800 µl/well for 30 min with gentle shaking). The loosely attached monolayers were scrapped from each well and the acetic acid and monolayer mixtures were transferred to 1.5 ml microcentrifuge tubes, vortexed vigorously for 30 sec, and exposed to heating (85°C for 10 min)/cooling (on ice for 5 min) condition. After centrifugation (at 20,000 x g for 15 min), each supernatant was pH-adjusted to 4.1-4.5 by mixing 400 µl of supernatant with ~150 µl of 10% *(w/v)* ammonium hydroxide, transferred to a 96-well plate, and read the absorbance using a microplate reader at a wavelength of 405 nm.

Quantification of eicosanoids and growth factors in conditioned media

Eicosanoids such as PGE_2 and LTB_4, and growth factors including IGF-1, IGFBP-3, TGF-β, and BMP-2, were measured in duplicate by using commercially available enzyme immunoassay (EIA) kits. The PGE_2, LTB_4, and TGF-β were measured using respective EIA kits (mouse) purchased from Assay Designs (Ann Arbor, MI). The IGF-1, IGFBP-3, and BMP-2 were measured by respective EIA kits (mouse) obtained from R&D Systems, Inc. (Minneapolis, MN). All EIAs were performed using cell culture supernatants following the manufacturer's instructions.

Statistical Analysis

Results are expressed as means ± S.E.M. including at least three replicates in each group. Differences in treatment groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Data were analyzed using SPSS 19.0 (Somers, NY) or GraphPad Prism Software 4 (La Jolla, CA), where applicable. The $p<0.05$ was considered statistically significant.

CHAPTER 4

RESULTS

Specific Aim 1. To investigate the effect of ALA with n-6:n-3 PUFA ratios=1-5:1 on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 1*: It is hypothesized that n-6:n-3 PUFA ratios with a range of 1-5:1 will facilitate the commitment of MSC toward osteoblast lineage, decrease the growth (hyperplasia) of adipocytes, and increase the growth of osteoblasts, favoring bone development and inhibiting fat cell formation.*

Cell Proliferation

The effect of ALA with relatively low LA:ALA ratios (1-5:1) on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell proliferation is shown in Figure 3. For ST2 cells, only the LA:ALA ratio=1:1 significantly inhibited proliferation (~25.8%) that was, however, returned to the control (DMSO) level as the ratios increased (Figure 3.A). In contrast, proliferation of both MC3T3-L1 and MC3T3-El cells was significantly inhibited by all ranges of LA:ALA ratios. Although MC3T3-L1 cell proliferation was consistently suppressed by all different LA:ALA ratios (Figure 3.B), it was of interest that MC3T3-E1 cell proliferation had a tendency to increase gradually as the ratios increased (Figure 3.C).

Quantification of Cell Differentiation

To determine the effect of ALA with relatively low LA:ALA ratios (1-5:1) on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell differentiation, the

amount of oil red O or alizarin red S staining taken up by differentiating cells was measured. As shown in Figure 4.A-1 and 4.A-2, compared with vehicle, ALA at the LA:ALA=1:1 (containing too high n-3 PUFA) significantly facilitated both adipogenic and osteoblastic differentiation of ST2 cells, representing much higher lipid droplet accumulation and calcium deposition. Similar to the proliferative phase, MC3T3-L1 adipocyte-like cell differentiation was consistently lowered by the treatment of all ranges of LA:ALA ratios (Figure 4.B), indicating that relatively low LA:ALA (or n-6:n-3 PUFA) ratios exert an inhibitory effect on both proliferation and differentiation of adipocytes ($P<0.05$). For MC3T3-E1 osteoblast-like cells, unlike the proliferative stage, the LA:ALA ratio of 3-5:1 resulted in significantly higher calcium deposition (up to 1.33-fold, $P<0.05$) compared with vehicle, showing increased osteoblastic differentiation (Figure 4.C). Overall, in this experiment, the LA:ALA ratio=4:1 appeared to be the best in that, at this ratio, osteoblastogenesis was maximized whereas adipogenesis was minimized.

Quantification of Regulatory Factors Related to MC3T3-L1 and MC3T3-E1 Cell Differentiation

To determine regulatory factors associated with the effect of ALA with relatively low LA:ALA ratios (1-5:1) on MC3T3-L1 adipocyte-like cell and MC3T3-E1osteoblast-like cell differentiation, the secretion of eicosanoids (PGE_2, LTB_4) and local growth factors (IGF-1, IGFBP-3, TGF-β, BMP-2) was measured from conditioned media. First, in MC3T3-L1 adipocytes, PGE_2 production was prone to increase in all ranges of ALA ratios but the result was not significant (Figure 5.1.A). Production of LTB_4 was not significantly altered by all LA:ALA ratios (Figure 5.1.B). IGF-1 is a potent anabolic agent that largely binds to one of its receptors, IGFBP-3, in circulation. In this study, secretion of both IGF-1 and IGFBP-3 were altered with a

similar pattern, in which at the LA:ALA=4:1, production of IGF-1 and IGFBP-3 were lowest (Figure 5.1.C, 5.1.D). It implies that n-3 ALA, especially at the level of LA:ALA=4:1, may inhibit adipocytes' differentiation via IGF-1/IGFBP-3 signaling pathway. TGF-β is known to be a potent inhibitor of adipocyte differentiation and in this study, even though the secretion of TGF-β was not significantly influenced by different LA:ALA ratios, it was changed in a similar pattern to the secretion of IGF-1 and IGFBP-3, showing a nadir at LA:ALA=3-4:1 (Figure 5.1.E). This data, hence, implied the possible involvement of TGF-β, as well, in suppressing adipocyte differentiation by ALA (at LA:ALA=3-4:1) (Figure 5.1.E). The role of BMP-2 in adipocyte differentiation is not well understood. This study showed that different ratios of LA:ALA did not affect the production of BMP-2 from differentiating adipocytes (Figure 5.1.F). Meanwhile, during MC3T3-E1 osteoblast-like cell differentiation, addition of ALA tended to increase PGE_2 production, which was not statistically significant except at the ratio of LA:ALA=5:1 (Figure 5.2.A). Compared with control, relative production of IGF-1 was significantly higher at the LA:ALA=2:1 (Figure 5.2.C). Other local mediators, such as LTB_4, IGFBP-3, TGF-β, and BMP-2, were not influenced by different LA:ALA ratios (Figure 5.2.B, 5.2.D-F).

Overall, ALA with the LA:ALA=1-5:1 decreased MC3T3-L1 adipocyte-like cell proliferation/differentiation and increased MC3T3-E1 osteoblast-like cell differentiation, showing maximal osteoblastogenesis and minimal adipogenesis at the LA:ALA=4:1. At this ratio, ALA appears to attenuate adipocyte differentiation via IGF-1/IGFBP-3 signaling pathway.

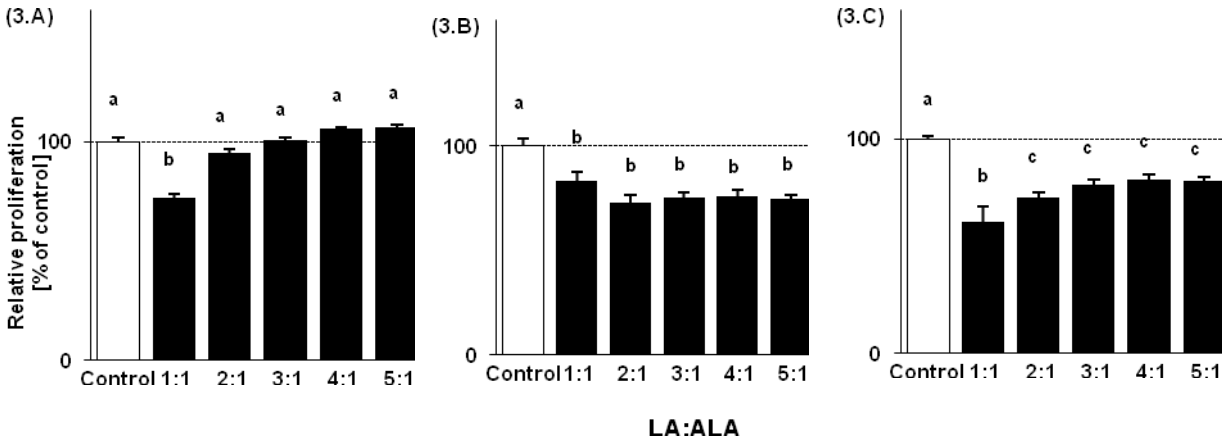


Figure 3. Effects of ALA with low LA:ALA ratios (1-5:1) on proliferation of ST2 (3.A), MC3T3-L1 (3.B), and MC3T3-E1 cells (3.C). Cells were cultured for 8 days in the presence of vehicle (1 µl/ml DMSO; control) or various concentrations of ALA (40-200 µM for ST2 and MC3T3-E1 cells; 7-35 µM for MC3T3-L1 cells) to develop LA:ALA ratios of 1-5:1. Prior to ALA treatment, cells were preincubated for ~10 days (3 passages) with the n-3 PUFA mixture (ALA:EPA:DHA=1:3:6) developed for exposing cells to LA:ALA=20:1. Cell proliferation was measured by Resazurin sodium salt-based assay and is presented as % of change from the control where no ALA was added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

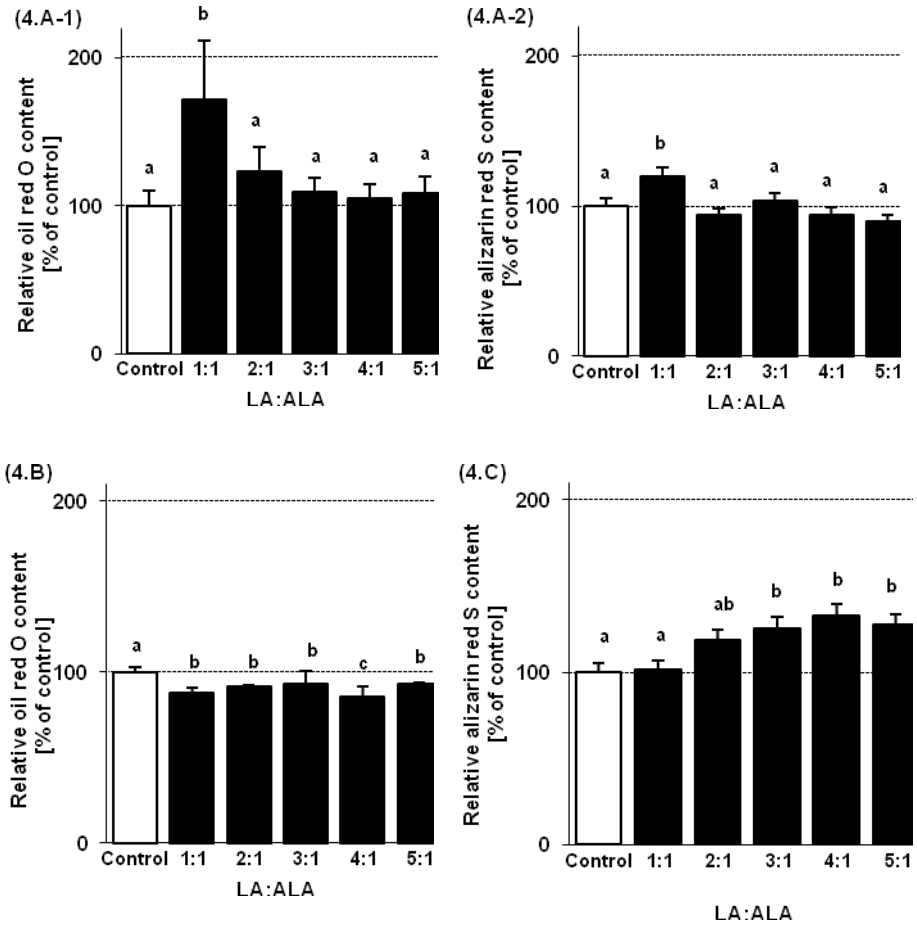
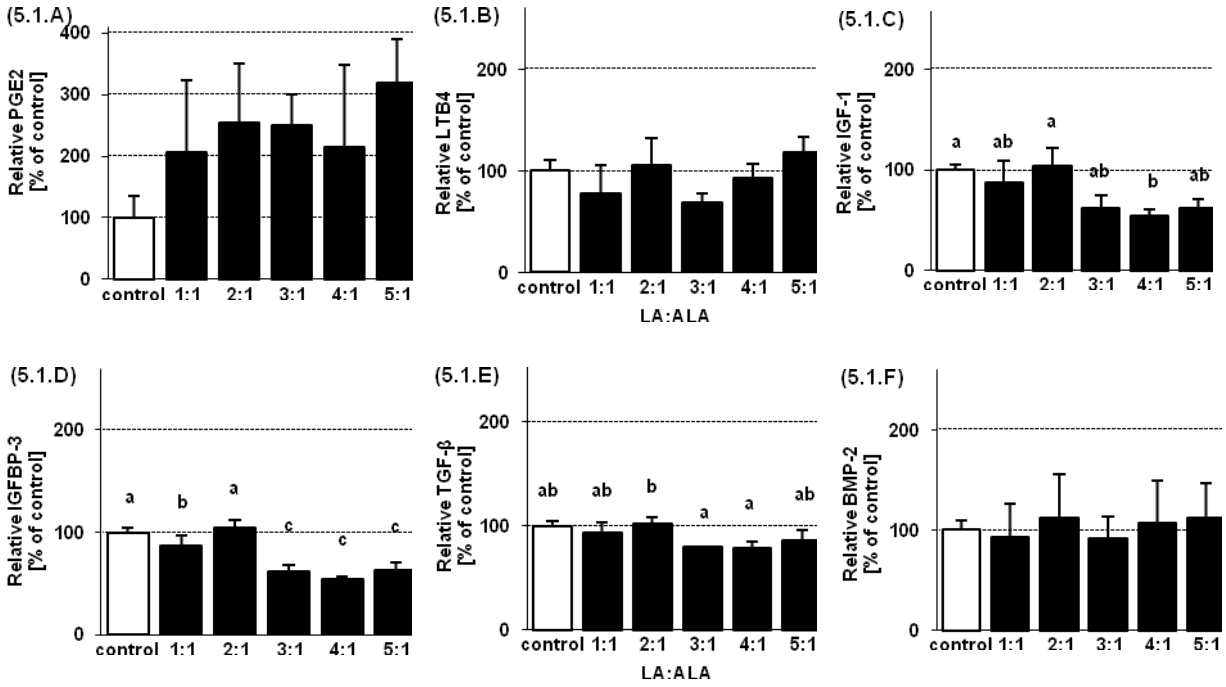


Figure 4. Effects of ALA with low LA:ALA ratios (1-5:1) on differentiation of ST2 (4.A-1, 4.A-2), MC3T3-L1 (4.B), and MC3T3-E1 cells (4.C). Following the pretreatment of the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, cell differentiation was induced for 6 days in the presence of vehicle (1 µl/ml DMSO; control) or various concentrations of ALA (40-200 µM for ST2 and MC3T3-E1 cells; 7-35 µM for MC3T3-L1 cells) developed for exposing cells to LA:ALA=1-5:1. Quantification of triglyceride accumulation was performed in oil red O stained ST2 and MC3T3-L1 cells that underwent adipogenesis. Quantification of calcium deposition was performed in alizarin red S stained ST2 and MC3T3-E1 cells that underwent osteogenesis. Results are expressed as % of change from the control where no ALA was added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.


(5.1.A)
Relative PGE2 [% of control]
400
300
200
100
0
control 1:1 2:1 3:1 4:1 5:1
(5.1.B)
Relative LTB4 [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1
LA:ALA
(5.1.C)
Relative IGF-1 [% of control]
200
100
0
a ab a ab b ab
control 1:1 2:1 3:1 4:1 5:1
(5.1.D)
Relative IGFBP-3 [% of control]
200
100
0
a b a c c c
control 1:1 2:1 3:1 4:1 5:1
(5.1.E)
Relative TGF-β [% of control]
200
100
0
ab ab b a a ab
control 1:1 2:1 3:1 4:1 5:1
LA:ALA
(5.1.F)
Relative BMP-2 [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1

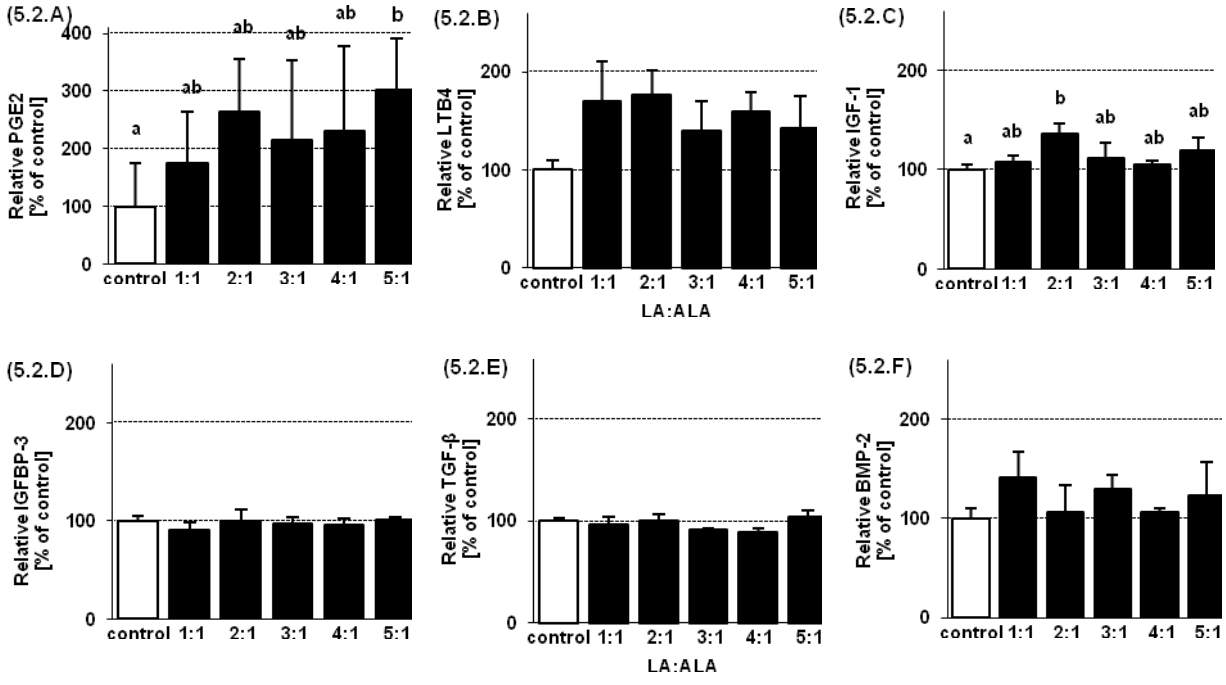

(5.2.A)
Relative PGE2 [% of control]
400
300
200
100
0
a ab ab ab ab b
control 1:1 2:1 3:1 4:1 5:1
(5.2.B)
Relative LTB4 [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1
LA:ALA
(5.2.C)
Relative IGF-1 [% of control]
200
100
0
a ab b ab ab ab
control 1:1 2:1 3:1 4:1 5:1
(5.2.D)
Relative IGFBP-3 [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1
(5.2.E)
Relative TGF-β [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1
LA:ALA
(5.2.F)
Relative BMP-2 [% of control]
200
100
0
control 1:1 2:1 3:1 4:1 5:1

Figure 5. Effects of ALA with low LA:ALA ratios (1-5:1) on secretion of PGE_2 (5.1.A; 5.2.A), LTB_4 (5.1.B; 5.2.B), IGF-1 (5.1.C; 5.2.C), IGFBP-3 (5.1.D; 5.2.D), TGF-β (5.1.E; 5.2.E), and BMP-2 (5.1.F; 5.2.F) by MC3T3-L1 and MC3T3-E1 cells (1 for former; 2 for latter). Analysis of these protein levels from MC3T3-L1 or MC3T3-E1 cells that were precultured in the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, followed by 6 days of exposure to adipogenic or osteogenic induction media in the presence of vehicle (1 µl/ml DMSO; control) or various concentrations of ALA (40-200 µM for MC3T3-E1 cells; 7-35 µM for MC3T3-L1 cells) developed for exposing cells to LA:ALA=1-5:1. Secretion of these proteins was measured by ELISA from the conditioned media and is presented as % of change from the control where no ALA was added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

Specific Aim 2. To examine the effect of CLA (9,11- and 10,12-CLAs) on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 2: *It is hypothesized that individual and/or a combination of CLA isomers (80-90% 9,11- and 5-10% 10,12-CLAs; the ratio of 9,11- and 10,12-CLAs=100:0, 0:100, 80:10, 90:10, and 90:5) will trigger the trans-differentiation of MSC toward osteoblasts, attenuate the growth (proliferation/differentiation) of adipocytes, and facilitate the growth of osteoblasts by modulating lipid signaling pathways which may involve local regulatory factors (some eicosanoids and growth factors).*

Cell Proliferation

It was determined how individual or combinations of 20 μM 9,11- and 10,12-CLA isomers (80:10%, 90:10%, or 90:10%) affect ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell proliferation and the results are Figure 6. Overall, proliferation of MC3T3-L1 and MC3T3-E1 cells was significantly inhibited by most combinations of CLA isomers. Especially, the 90:5% CLA mixture appeared to have the least benefit by greatly suppressing ST2 and MC3T3-E1 but relatively (insignificant) increasing MC3T3-L1 cell proliferation.

Quantification of Cell Differentiation

The effect of individual or combinations of 20 μM 9,11- and 10,12-CLAs (80:10%, 90:10%, and 90:5%) on ST2, MC3T3-L1, and MC3T3-E1 cell differentiation was determined. As shown in Figure 7.A-1 and 7.A-2, various combinations of 9,11- and 10,12-CLAs were, in general, likely to boost adipogenic but not osteoblastic differentiation of ST2 cells, despite a

slight difference among individual CLA isomer mixtures. Individual 9,11- and 10,12-CLA isomers did not exhibit any obvious effects on MC3T3-L1 adipocyte-like or MC3T3-E1 osteoblast-like cell differentiation. When combined, however, they resulted in a further decrease in adipocyte differentiation (~21.0% from 90:10%; ~26.5% from 90:5%) and a further increase in osteoblast differentiation (~38.1% from 80:10%) (Figure 7.B, 7.C). It is assumed from the result that 9,11- and 10,12-CLA isomers interact together, which may produce a different effect on adiposity and bone formation.

Quantification of Regulatory Factors Related to MC3T3-L1 and MC3T3-E1 Cell Differentiation

To investigate the regulatory mechanism of 9,11- and 10,12-CLAs (individual or combinations of 80:10%, 90:10%, or 90:5%) in adipocyte and osteoblast differentiation, various local factors were measured from cell culture media. First, in MC3T3-L1 adipocyte-like cells, PGE_2 production was dramatically, but insignificantly increased (~ up to 8.6-fold) by all CLA treatments except with 9,11-CLA (100%) (Figure 8.1.A). In contrast, compared with control, secretion of IGF-1 and IGFBP-3 was relatively lowered by most of CLA isomer mixtures (80:10%, 90:10%, and 90:5%) but the result was not statistically significant (Figure 8.1.C-D). Other regulatory factors such as LTB_4, TGF-β, and BMP-2 did not show any noticeable changes by these CLA supplementations (Figure 8.1.B, E, F). In MC3T3-E1 osteoblast-like cells, all various CLA treatments resulted in a greater increase in PGE_2 secretion (~ up to 5.9-fold) but the result was insignificant except with 100% 10,12-CLA (Figure 8.2.A). In this study, no obvious changes were observed in the production of LTB_4, IGF-1, IGFBP-3, TGF-β, and BMP-2 by individual and/or mixtures of 9,11- and 10,12-CLAs (Figure 8.2.B-F).

Overall findings indicate that individual and/or various combinations of 9,11- (80-90%) and 10,12-CLA (5-10%) isomers had a tendency to inhibit both MC3T3-L1 adipocyte-like and MC3T3-E1 osteoblast-like cell proliferation. However, during differentiation, 9,11- and 10,12-CLA mixtures, unlike individual CLA isomers having a negligible effect on both cell growth, exerted a promising outcome by further decreasing adipocytic and increasing osteoblastic differentiation. In both cell lines, most CLA isomer blends showed an increase in PGE_2 production but the result was not statistically significant.

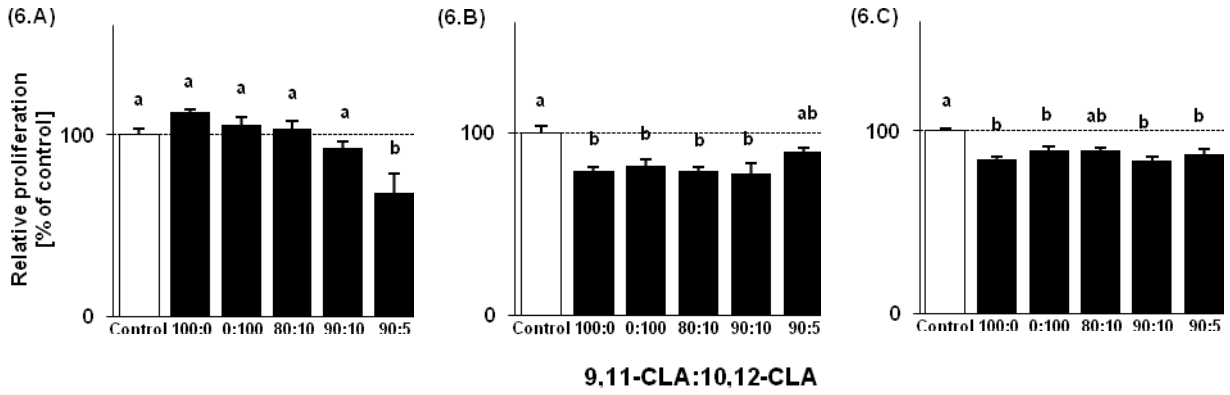


Figure 6. Effects of individual or combinations of 9,11- and 10,12-CLA isomers on proliferation of ST2 (6.A), MC3T3-L1 (6.B), and MC3T3-E1 cells (6.C). Cells were cultured for 8 days in the presence of vehicle (1 µl/ml DMSO; control) or various combinations of 20 µM 9,11-CLA and 10,12-CLA in the ratio of 100:0%, 0:100%, 80:10%, 90:10%, and 90:5%, respectively, which blends are predominantly found in milk products. Prior to CLA treatment, cells were preincubated for ~10 days (3 passages) with the n-3 PUFA mixture (ALA:EPA:DHA=1:3:6) developed for exposing cells to LA:ALA=20:1. Cell proliferation was measured by Resazurin sodium salt-based assay and is presented as % of change from the control where no CLA was added. Results are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

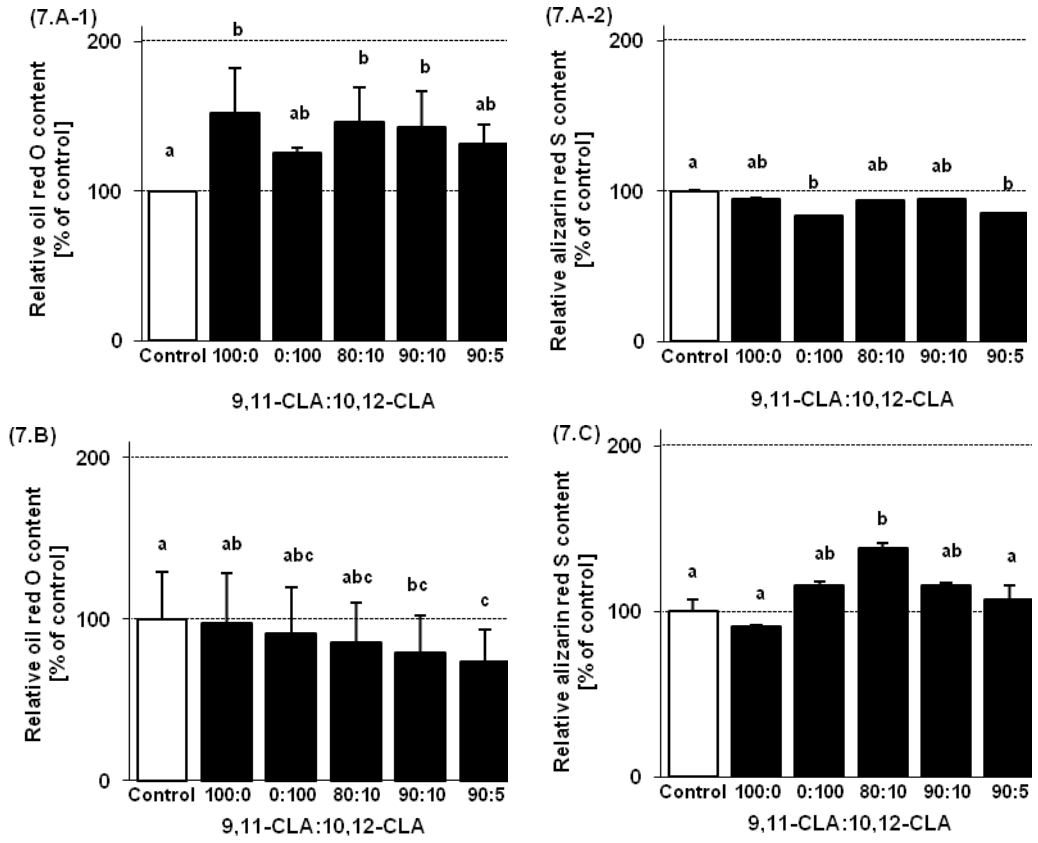
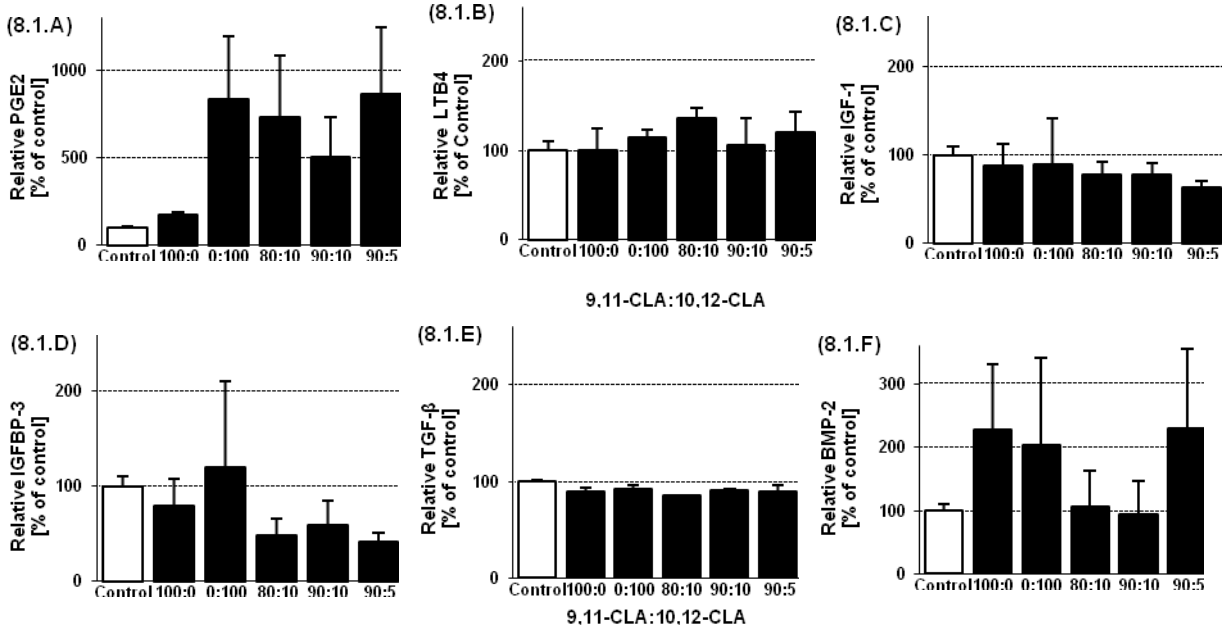


Figure 7. Effects of individual or combinations of 9,11- and 10,12-CLA isomers on differentiation of ST2 (7.A-1, 7.A-2), MC3T3-L1 (7.B), and MC3T3-E1 cells (7.C). Following the pretreatment of the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, cell differentiation was induced for 6 days in the presence of vehicle (1 μl/ml DMSO; control) or various combinations of 20 μM 9,11-CLA and 10,12-CLA in the ratio of 100:0%, 0:100%, 80:10%, 90:10%, and 90:5%, respectively, which blends are predominantly found in milk products. Quantification of triglyceride accumulation was performed in oil red O stained ST2 and MC3T3-L1 cells that underwent adipogenesis. Quantification of calcium deposition was performed in alizarin red S stained ST2 and MC3T3-E1 cells that underwent osteogenesis. Results are expressed as % of change from the control where no CLA was added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.


(8.1.A)
Relative PGE2 [% of control]
1000
500
0
Control 100:0 0:100 80:10 90:10 90:5
(8.1.B)
Relative LTB4 [% of Control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.1.C)
Relative IGF-1 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
9,11-CLA:10,12-CLA
(8.1.D)
Relative IGFBP-3 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.1.E)
Relative TGF-β [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.1.F)
Relative BMP-2 [% of control]
300
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
9,11-CLA:10,12-CLA

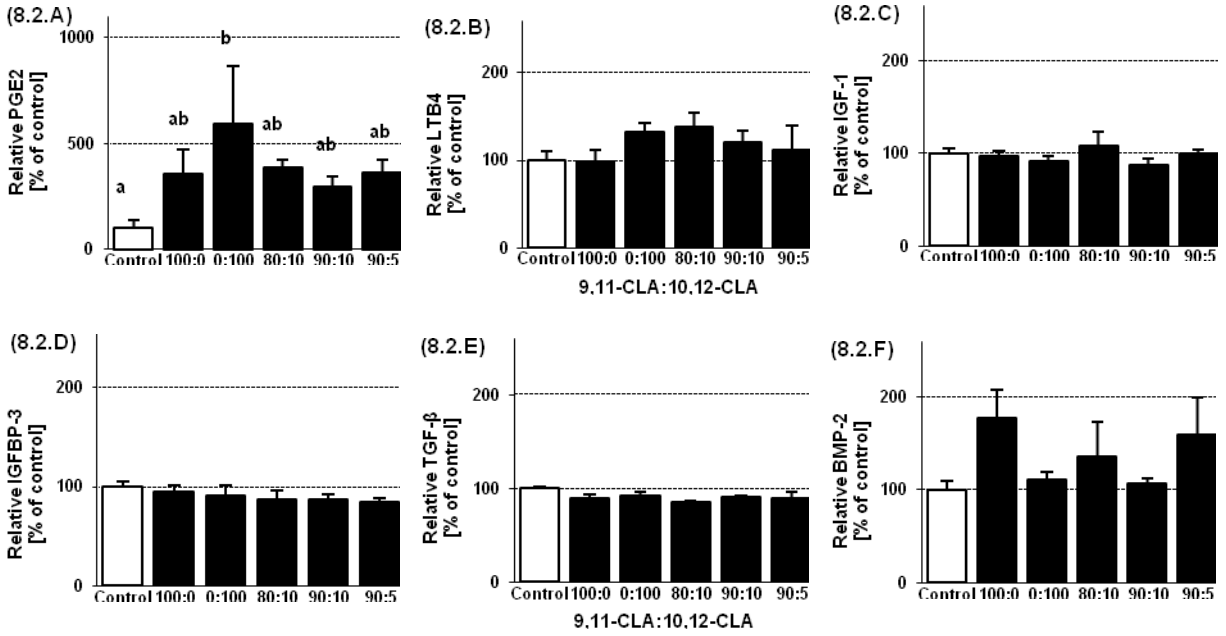

(8.2.A)
Relative PGE2 [% of control]
1000
500
0
a
ab
b
ab
ab
ab
Control 100:0 0:100 80:10 90:10 90:5
(8.2.B)
Relative LTB4 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.2.C)
Relative IGF-1 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
9,11-CLA:10,12-CLA
(8.2.D)
Relative IGFBP-3 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.2.E)
Relative TGF-β [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
(8.2.F)
Relative BMP-2 [% of control]
200
100
0
Control 100:0 0:100 80:10 90:10 90:5
9,11-CLA:10,12-CLA

Figure 8. Effects of individual or combinations of 9,11- and 10,12-CLA isomers on secretion of PGE_2 (8.1.A; 8.2.A), LTB_4 (8.1.B; 8.2.B), IGF-1 (8.1.C; 8.2.C), IGFBP-3 (8.1.D; 8.2.D), TGF-β (8.1.E; 8.2.E), and BMP-2 (8.1.F; 8.2.F) by MC3T3-L1 and MC3T3-E1 cells (1 for former; 2 for latter). Analysis of these protein levels from MC3T3-L1 or MC3T3-E1 cells that were precultured in the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, followed by 6 days of exposure to adipogenic or osteogenic induction media in the presence of vehicle (1 μl/ml DMSO; control) or various combinations of 20 μM 9,11-CLA and 10,12-CLA in the proportion of 100:0%, 0:100%, 80:10%, 90:10%, and 90:5%, respectively, which blends are predominantly found in milk products. Secretion of these proteins was measured by ELISA from the conditioned media and is presented as % of change from the control where no CLA was added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

Specific Aim 3. To investigate the effect of different levels of calcium on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 3: *It is hypothesized that higher levels of calcium (calcium phosphate found in milk) favor the commitment of MSCs' lineage to osteoblasts, increase osteoblast formation (osteoblastogenesis), and decrease adipocyte formation (adipogenesis).*

Cell Proliferation

A range of different calcium levels (0.5-3.0 mM) was tested in ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cells to determine whether extracellular calcium concentration influences the growth of these cells. As shown in Figure 9, at extreme calcium conditions such as at very low (0.5 mM) or very high (3.0 mM) calcium, ST2 and MC3T3-E1 cell proliferation was prone to be slightly inhibited. For MC3T3-El cells, especially, the optimal calcium level was shown as at 1.5-2.5 mM. The aspect of MC3T3-L1 cell proliferation was somewhat different from these two cell lines in that within the range of 0.5-3.0 mM calcium, the higher the calcium level, the lower the growth of MC3T3-L1 adipose-like cells.

Overall, at 1.5-2.5 mM calcium level, ST2 stromal and MC3T3-E1 osteoblast-like cell proliferation was increased, whereas MC3T3-L1 adipocyte-like cell proliferation was decreased. A higher level of calcium was fatal to the growth of MC3T3-L1 adipocyte-like cells.

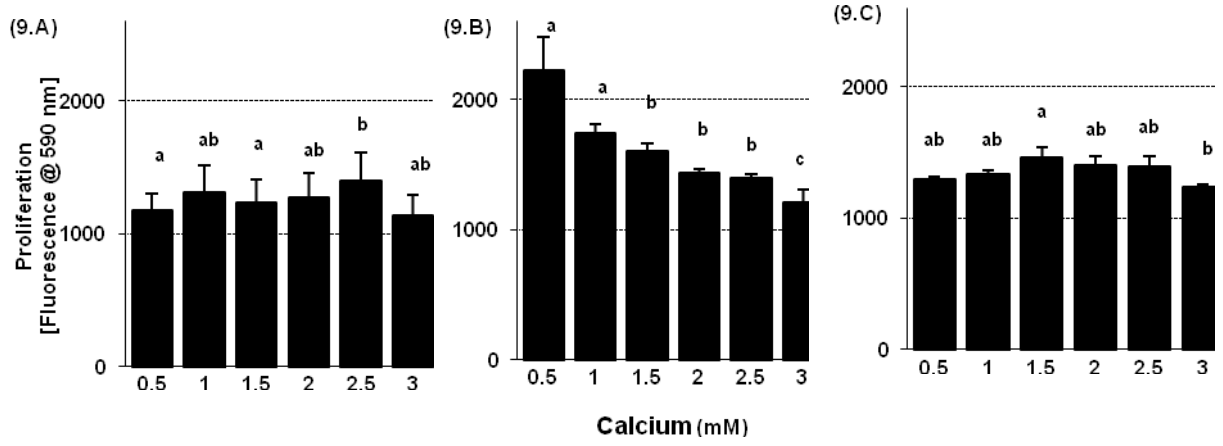


Figure 9. Effects of various calcium concentrations on proliferation of ST2 (9.A), MC3T3-L1 (9.B), and MC3T3-E1 cells (9.C). Cells were cultured for 6 days in the presence of different levels of calcium (0.5-3.0 mM), following the preincubation for ~10 days (3 passages) with the n-3 PUFA mixture (ALA:EPA:DHA=1:3:6) developed for exposing cells to LA:ALA=20:1. Cell proliferation was measured by Resazurin sodium salt-based assay and is presented as fluorescence detected at the wavelength of 590 nm. Results are shown as means ± S.E.M., $P <$ 0.05. The experiment was repeated in triplicate, each experiment yielding comparable data.

Specific Aim 4. To examine the possible interaction among ALA, CLA, and calcium on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell metabolism.

Hypothesis 4: *It is hypothesized that the incorporation of major milk components, such as ALA with n-6:n-3 PUFA ratios=1-5:1, CLA, and calcium, may further promote the potential of each component's capability to increase osteoblastogenesis and inhibit adipogenesis.*

Cell Proliferation

It was examined whether there is a possible interaction among ALA, CLA, and calcium in modulating ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell proliferation. For this experiment, ALA (LA:ALA=4:1), CLA isomer mixtures (80:10%, 90:10%, or 90:5%), and calcium (0.5 mM vs. 2.0 mM) were chosen because at these conditions, overall growth (proliferation and differentiation; see the results) of MC3T3-L1 adipocyte-like cells was minimized and, at the same time, that of MC3T3-E1 osteoblast-like cells was maximized. As shown in Figure 10.A, at both low (0.5 mM) and high (2.0 mM) calcium levels, overall ST2 cell proliferation was constantly suppressed by all treatments (except with 4:1 ALA at low calcium) compared with vehicle. Specifically, at low calcium (0.5 mM), individual effects of ALA and CLA were offset when they were added together. However, co-treatment of ALA and CLA did not influence MC3T3-L1 cell proliferation at both calcium levels (Figure 10.B). By the way, with all ALA and CLA treatments, overall proliferation of MC3T3-E1 cells was far decreased when they were exposed to high calcium condition. At low calcium level, MC3T3-E1 cell proliferation was dramatically increased (~ 2.9-fold) by ALA at the LA:ALA=4:1, which effect was, however, markedly offset by the addition of CLA isomers (Figure 10.C). From the result,

ALA at the LA:ALA=4:1 may be helpful to sustain osteoblast-like cell growth when calcium level is low in our body.

Quantification of Cell Differentiation

Possible interactive effects of ALA (at LA:ALA=4:1), CLA isomer blends (9,11:10:12=80:10% or 90:10%), and calcium (2.0 mM) on ST2 stromal, MC3T3-L1 adipocyte-like, and MC3T3-E1 osteoblast-like cell differentiation was measured. To perform this study, all cell lines were grown in media containing 2.0 mM calcium supplemented with ALA (LA:ALA = 4:1), CLA (80:10% or 90:10%), or ALA+CLA combinations. As a result, while osteoblastic differentiation of ST2 cells was significantly attenuated by all combined treatments, adipogenic differentiation of ST2 cells was not influenced by any of these co-treatments (Figure 11.A-1, 11.A-2). Meanwhile, co-treatment of ALA (4:1) + CLA (90:10%) + calcium (2.0 mM) significantly inhibited lipid droplet accumulation (~10.3%) in differentiating adipocytes and increased calcium deposition (~19.7%) in differentiating osteoblasts (Figure 11.B, 11.C), implying that this dietary combination may be beneficial to promote bone health by facilitating bone formation and, at the same time, reducing adiposity.

Quantification of Regulatory Factors Related to MC3T3-L1 and MC3T3-E1 Cell Differentiation

The combined effect of LA:ALA ratios (4:1), calcium (2.0 mM), and CLA (9,11:10:12=80:10% or 90:10%) on regulatory factors mediating adipocyte and osteoblast differentiation was also determined. During adipocytes' differentiation, CLA (80:10% and 90:10%) greatly increased the production of PGE_2 when co-treated with 2.0 mM calcium, which

was modestly, but insignificantly negated by addition of 4:1 ALA (Figure 12.1.A). However, this effect was not seen in differentiating osteoblast-like cells (Figure 12.2.A). Likewise, increased production of LTB_4 was also observed in differentiating MC3T3-L1 adipocyte-like cells when co-supplemented with calcium (2.0 mM) + 90:10% CLA, as well as ALA (4:1) + CLA (90:10%) + calcium (2.0 mM) (Figure 12.1.B), which result was not shown in MC3T3-E1 osteoblast-like cells. Overall secretion of IGF-1 in MC3T3-E1 osteoblast-like cells was lowered by these oil and calcium co-treatments (Figure 12.2.C). Other types of local modulators, such as IGFBP-3, TGF-β, and BMP-2, were not influenced by these treatments in both MC3T3-L1 and MC3T3-E1 cells (Figure 12.1.D-F; 12.2.D-F).

From these results, incorporation of ALA, CLA isomers, and calcium generally decreased ST2 stromal and MC3T3-E1 osteoblast-like, but not MC3T3-L1 adipocyte-like cell proliferation. In particular, ALA at the LA:ALA=4:1 markedly increased MC3T3-E1 cell proliferation at a low calcium level, implying a possible benefit of ALA (LA:ALA=4:1) in sustaining osteoblast-like cell growth when calcium level is very low in our body. During differentiation, the treatment of ALA (4:1) + CLA (90:10%) + calcium (2.0 mM) resulted in a best outcome by decreasing MC3T3-L1 adipocyte-like and increasing MC3T3-E1 osteoblast-like cell differentiation. In these cell lines, the production of PGE_2 and LTB_4 was increased in MC3T3-L1 cells and IGF-1 secretion was decreased in MC3T3-E1 cells by ALA (4:1) + CLA (90:10%) + calcium (2.0 mM), indicating a possible involvement of these local mediators in the differentiation of adipocyte-like and osteoblast-like cells.

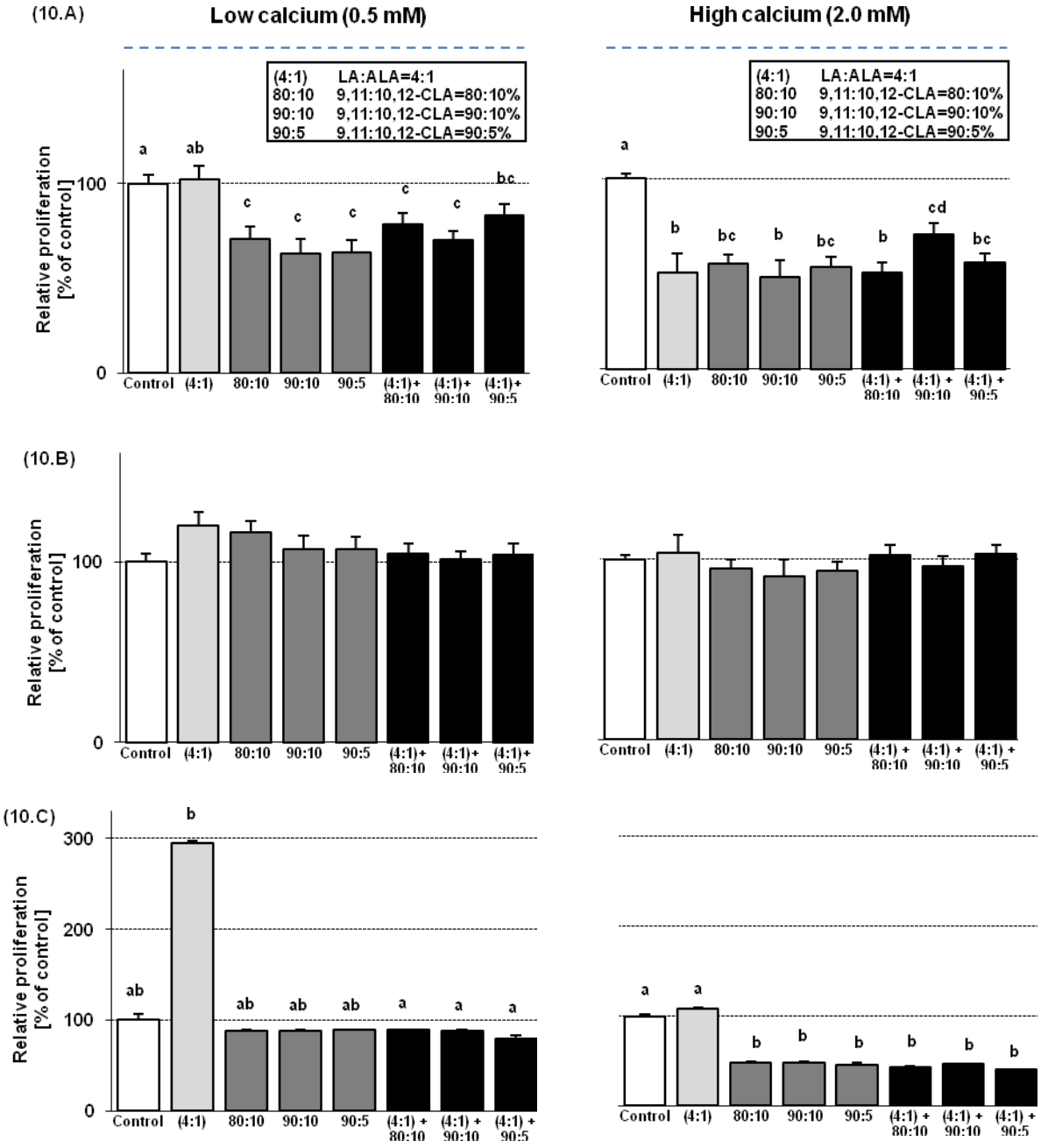

(10.A)
Low calcium (0.5 mM)
High calcium (2.0 mM)
(4:1) LA:ALA=4:1
80:10 9,11:10,12-CLA=80:10%
90:10 9,11:10,12-CLA=90:10%
90:5 9,11:10,12-CLA=90:5%
Relative proliferation [% of control]
Control
(4:1)
80:10
90:10
90:5
(4:1)+ 80:10
(4:1)+ 90:10
(4:1)+ 90:5
(10.B)
(10.C)

Figure 10. Interactive effects of ALA and 9,11-/10,12-CLAs on proliferation of ST2 (10.A), MC3T3-L1 (10.B), and MC3T3-E1 cells (10.C) at low or high calcium level. Cells were cultured for 6 days in the presence of vehicle (1 µl/ml DMSO; control) or individual/combination of ALA (LA:ALA = 4:1) or 20 µM CLA (80:10%, 90:10%, and 90:5%, respectively) at low (0.5 mM) or high (2.0 mM) calcium level. Before the oil treatment, cells were pretreated for ~10 days (3 passages) with the n-3 PUFA mixture (ALA:EPA:DHA=1:3:6) developed for exposing cells to LA:ALA=20:1. Cell proliferation was measured by Resazurin sodium salt-based assay and is presented as % of change from the control where no oil was added. Results are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

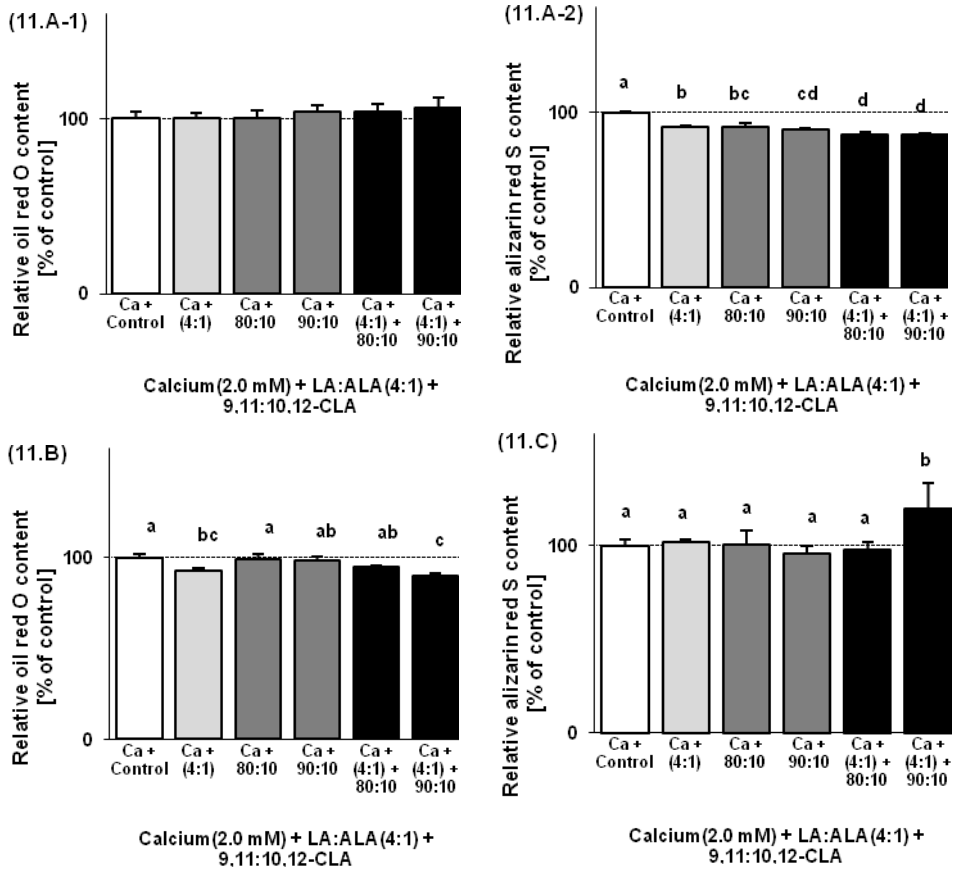
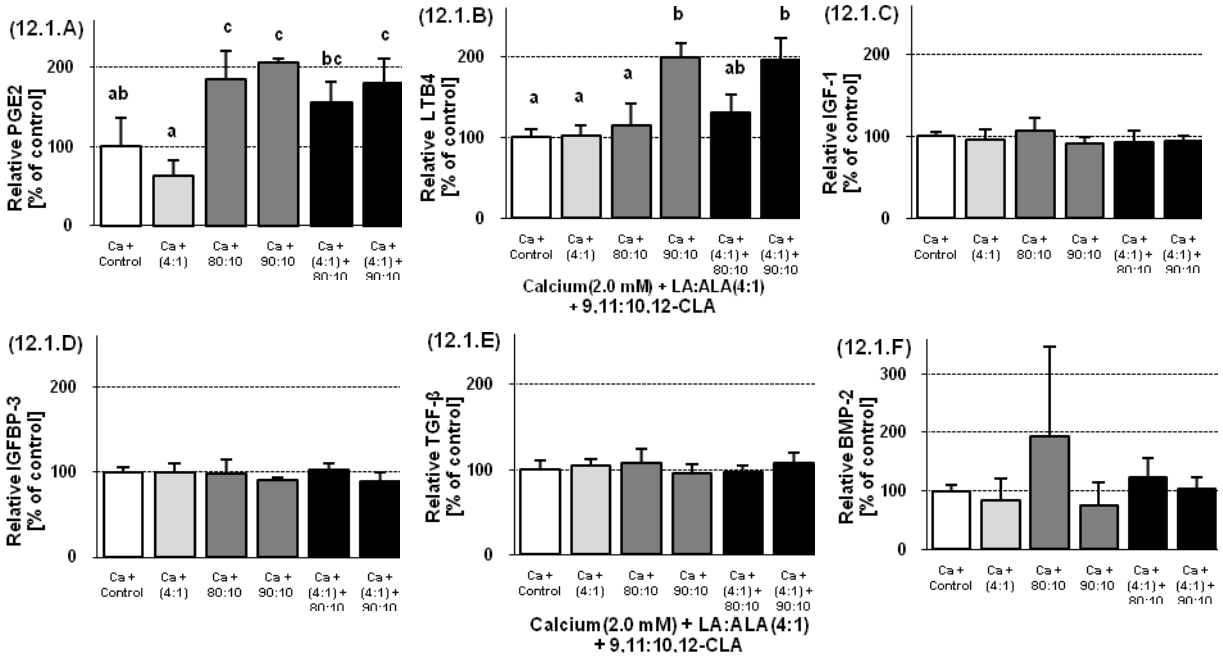


Figure 11. Interactive effects of ALA, CLA, and calcium on differentiation of ST2 (11.A-1, 11.A-2), MC3T3-L1 (11.B), and MC3T3-E1 cells (11.C). Following the pretreatment of the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, cell differentiation was induced for 6 days in the presence of vehicle (1 µl/ml DMSO; control) or individual/combination of ALA (LA:ALA=4:1), 20 µM CLA (80:10%, 90:10%), or calcium (2.0 mM). Quantification of triglyceride accumulation was performed in oil red O stained ST2 and MC3T3-L1 cells that underwent adipogenesis. Quantification of calcium deposition was performed in alizarin red S stained ST2 and MC3T3-E1 cells that underwent osteogenesis. Results are expressed as % of change from the control where no oils were added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.


(12.1.A)
Relative PGE2 [% of control]
ab
a
c
c
bc
c
(12.1.B)
Relative LTB4 [% of control]
a
a
a
b
ab
b
(12.1.C)
Relative IGF-1 [% of control]
Ca + Control
Ca + (4:1)
Ca + 80:10
Ca + 90:10
Ca + (4:1) + 80:10
Ca + (4:1) + 90:10
Calcium (2.0 mM) + LA:ALA (4:1) + 9,11:10,12-CLA
(12.1.D)
Relative IGFBP-3 [% of control]
(12.1.E)
Relative TGF-β [% of control]
(12.1.F)
Relative BMP-2 [% of control]

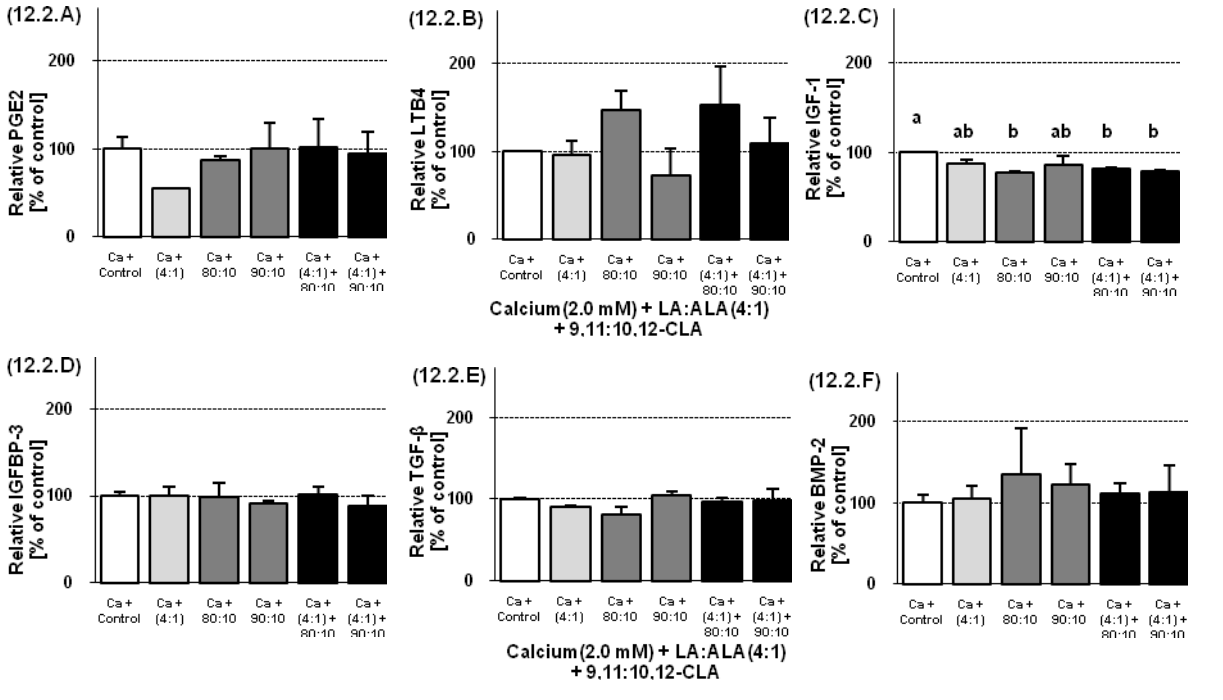

(12.2.A)
Relative PGE2 [% of control]
(12.2.B)
Relative LTB4 [% of control]
(12.2.C)
Relative IGF-1 [% of control]
a
ab
b
ab
b
b
Ca + Control
Ca + (4:1)
Ca + 80:10
Ca + 90:10
Ca + (4:1) + 80:10
Ca + (4:1) + 90:10
Calcium (2.0 mM) + LA:ALA (4:1) + 9,11:10,12-CLA
(12.2.D)
Relative IGFBP-3 [% of control]
(12.2.E)
Relative TGF-β [% of control]
(12.2.F)
Relative BMP-2 [% of control]

Figure 12. Interactive effects of ALA, CLA, and calcium on secretion of PGE_2 (12.1.A; 12.2.A), LTB_4 (12.1.B; 12.2.B), IGF-1 (12.1.C; 12.2.C), IGFBP-3 (12.1.D; 12.2.D), TGF-β (12.1.E; 12.2.E), and BMP-2 (12.1.F; 12.2.F) by MC3T3-L1 and MC3T3-E1 cells (1 for former; 2 for latter). Analysis of these protein levels from MC3T3-L1 or MC3T3-E1 cells that were precultured in the n-3 mixture (ALA:EPA:DHA=1:3:6) for ~10 days, followed by 6 days of exposure to adipogenic or osteogenic induction media in the presence of vehicle (1 μl/ml DMSO; control) or individual/combination of ALA (LA:ALA=4:1), 20 μM CLA (80:10%, 90:10%), and calcium (2.0 mM). Secretion of these proteins was measured by ELISA from the conditioned media and is presented as % of change from the control where no oils were added. Data are shown as means ± S.E.M., $P < 0.05$. The experiment was repeated in triplicate, each experiment yielding comparable data.

CHAPTER 5

DISCUSSION

To our knowledge, this is the first study to investigate the interaction among bioactive milk components, including ALA with relatively low LA:ALA ratios, CLA, and calcium, in modulating osteoblastic and adipocytic cells. Osteoblasts and adipocytes share a progenitor MSC as their common origin and there is growing evidence supporting an inverse association between bone marrow adiposity and bone mass. Therefore, it is crucial to identify factors capable of stimulating MSC's lineage commitment from adipocytes to osteoblasts, or directly enhancing osteoblast differentiation and inhibiting adipocyte differentiation, all of which will ultimately favor bone mass. Because bone formation is tightly regulated by a variety of local and systemic factors (i.e., eicosanoids, growth factors, hormones), this study also determined some of these regulatory agents to unravel the mechanisms responsible for bone homeostasis.

n-6 LA and n-3 ALA Ratios

Recently, the effect of some types of PUFA (i.e., n-3 and n-6 PUFA, CLA) on bone homeostasis has been widely investigated. However, compared with EPA and DHA, the role of ALA has not been equally dealt with due to the assumption that ingested ALA is endogenously metabolized to EPA and DHA in our body. In spite of it, more recent evidence has demonstrated that this conversion process rarely occurs in the mammalian systems and if so, the efficiency is very low [Plourde and Cunnane, 2007]. During the past decades, ALA consumption has decreased by ~40% with an increase in LA intake, resulting in a 4.2-fold increase in the LA:ALA ratio (10-41:1) for typical US diets [Taber et al., 1998]. In respect to the role of ALA in adiposity,

it has been known that LA is more potent than ALA to promote adipogenesis *in vivo* [Massiera et al., 2003]. It was consistent with the current study that relatively low ratios of LA:ALA (1-5:1) significantly attenuated the proliferation as well as differentiation of adipocytes. However, the ratio=1:1 (very high LA:ALA) was unfavorable because at this ratio, adipogenic differentiation of stromal cells was enhanced and, at the same time, proliferation and differentiation of osteoblasts were also suppressed. In this study, overall, the LA:ALA ratio=4:1 was the most promising in terms of minimizing adipogenesis and maximizing osteoblastogenesis. This finding is of worth because there is no study examining the direct role of ALA (using purified ALA) in adipogenesis and bone formation *in vitro*. In particular, in this experiment, the level of ALA added to adipocytes was 7-35 μM, which is a physiologically relevant level, indicating an achievable level of ALA from our diets. To date, the optimal ratio of dietary n-6 to n-3 PUFA is still under debating because it can vary in different physiological and pathological conditions [Cook, 1996; de Lorgeril et al., 1994]. Nevertheless, the optimal ratio found in this study (LA:ALA=4:1) was in accordance with the general guidelines provided by Health Canada as low as 4:1 and was very close to the US DRIs as ~5-10:1 [Kris-Etherton et al., 2002].

9,11- and 10,12-CLA Isomers

CLA is known to have variable effects on bone formation and adiposity *in vivo* and *in vitro*, which is possibly due to individually diverse effects of two major CLA isomers, 9,11- and 10,12-CLAs. Therefore, a wide range of CLA activities can result from interactions between these two CLA isomers with additive (or synergistic), independent, or antagonistic effects [Park and Pariza, 2001]. Platt and El-Sohemy (2009) have reported that 9,11-CLA (6.25-12.5 μM) decreased osteoblast differentiation and increased adipocyte differentiation from human MSC,

whereas 10,12-CLA (6.25-50.0 μM) showed an opposite result, suggesting 10,12-CLA as useful to promote bone formation. A similar result was found in this study that 20 μM 9,11-CLA significantly increased adipogenic differentiation of mouse ST2 cells but did not affect either adipocyte or osteoblast differentiation. The bone-favorable effect of 10,12-CLA was not confirmed in this study that 20 μM 10,12-CLA rather attenuated osteoblastic differentiation of ST2 cells. However, 20 μM 10,12-CLA resulted in a slight, but not significant reduction in adipocyte differentiation as well as a slight (not significant) increase in osteoblast differentiation, which partially proved the anabolic effect of 10,12-CLA on bone. Intriguingly, incorporation of 9,11- and 10,12-CLAs, at specific combining ratios, exerted more optimistic results by reducing adipocyte differentiation (at 90:10 and 90:5%) and stimulating osteoblast differentiation (at 80:10%). Therefore, it is postulated from this study that 9,11- and 10,12-CLAs possess different functions in modulating bone formation and adiposity and when incorporated, they possibly interact together, which, nevertheless, may not result in simple additive or compromising net outcomes. Rather, the net effect might depend on different combining ratios of CLA isomers or different tissue types. Overall, from the data, 10,12-CLA was more prominent than 9,11-CLA to promote bone mass by reducing bone marrow adiposity and increasing bone formation. The major regulation by 10,12-CLA may occur in the differentiation stage of adipocytes and osteoblasts, and not in the step of lineage commitment of stromal cells in the bone microenvironment.

Calcium

Calcium is a vital nutrient for bone health but it may play a role in body weight maintenance. As a plausible anti-adipogenic mechanisms of calcium, it has been proposed that

low calcium intake stimulates its influx into adipocytes via PTH and calcitriol, which, in turn, upregulates lipogenesis and downregulates lipolysis. By contrast, high calcium intake drives an opposite response, leading to an upregulation of lipolysis [Zemel et al., 2000]. Additionally, the capacity of calcium to bind fatty acids from the intestinal tract, thus rendering them unavailable for absorption, is regarded as another anti-adipogenic mechanism of calcium. In fact, an inverse relationship between calcium and body weight/body fat has been revealed in several epidemiological and clinical studies [Davies et al., 2000; Heaney, 2003; Parikh and Yanovski, 2003; Teegarden, 2003]. It has also been reported that calcium consumption was inversely associated with adipocyte size and in actual, high calcium intake (> 2 servings of dairy products) reduced adipocyte size in women [Caron-Jobin et al., 2011]. By contrast, calcium restriction in a diet led to the impairment of adipocyte apoptosis and dysregulation of glucocorticoid metabolism in adipose tissues [Centeno et al., 2009]. A result observed in the study was consistent with such findings that higher calcium levels (up to 3.0 mM) offered unfavorable condition for MC3T3-L1 cells to survive. The proliferation of MC3T3-L1 cells was much enhanced at lower calcium concentrations. It is known that within a typical cell, intracellular calcium concentration is relatively low as ~100 nM, compared with extracellular calcium concentration as high as ~12,000-fold (=~1.2 mM). In ST2 and MC3T3-El cells tested in this study, additionally, too low (0.5 mM) or too high (3.0 mM) calcium was not ideal for their growth, which was, perhaps, due to the reason. Overall, the optimum calcium level enough to suppress adipogenesis and increase osteoblastogenesis was the range of 1.5-2.5 mM calcium.

Interactions among ALA with LA:ALA=1-5:1, 9,11-/10,12-CLAs, and Calcium

In the experiments measuring the synergism among ALA with LA:ALA=4:1, 9,11- and 10,12-CLAs (80:10%, 90:10%, 90:5%), and calcium (0.5 or 2.0 mM), it was apparent that at 0.5 mM calcium, ALA dramatically facilitated osteoblast proliferation, which effect, however, disappeared at 2.0 mM calcium. Hence, it was assumed that this level of ALA with LA:ALA=4:1 may be important to sustain osteoblast growth when calcium level is low. To date, only a few *in vivo* data are available to support the positive role of n-3 PUFA in calcium metabolism. Kelly et al. (2003) demonstrated that n-3 EPA and DHA stimulated calcium absorption. Administrations of n-6 γ-linolenic acid (GLA) and n-3 EPA, in the ratios of 3:1, 1:1, and 1:3, increased intestinal calcium absorption and decreased urinary calcium excretion [Claassen et al., 1995]. Similarly, EPA+DHA (tuna oil) resulted in a higher calcium absorption, *ex vivo* BMD, and bone calcium content [Kruger and Schollum, 2005].

It seems that there is no direct evidence to support our hypothesis that ALA may have a positive role in enhancing calcium availability in osteoblasts, especially in low calcium environment, because as mentioned earlier, most of studies investigating n-3 PUFA have focused on long-chain n-3 EPA and DHA, not ALA. Studies have shown that long-term treatment of 10,12-, but not 9,11-CLA, increased paracellular transport of calcium in Caco-2 cells (a human epithelial colorectal cancer cells) [Jewell and Cashman, 2003]. A similar finding was reported by Park et al. (2008) indicating that co-supplementation of CLA and calcium improved bone mass *in vivo*.

It can be postulated from these results that 10,12-CLA may have a beneficial role in promoting calcium availability and therefore, co-treatment of 10,12-CLA and calcium, and possibly with an addition of ALA=4:1, could further enhance calcium availability, leading to increased osteoblastogenesis. The results from our study showed that 2.0 mM calcium + ALA

(LA:ALA=4:1) + 90:10% 9,11:10,12-CLA improved osteoblast differentiation and, at the same time, significantly attenuated adipocyte differentiation, compared with any combination of each compound. Because reduced bone marrow adiposity can further contribute to improved bone formation, our finding is prominent in suggesting a practical dietary strategy to promote bone health.

Mechanisms: Eicosanoids and Growth Factors

Proposed mechanisms related to the effects of ALA (or LA:ALA ratios), CLA, and calcium on adiposity and bone formation have already been described above. To reveal the mechanisms, this study focused on, 1) PGE_2 and LTB_4, two major eicosanoids closely associated with ALA and CLA metabolic processes as well as many physiological functions on bone metabolism, and 2) IGF-1, IGFBP-3, TGF-β, and BMP-2, all of which are local growth factors related to the development of stromal cells, adipocytes, and osteoblasts.

PGE_2 is produced by osteoblastic lineage and is a potent local factor that stimulates bone resorption *in vivo* and *in vitro* [Kobayashi et al., 2005; Okada et al., 2003]. However, in *in vitro* studies, PGE_2 has shown biphasic and concentration-dependent effects – it stimulated bone formation at low concentration and inhibited bone formation at high concentration [Shen et al., 2004]. According to Hakeda et al. (1985), PGE_2 elevated collagen and non-collagen protein syntheses in a dose-dependent manner up to 2 μg/ml. PGE_2 production observed in this study was relatively low, ranged from 0.2-0.8 ng/ml by ALA and 0.05-0.5 ng/ml by CLA treatments (data not shown) and overall, it was increased (but mostly not significantly) in both differentiating adipocytes and osteoblasts. Although a line of recent evidence has documented that n-3 PUFA (EPA and DHA) and CLA attenuated *ex vivo* PGE_2 production [Cusack et al.,

2005; Li et al., 2008], it was not confirmed in this study. The controversy may be due to the fact that LA and ALA, the parent molecules of n-6 and n-3 PUFA, respectively, were not efficiently metabolized to AA and EPA, compounds directly related to the regulation of PGE_2 synthesis. In spite of it, this study showed elevated PGE_2 secretion when adipocyte differentiation was reduced and osteoblast differentiation was enhanced. It is supported by recent evidence that PGE_2 inhibited adipocyte differentiation via acting on the EP4 receptor in adipocytes [Sugimoto et al., 2004; Tsuboi et al., 2004].

Apart from PGE_2, LTB_4 is also known as a strong (but weaker than PGE_2) stimulator of bone resorption [Gaillard et al., 1989]. LTB_4 receptors are present in osteoblasts. Although Paredes et al. (2002) reported that LTB_4 dose-dependently stimulated osteocalcin release in osteoblasts, the stimulatory effect of LTB_4 on osteoclast differentiation was also documented [Chen et al., 2010]. In this study, LTB_4 secretion was not significantly changed by any of treatments except with 2.0 mM calcium + ALA=4:1 + CLA (90:10%), at which adipocyte differentiation was attenuated, suggesting that LTB_4 may induce an anti-adipogenic effect like PGE_2.

IGF-1 is a structural homolog to pro-insulin that is produced by many tissues. It is abundant in the circulation, in which over 80% of IGF-1 is bound to IGFBP-3, whereas most of the remainder is bound to other types of binding proteins (IGFBP-1, 2, 4, 5, 6), thereby extending its half-life. IGF-1 is a potent anabolic agent that has been known to trigger adipogenesis. According to recent studies, IGF-1 stimulated cell growth and differentiation of human MSC's into adipocytes *in vitro* [Scavo et al., 2004] and increased preadipocyte differentiation [Smith et al., 1988]. Additionally, in many studies, adiposity (or body fat mass) was positively correlated with serum IGF levels [Alderete et al., 2011]. IGFBP-3 regulates the

pro-mitogenic and anti-apoptogenic functions of the IGFs but also has independent functions. In spite of it, the role of IGFBP-3 in regulating cell growth is still ambiguous: IGFBP-3 can stimulate the proliferation of IGFs or inhibit IGF actions. In cultured cells, IGFBP-3 has also been shown to enhance both apoptosis and survival, indicating that IGFBP-3 may promote or suppress cell growth depending on specific conditions [Granata et al., 2004; Liu et al., 2007; Spagnoli et al., 2001]. Recently, Grohmann et al. (2005) reported that IGF-1 stimulated differentiation of visceral adipocytes and IGFBP-3 enhanced their differentiation, implying a close interaction between IGF-1 and IGFBP-3 in increasing adiposity. The role of IGF-1 in bone formation has been reported by Yakar et al. (2010) showing that IGF-1 axis acted as a strong determinant of bone mineralization in osteoblasts. Such findings were partially proven in this study; secretion of both IGF-1 and IGFBP-3 in adipocyte differentiation was altered in a similar manner by treatment with ALA (LA:ALA=1-5:1). Specifically, when adipocyte differentiation was minimized by LA:ALA=4:1, both secretion of IGF-1 and IGFBP-3 was the lowest, indicating that n-3 ALA may modulate adipocye differentiation via IGF-1/IGFBP-3 signaling axis. Nevertheless, no significant change in IGF-1 and/or IGFBP-3 was observed in other treatments and the involvement of IGF-1/IGFBP-3 in the control of osteoblast differentiation was not shown in this study.

BMP-2 is implicated as a potent stimulator of osteogenesis but in regards to the effect on adipogenesis, both stimulatory and inhibitory effects have been described [Gimble et al., 2006]. The action of BMP-2 may be dose-dependent: at low concentration, it increases adipogenesis by interacting with TGF-β and insulin but at high concentration, it supports osteoblastogenesis [Cao, 2011]. No significant change in BMP-2 by any PUFA and calcium treatments was observed in this study.

TGF-β, another type of local growth factor, is also crucial in a variety of cellular functions. However, its signaling pathway is tremendously complex, which makes its exact role remain elusive [Clarke and Liu, 2008]. TGF-β is regarded as important in maintaining MSCs' population and coordinating bone remodeling cycle, although the concentrations required are ambiguous. To date, it has been documented that TGF-β mediates MSC's differentiation and inhibits both osteoblast and adipocyte differentiation [Choy and Derynck, 2003]. In accordance with these findings, a slight, but not significant reduction of TGF-β was observed in adipocytes treated with ALA=3-4:1, which is the LA:ALA levels significantly decreasing adipocyte differentiation. This outcome, therefore, confirmed the anti-adipogenic effect of TGF-β but similar changes of TGF-β secretion were not seen by CLA or PUFA (ALA or CLA) + calcium combinations.

Summary and Conclusions

Osteoporosis and obesity are global health concerns and research elucidating the link between these two pathophysiological conditions is a new area of investigation. Milk and dairy products are high in ALA having relatively low n-6 LA:n-3 ALA ratios of 1-5:1 (compared to 20:1 found in a typical Western diet), CLA, and calcium, all of which are implicated to be health-beneficial with a potential to promote bone formation and decrease adiposity. Based on the evidence supporting an inverse relationship between bone marrow adiposity and bone mass, reducing adiposity may further contribute to increased bone mass. In conclusion, the present study showed that LA:ALA=4:1 resulted in maximal osteoblastogenesis and minimal adipogenesis, which may be partially mediated via IGF-1/IGFBP-3 signaling pathway. This effect was further enhanced when LA:ALA=4:1 was incorporated with CLA

(9,11:10,12=90:10%) and high calcium (2.0 mM), implying the possible benefit of this dietary regimen in promoting bone health by increasing bone formation and at the same time, reducing bone marrow adiposity. Consequently, this *in vitro* study provides supporting evidence that milk products can be used as an efficient delivery system for nutraceuticals to possibly modulate clinical pathological conditions including obesity and osteoporosis.

REFERENCES

Alderete TL, Byrd-Williams CE, Toledo-Corral CM, Conti DV, Weigensberg MJ, Goran MI. Relationships between IGF-1 and IGFBP-1 and adiposity in obese African-American and Latino adolescents. Obesity (Silver Spring). 2011;19:933-8.

Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004;8:301-16.

Bassey EJ, Littlewood JJ, Rothwell MC, Pye DW. Lack of effect of supplementation with essential fatty acids on bone mineral density in healthy pre- and postmenopausal women: two randomized controlled trials of Efacal v. calcium alone. Br J Nutr. 2000;83:629-35.

Begg DP, Sinclair AJ, Stahl LA, Premaratna SD, Hafandi A, Jois M, Weisinger RS. Hypertension induced by omega-3 polyunsaturated fatty acid deficiency is alleviated by alpha-linolenic acid regardless of dietary source. Hypertens Res. 2010;33:808-13.

Berkey KS, Rockett HRH, Willett WC, Colditz GA. Milk, dairy fat, dietary calcium, and weight gain: a longitudinal study of adolescents. Arch Pediatr Adolesc Med. 2005;159:543-50.

Bhattacharya A, Rahman M, Sun D, Fernandes G. Effect of fish oil on bone mineral density in aging C57BL/6 female mice. J Nutr Biochem. 2007;18:372-9.

Brooks BM, Rajeshwari R, Nicklas TA, Yang SJ, Berenson GS. Association of calcium intake, dairy product consumption with overweight status in young adults (1995-1996): the Bogalusa Heart Study. J Am Coll Nutr. 2006;25:523-32.

Brown JM, Boysen MS, Chung S, Fabiyi O, Morrison RF, Mandrup S, McIntosh MK. Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem. 2004;279:26735-47.

Brown JM, McIntosh MK. Conjugated linoleic acid in humans: regulation of adiposity and insulin sensitivity. J Nutr. 2003;133:3041-6.

Brownbill RA, Petrosian M, Ilich JZ. Association between dietary conjugated linoleic acid and bone mineral density in postmenopausal women. J Am Coll Nutr. 2005;24:177-81.

Burr LL, Taylor CG, Weiler HA. Dietary conjugated linoleic acid does not adversely affect bone mass in obese fa/fa or lean Zucker rats. Exp Biol Med (Maywood). 2006;231:1602-9.

Cao JJ. Effects of obesity on bone metabolism. J Orthop Surg Res. 2011;6:30.

Caron-Jobin M, Morisset AS, Tremblay A, Huot C, Légaré D, Tchernof A. Elevated serum 25(OH)D concentrations, vitamin D, and calcium intakes are associated with reduced adipocyte size in women. Obesity (Silver Spring). 2011;19:1335-41.

Carruth BR, Skinner JD. The role of dietary calcium and other nutrients in moderating body fat in preschool children. Int J Obesity. 2001;25:559-66.

Cartwright MJ, Tchkonia T, Kirkland JL. Aging in adipocytes: potential impact of inherent, depot-specific mechanisms. Exp Gerontol. 2007;42:463-71.

Cashman KD. Calcium intake, calcium bioavailability and bone health. Br J Nutr. 2002;87:S169-S77.

Cashman K. Prebiotics and calcium bioavailability. Curr Issues Intest Microbiol. 2003;4:21-32.

Cashman KD. Diet, nutrition, and bone health. J Nutr. 2007;137:S2507-S12.

Cashman KD, Flynn A. Optimal nutrition: calcium, magnesium and phosphorus. Proc Nutr Soc. 1999;58:477-87.

Centeno V, de Barboza GD, Marchionatti A, Rodríguez V, de Talamoni NT. Molecular mechanisms triggered by low-calcium diets. Nutr Res Rev. 2009;22:163-74.

Chen ZK, Lv HS, Jiang J. LTB4 can stimulate human osteoclast differentiation dependent of RANKL. Artif Cells Blood Substit Immobil Biotechnol. 2010;38:52-6.

Chicco AG, D'Alessandro ME, Hein GJ, Oliva ME, Lombardo YB. Dietary chia seed (Salvia hispanica L.) rich in alpha-linolenic acid improves adiposity and normalizes hypertriacylglycerolaemia and insulin resistance in dyslipaemic rats. Br J Nutr. 2009;101:41-50.

Choy L, Derynck R. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. J Biol Chem. 2003;278:9609-19.

Churruca I, Fernández-Quintela A, Portillo MP. Conjugated linoleic acid isomers: differences in metabolism and biological effects. Biofactors. 2009;35:105-11.

Claassen N, Coetzer H, Steinmann CM, Kruger MC. The effect of different n-6/n-3 essential fatty acid ratios on calcium balance and bone in rats. Prostaglandins Leukot Essent Fatty Acids. 1995;53:13-9.

Clarke DC, Liu X. Decoding the quantitative nature of TGF-beta/Smad signaling. Trends Cell Biol. 2008;18:430-42.

Cohen SL, Moore AM, Ward WE. Flaxseed-oil and inflammation-associated bone abnormalities in interleukin-10 knockout mice. J Nutr Biochem. 2005;16:368-74.

Cook HW. Fatty acid desaturation and chain elongation in eukaryotes. P 129 in Biochemistry of Lipids, Lipoproteins and Membranes. Vance DE and Vance JE, ed. Elsevier, Amsterdam, the Netherlands, 1996.

Corino C, Mourot J, Magni S, Pastorelli G, Rosi F. Influence of dietary conjugated linoleic acid on growth, meat quality, lipogenesis, plasma leptin and physiological variables of lipid metabolism in rabbits. J Anim Sci. 2002;80;1020-8.

Cusack S, Jewell C, Cashman KD. The effect of conjugated linoleic acid on the viability and metabolism of human osteoblast-like cells. Prostaglandins Leukot Essent Fatty Acids. 2005;72:29-39.
Davies KM, Heaney RP, Recker RR, Lappe JM, Barger-Lux MJ, Rafferty K, Hinders S. Calcium intake and body weight. Clin Endocr Metab. 2000;85:4635-42.

de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, Guidollet J, Touboul P, Delaye J. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet. 1994;343:1414-19.

Dhiman TR, Nam SH, Ure AL. Factors affecting conjugated linoleic acid content in milk and meat. Crit Rev Food Sci Nutr. 2005;45:463-82.

Dixon LB, Pellizzon MA, Jawad AF, Tershakovec AM. Calcium and dairy intake and measures of obesity in hyperand normocholesterolemic children. Obes Res. 2005;13:1727-38.

dos Santos LC, de Pádua Cintra I, Fisberg M, Martini LA. Calcium intake and its relationship with adiposity and insulin resistance in post-pubertal adolescents. J Hum Nutr Diet. 2008;21:109-16.

Doyle L, Jewell C, Mullen A, Nugent AP, Roche HM, Cashman KD. Effect of dietary supplementation with conjugated linoleic acid on markers of calcium and bone metabolism in healthy adult men. Eur J Clin Nutr. 2005;59:432-40.

Drapeau V, Després JP, Bouchard C, Allard L, Fournier G, Leblanc C, Tremblay A. Modifications in food-group consumption are related to long-term body weight changes. Am J Clin Nutr. 2004;80:29-37.

Duque G. Bone and fat connection in aging bone. Curr Opin Rheumatol. 2008;20:429-34.

Dyerberg J, Bang HO, Stoffersen E, Moncada S, Vane JR. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? Lancet. 1978;2:117-9.

Eagan MS, Lyle RM, Gunther CW, Peacock M, Teegarden D. Effect of 1-year dairy product intervention on fat mass in young women: 6-month follow-up. Obesity (Silver Spring). 2006;14:2242-8.

Ebringer L, Ferencík M, Krajcovic J. Beneficial health effects of milk and fermented dairy

products--review. Folia Microbiol (Praha). 2008;53:378-94.

Elwood PC, Strain JJ, Robson PJ, Fehily AM, Hughes J, Pickering J, Ness A. Milk consumption, stroke, and heart attack risk: evidence from the Caerphilly cohort of older men. J Epidemiol Community Health. 2005;59:502-5.

Evans M, Lin X, Odle J, McIntosh M. Trans-10, cis-12 conjugated linoleic acid increases fatty acid oxidation in 3T3-L1 preadipocytes. J Nutr. 2002;132:450-5.
Evans M, Park Y, Pariza M, Curtis L, Kuebler B, McIntosh M. Trans-10,cis-12 conjugated linoleic acid reduces triglyceride content while differentially affecting peroxisome proliferator activated receptor gamma2 and aP2 expression in 3T3-L1 preadipocytes. Lipids. 2001;36:1223-32.

Flachs P, Horakova O, Brauner P, Rossmeisl M, Pecina P, Franssen-van Hal N, Ruzickova J, Sponarova J, Drahota Z, Vlcek C, Keijer J, Houstek J, Kopecky J.Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. Diabetologia. 2005;48:2365-75.

Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. JAMA. 2010;303:235-41.

Foo C, Frey S, Yang HH, Zellweger R, Filgueira L. Downregulation of beta-catenin transdifferentiation of human osteoblasts to adipocytes under estrogen deficiency. Gynecol Endocrinol. 2007;23:535-40.

Fujita T, Palmieri GMA. Calcium paradox disease: calcium deficiency prompting secondary hyperparathyroidism and cellular calcium overload. J Bone Miner Res. 2000;18109-25.

Gaillard D, Negrel R, Lagarde M, Ailhaud G. Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. Biochem J. 1989;257:389-97.

Gaiva MH, Couto RC, Oyama LM, Couto GE, Silveira VL, Nascimento CM. Polyunsaturated fatty acid-rich diets: effect on adipose tissue metabolism in rats. Br J Nutr. 2001;86:371-7.

Gaullier JM, Halse J, Hoye K, Kristiansen K, Fagertun H, Vik H, Gudmundsen O. Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. Am J Clin Nutr. 2004;79:1118-25.

Gimble JM, Zvonic S, Floyd ZE, Kassem M, Nuttall M. Playing with bone and fat. J Cell Biochem. 2006;98:251-66.

Goldberg TB, da Silva CC, Peres LN, Berbel MN, Heigasi MB, Ribeiro JM, Suzuki K, Josué LM, Dalmas JC. Calcium intake and its relationship with risk of overweight and obesity in adolescents. Arch Latinoam Nutr. 2009;59:14-21.

Goodpaster BH, Brown NF. Skeletal muscle lipid and its association with insulin resistance:

what is the role for exercise? Exerc Sport Sci Rev. 2005;33:150-4.

Goulding A, Jones IE, Taylor RW, Williams SM, Manning PJ. Bone mineral density and body composition in boys with distal forearm fractures: a dual-energy x-ray absorptiometry study. J Pediatr. 2001;139:509-15.

Granata R, Trovato L, Garbarino G, Taliano M, Ponti R, Sala G, Ghidoni R, Ghigo E. Dual effects of IGFBP-3 on endothelial cell apoptosis and survival: involvement of the sphingolipid signaling pathways. FASEB J. 2004;18:1456-8.

Granlund L, Pedersen JI, Nebb HI. Impaired lipid accumulation by trans10, cis12 CLA during adipocyte differentiation is dependent on timing and length of treatment. Biochim Biophys Acta. 2005;1687:11–22.

Grohmann M, Sabin M, Holly J, Shield J, Crowne E, Stewart C. Characterization of differentiated subcutaneous and visceral adipose tissue from children: the influences of TNF-alpha and IGF-I. J Lipid Res. 2005;46:93-103.

Gunther CW, Legowski PA, Lyle RM, McCabe GP, Eagan MS, Peacock M, Teegarden D. Dairy products do not lead to alterations in body weight or fat mass in young women in a 1-y intervention. Am J Clin Nutr. 2005;81:751-6.

Guo W, Xie W, Lei T, Hamilton JA. Eicosapentaenoic acid, but not oleic acid, stimulates beta-oxidation in adipocytes. Lipids. 2005;40:815-21.

Haug A, Hostmark AT, Harstad OM. Bovine milk in human nutrition--a review. Lipids Health Dis. 2007;6:25.

Hakeda Y, Nakatani Y, Kurihara N, Ikeda E, Maeda N, Kumegawa M. Prostaglandin E2 stimulates collagen and non-collagen protein synthesis and prolyl hydroxylase activity in osteoblastic clone MC3T3-E1 cells. Biochem Biophys Res Commun. 1985;126:340-5.

Harris WS. Are omega-3 fatty acids the most important nutritional modulators of coronary heart disease risk? Curr Atheroscler Rep. 2004;6:447-52.

Harvey-Berino J, Casey Gold B, Lauber R, Starinski A. The impact of calcium and dairy product consumption on weight loss. Obes Res. 2005;13:1720-6.

Haug A, Hostmark AT, Harstad OM. Bovine milk in human nutrition--a review. Lipids Health Dis. 2007;6:25.

Heaney RP. Normalizing calcium intake: projected population effects for body weight. J Nutr. 2003;133:S268-S70.

Heiss CJ, Shaw SE, Carothers L. Association of calcium intake and adiposity in postmenopausal women. J Am Coll Nutr. 2008;27:260-6.

Högström M, Nordström P, Nordström A. n-3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the NO2 Study. Am J Clin Nutr. 2007;85:803-7.

Huth PJ, DiRienzo DB, Miller GD. Major scientific advances with dairy foods in nutrition and health. J Dairy Sci. 2006;89:1207-21.

Hur SJ, Park Y. Effect of conjugated linoleic acid on bone formation and rheumatoid arthritis. Eur J Pharmacol. 2007;568:16-24.

Insel P, Turner RE, Ross D. Nutrition Second edition. American dietetic association. Jones and Bartlett, USA; 2004.

Jenkins TC, McGuire MA. Major advances in nutrition: impact on milk composition. J Dairy Sci. 2006;89:1302-10.

Jensen RG, Newburg DS. Bovine milk lipids. In Handbook of milk composition. Edited by: Jensen RG. Academic Press, USA. 1995;543-75.

Jewell C, Cashman KD. The effect of conjugated linoleic acid and medium-chain fatty acids on transepithelial calcium transport in human intestinal-like Caco-2 cells. Br J Nutr. 2003;89:639-47.

Kagawa Y, Nishizawa M, Suzuki M, Miyatake T, Hamamoto T, Goto K, Motonaga E, Izumikawa H, Hirata H, Ebihara A. Eicosapolyenoic acids of serum lipids of Japanese islanders with low incidence of cardiovascular diseases. J Nutr Sci Vitaminol (Tokyo). 1982;28:441-53.

Kang K, Liu W, Albright KJ, Park Y, Pariza MW. Trans-10,cis-12 CLA inhibits differentiation of 3T3-L1 adipocytes and decreases PPAR gamma expression. Biochem Biophys Res Commun. 2003;303:795-9.

Kelly O, Cashman KD. The effect of conjugated linoleic acid on calcium absorption and bone metabolism and composition in adult ovariectomised rats. Prostaglandins Leukot Essent Fat Acids. 2004;71:295-301.

Kelly O, Cusack S, Jewell C, Cashman KD. The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats. Br J Nutr. 2003;90:743-50.

Kepler CR, Hirons KP, McNeill JJ, Tove SB. Intermediates and products of the biohydrogenation of linoleic acid by Butyrinvibrio fibrisolvens. J Biol Chem. 1966;241:1350-4.

Kim H, Choi S, Lee HJ, Lee JH, Choi H. Suppression of fatty acid synthase by dietary polyunsaturated fatty acids is mediated by fat itself, not by peroxidative mechanism. J Biochem Mol Biol. 2003;36:258-64.

Kim HK, Della-Fera M, Lin J, Baile CA. Docosahexaenoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. J Nutr. 2006;136:2965-9.

Kim Y, Ilich JZ. Implications of dietary α-linolenic acid in bone health. Nutrition. 2011; 27:1101-7.

Kloss R, Linscheid J, Johnson A, Lawson B, Edwards K, Linder T, Stocker K, Petitte J, Kern M. Effects of conjugated linoleic acid supplementation on blood lipids and adiposity of rats fed diets rich in saturated versus unsaturated fat. Pharmacol Res. 2005;51:503-7.

Kobayashi Y, Take I, Yamashita T, Mizoguchi T, Ninomiya T, Hattori T, Kurihara S, Ozawa H, Udagawa N, Takahashi N. Prostaglandin E2 receptors EP2 and EP4 are down-regulated during differentiation of mouse osteoclasts from their precursors. J Biol Chem. 2005;280:24035-42.

Kreider RB, Ferreira MP, Greenwood M, Wilson M, Almada AL. Effects of conjugated linoleic acid supplementation during resistance training on body composition, bone density, strength, and selected hematological markers. J Strength Cond Res. 2002;16:325-34.

Kris-Etherton PM, Harris WS, Appel LJ; American Heart Association. Nutrition Committee. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation. 2002;106:2747-57.

Kris-Etherton PM, Hill AM. N-3 fatty acids: food or supplements? J Am Diet Assoc. 2008;108:1125-30.

Kruger MC, Coetzer H, de Winter R, Gericke G, van Papendorp DH. Calcium, gamma-linolenic acid and eicosapentaenoic acid supplementation in senile osteoporosis. Aging (Milano). 1998;10:385-94.

Kruger MC, Schollum LM. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins Leukot Essent Fatty Acids. 2005;73:327-4.

Kunesová M, Braunerová R, Hlavatý P, Tvrzická E, Stanková B, Skrha J, Hilgertová J, Hill M, Kopecký J, Wagenknecht M, Hainer V, Matoulek M, Parízková J, Zák A, Svacina S. The influence of n-3 polyunsaturated fatty acids and very low calorie diet during a short-term weight reducing regimen on weight loss and serum fatty acid composition in severely obesewomen. Physiol Res. 2006;55:63-72.

Larsen T, Toubro S, Astrup A. Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies. J Lipid Res. 2003;44:2234-41.

Lee Y. Isomer specificity of conjugated linoleic acid (CLA): 9E,11E-CLA. Nutr Res Pract. 2008;2:326-30.

Li JJ, Huang CJ, Xie D. Anti-obesity effects of conjugated linoleic acid, docosahexaenoic acid, and eicosapentaenoic acid. Mol Nutr Food Res. 2008;52:631-45.

Li Y, Seifert MF, Ney DM, Grahn M, Grant AL, Allen KG, Watkins BA. Dietary conjugated linoleic acids alter serum IGF-I and IGF binding protein concentrations and reduce bone formation in rats fed (n-6) or (n-3) fatty acids. J Bone Miner Res. 1999;14:1153-62.

Liu LQ, Sposato M, Liu HY, Vaudrain T, Shi MJ, Rider K, Stevens Z, Visser J, Deng HK, Kraus M. Functional cloning of IGFBP-3 from human microvascular endothelial cells reveals its novel role in promoting proliferation of primitive CD34+CD38- hematopoietic cells in vitro. Oncol Res. 2003;13:359-71.

Loos RJ, Rankinen T, Leon AS, Skinner JS, Wilmore JH, Rao DC, Bouchard C. Calcium intake is associated with adiposity in Black and White men and White women of the HERITAGE Family Study. J Nutr. 2004;134:1772-8.

MacDonald HB. Conjugated linoleic acid and disease prevention: a review of current knowledge. J Am Coll Nutr. 2000;19:S111-S8.

Mallamaci F, Tripepi G, Zoccali C. Leptin in end stage renal disease (ESRD): a link between fat mass, bone and the cardiovascular system. J Nephrol. 2005;18:464-8.
Marques-Vidal P, Goncalves A, Dias CM. Milk intake is inversely related to obesity in men and in young women: data from the Portuguese Health Interview Survey 1998-1999. Int J Obes. 2006;30:88-93.

Martin JC, Valeille K. Conjugated linoleic acids: all the same or to everyone its function? Reprod Nutr Develop. 2002;42:525-36.

Massiera F, Saint-Marc P, Seydoux J, Murata T, Kobayashi T, Narumiya S, Guesnet P, Amri EZ, Negrel R, Ailhaud G. Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? J Lipid Res. 2003;44:271-9.

Meadus WJ, MacInnis R, Dugan ME. Prolonged dietary treatment with conjugated linoleic acid stimulates porcine muscle peroxisome proliferator activated receptor gamma and glutamine-fructose aminotransferase gene expression in vivo. J Mol Endocrinol. 2002;28:79-86.

Meyers VE, Zayzafoon M, Douglas JT, McDonald JM. RhoA and cytoskeletal disruption mediate reduced osteoblastogenesis and enhanced adipogenesis of human mesenchymal stem cells in modeled microgravity. J Bone Miner Res. 2005;20:1858-66.

Minami A, Ishimura N, Sakamoto S, Takishita E, Mawatari K, Okada K, Nakaya Y. Effect of eicosapentaenoic acid ethyl ester v. oleic acid-rich safflower oil on insulin resistance in type 2 diabetic model rats with hypertriacylglycerolaemia. Br J Nutr. 2002;87:157-62.

Mirand PP, Arnal-Bagnard MA, Mosoni L, Faulconnier Y, Chardigny JM, Chilliard Y. Cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid isomers do not modify body composition in adult sedentary or exercised rats. J Nutr. 2004;134:2263-9.

Mollard RC, Gillam ME, Wood TM, Taylor CG, Weiler HA. (n-3) fatty acids reduce the release of prostaglandin E2 from bone but do not affect bone mass in obese (fa/fa) and lean Zucker rats. J Nutr. 2005;135:499-504.

Nanes MS. Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology. Gene. 2003;321:1-15.

Navarro V, Fernández-Quintela A, Churruca I, Portillo MP. The body fat-lowering effect of conjugated linoleic acid: a comparison between animal and human studies. Physiol Biochem. 2006;62:137-47.

Okada Y, Pilbeam C, Raisz L, Tanaka Y. Role of cyclooxygenase-2 in bone resorption. J UOEH. 2003;25:185-95.

Oudart H, Groscolas R, Calgari C, Nibbelink M, Leray C, Le Maho Y, Malan A. Brown fat thermogenesis in rats fed high-fat diets enriched with n-3 polyunsaturated fatty acids. Int J Obes Relat Metab Disord. 1997;21:955-62.

Paredes Y, Massicotte F, Pelletier JP, Martel-Pelletier J, Laufer S, Lajeunesse D. Study of the role of leukotriene B4 in abnormal function of human subchondral osteoarthritis osteoblasts: effects of cyclooxygenase and/or 5-lipoxygenase inhibition. Arthritis Rheum. 2002;46:1804-12.

Park Y, Pariza MW. Lipoxygenase inhibitors inhibit heparin-releasable lipoprotein lipase activity in 3T3-L1 adipocytes and enhance body fat reduction in mice by conjugated linoleic acid. Biochim Biophys Acta. 2001;1534:27-33.

Park Y, Pariza MW, Park Y. Cosupplementation of dietary calcium and conjugated linoleic acid (CLA) improves bone mass in mice. J Food Sci. 2008;73:C556-C60.

Parikh SJ, Yanovski JA. Calcium intake and adiposity. Am J Clin Nutr. 2003;77:281-7.

Pariza MW, Park Y, Cook ME. The biologically active isomers of conjugated linoleic acid. Prog Lipid Res. 2001;40:283-98.

Pariza MW, Park Y, Xu X, Ntambi J, Kang K. Chapter 14: Speculation on the mechanisms of action of conjugated linoleic acid. Pp. 251-258 in Advances in Conjugated Linoleic Acid Research, Vol. 2. J.L. Sebedio, W.W. Christie, and R. Adlof (eds.), AOCS Press, Champaign, IL. 2003.

Perez-Matute P, Perez-Echarri N, Martinez JA, Marti A, Moreno-Aliaga MJ. Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectin and tumour necrosis factor-alpha. Br J Nutr. 2007;97:389-98.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143-7.

Platt I, Rao LG, El-Sohemy A. Isomer-specific effects of conjugated linoleic acid on mineralized bone nodule formation from human osteoblast-like cells. Exp Biol Med (Maywood). 2007;232:246-52.

Plourde M, Cunnane SC. Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. Appl Physiol Nutr Metab 2007;32:619-34.

Pollock NK, Laing EM, Baile CA, Hamrick MW, Hall DB, Lewis RD. Is adiposity advantageous for bone strength? A peripheral quantitative computed tomography study in late adolescent females. Am J Clin Nutr. 2007;86:1530-8.

Pouteau E, Aprikian O, Grenot C, Reynaud D, Pace-Asciak C, Cuilleron CY, Castañeda-Gutiérrez E, Moulin J, Pescia G, Beysen C, Turner S, Macé K. A low alpha-linolenic intake during early life increases adiposity in the adult guinea pig. Nutr Metab (Lond). 2010;7:8.

Raclot T, Groscolas R, Langin D, Ferre P. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. J Lipid Res. 1997;38:1963-72.

Ragab AA, Nalepka JL, Bi Y, Greenfield EM. Cytokines synergistically induce osteoclast differentiation: support by immortalized or normal calvarial cells. Am J Physiol Cell Physiol. 2002;283:C679-C87.

Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest. 2005;115:3318-25.

Rodrı´guez E, Ribot J, Palou A. Trans-10,cis-12, but not cis-9,trnas-11 CLA isomer inhibits brown adipocyte thermogenic capacity. Am J Physiol. 2002;282:1789-97.

Roodman GD. Advances in bone biology: the osteoclast. Endocr Rev. 1996;17:308-32.

Rosell M, Johansson G, Berglund L, Vessby B, de Faire U, Hellenius ML. Associations between the intake of dairy fat and calcium and abdominal obesity. Int J Obes Relat Metab Disord. 2004;28:1427-34.

Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol. 2006;2:35-43.

Ruzickova J, Rossmeisl M, Prazak T, Flachs P, Sponarova J, Veck M, Tvrzicka E, Bryhn M, Kopecky J. Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. Lipids. 2004;39:1177-85.

Ryan-Harshman M, Aldoori W. Calcium and optimal bone health. Can Fam Physician. 2005;51:1205-6.

Sacco SM, Jiang JM, Reza-L_opez S, Ma DW, Thompson LU, Ward WE. Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats. Menopause. 2009;16:545-54.

Sacco SM, Jiang JM, Reza-Lopez S, Ma DW, Thompson LU, Ward WE. Flaxseed does not antagonize the effect of ultra-low-dose estrogen therapy on bone mineral density and biomechanical bone strength in ovariectomized rats. J Toxicol Environ Health A. 2009;72:1209-16.

Sakaguchi K, Morita I, Murota S. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. Prostaglandins Leukot Essent Fatty Acids. 1994;50:81-4.

Salas-Salvadó J, Márquez-Sandoval F, Bulló M. Conjugated linoleic acid intake in humans: a systematic review focusing on its effect on body composition, glucose, and lipid metabolism. Crit Rev Food Sci Nutr. 2006;46:479-88.

Scavo LM, Karas M, Murray M, Le Roith D. Insulin-like growth factor-I stimulates both cell growth and lipogenesis during differentiation of human mesenchymal stem cells into adipocytes. J Clin Endocrinol Metab. 2004;89:3543-53.

Schlemmer CK, Coetzer H, Claassen N, Kruger MC. Oestrogen and essential fatty acid supplementation corrects bone loss due to ovariectomy in the female Sprague Dawley rat. Prostaglandins Leukot Essent Fatty Acids. 1999;61:381-90.

Shen B, Mu JX, Pei FX. Relationship among bone mineral density, collagen composition, and biomechanical properties of callus in the healing of osteoporotic fracture. Chin J Traumatol. 2007;10:360-5.

Shen CL, Dunn DM, Henry JH, Li Y, Watkins BA. Decreased production of inflammatory mediators in human osteoarthritic chondrocytes by conjugated linoleic acids. Lipids. 2004;39:161-6.

Shirouchi B, Nagao K, Inoue N, Ohkubo T, Hibino H, Yanagita T. Effect of dietary omega 3 phosphatidylcholine on obesity-related disorders in obese Otsuka Long-Evans Tokushima fatty rats. J Agric Food Chem. 2007;55:7170-6.

Simopoulos AP. Human requirement for N-3 polyunsaturated fatty acids. Poult Sci. 2000;79:961-70.

Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother. 2002;56:365-79.

Simopoulos AP. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. Biomed Pharmacother. 2006;60:502-7.

Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3–L1 adipocytes. J Biol Chem. 1988;263:9402-8.

Spagnoli A, Hwa V, Horton WA, Lunstrum GP, Roberts CT Jr, Chiarelli F, Torello M, Rosenfeld RG. Antiproliferative effects of insulin-like growth factor-binding protein-3 in mesenchymal chondrogenic cell line RCJ3.1C5.18. relationship to differentiation stage. J Biol Chem. 2001; 276:5533-40.

Sugimoto Y, Tsuboi H, Okuno Y, Tamba S, Tsuchiya S, Tsujimoto G, Ichikawa A. Microarray evaluation of EP4 receptor-mediated prostaglandin E2 suppression of 3T3-L1 adipocyte differentiation. Biochem Biophys Res Commun. 2004;322:911-7.

Summerbell CD, Watts C, Higgins JP, Garrow JS. Randomized controlled trial of novel, simple, and well supervised weight reducing diets in outpatients. BMJ. 1998;317:1487-9.

Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. Bone Miner Res. 2003;18:1206-16.

Taber L, Chiu CH, Whelan J. Assessment of the arachidonic acid content in foods commonly consumed in the American diet. Lipids. 1998;33:1151-7.

Takahashi Y, Ide T. Dietary n-3 fatty acids affect mRNA level of brown adipose tissue uncoupling protein 1, and white adipose tissue leptin and glucose transporter 4 in the rat. Br J Nutr. 2000;84:175-84.

Teegarden D. Calcium intake and reduction in weight or fat mass. J Nutr. 2003;133:S249-S51.

Thompson WG, Holdman NR, Janzow DJ, Slezak JM, Morris KL, Zemel MB. Effects of energy-reduced diets high in dairy products and fiber on weight loss in obese adults. Obes Res. 2005;13:1344-53.

Thorsdottir I, Tomasson H, Gunnarsdottir I, Gisladottir E, Kiely M, Parra MD, Bandarra NM, Schaafsma G, Martinéz JA. Randomized trial of weight-loss-diets for young adults varying in fish and fish oil content. Int J Obes (Lond.). 2007;31:1560-6.

Tsuboi H, Sugimoto Y, Kainoh T, Ichikawa A. Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. Biochem Biophys Res Commun. 2004;322:1066-72.

Uenishi K, Ishida H, Toba Y, Aoe S, Itabashi A, Takada Y. Milk basic protein increases bone mineral density and improves bone metabolism in healthy young women. Osteoporos Int. 2007;18:385-90.

USDA National Nutrient Database for Standard Reference [http://www.nal.usda.gov/fnic/foodcomp/Data/].

van Papendorp DH, Coetzer H, Kruger MC. Biochemical profile of osteoporotic patients on essential fatty acid supplementation. Nutrition Research. 1995;15:325-34.

Wahle KW, Heys SD, Rotondo D. Conjugated linoleic acids: are they beneficial or detrimental to health? Prog Lipid Res. 2004;43:553-87.

Wang YW, Jones PJ. Conjugated linoleic acid and obesity control: efficacy and mechanisms. Int J Obes Relat Metab Disord. 2004;28:941-55.

Watkins BA, Feng S, Strom AK, DeVitt AA, Yu L, Li Y. Conjugated linoleic acids alter the fatty acid composition and physical properties of egg yolk and albumen. J Agric Food Chem. 2003;51:6870-6.

Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Prog Lipid Res. 2001;40:125-48.

Watkins BA, Reinwald S, Li Y, Seifert MF. Protective actions of soy isoflavones and n-3 PUFAs on bone mass in ovariectomized rats. J Nutr Biochem. 2005;16:479-88.

Weber PC, Fischer S, von Schacky C, Lorenz R, Strasser T. The conversion of dietary eicosapentaenoic acid to prostanoids and leukotrienes in man. Prog Lipid Res. 1986; 25:273-6.

Weiler HA, Janzen L, Green K, Grabowski J, Seshia MM, Yuen KC. Percent body fat and bone mass in healthy Canadian females 10 to 19 years of age. Bone. 2000;27:203-7.

Welch AA, Bingham SA, Ive J, Friesen MD, Wareham NJ, Riboli E, Khaw KT. Dietary fish intake and plasma phospholipid n-3 polyunsaturated fatty acid concentrations in men and women in the European Prospective Investigation into Cancer-Norfolk United Kingdom cohort. Am J Clin Nutr. 2006;84:1330-9.

Yakar S, Courtland HW, Clemmons D. IGF-1 and bone: New discoveries from mouse models. J Bone Miner Res. 2010;25:2543-52.

Yanovski JA, Parikh SJ, Yanoff LB, Denkinger BI, Calis KA, Reynolds JC, Sebring NG, McHugh T. Effects of calcium supplementation on body weight and adiposity in overweight and obese adults: a randomized trial. Ann Intern Med. 2009;150:821-9, W145-6.

Yu Y, Correll PH, Vanden Heuvel JP. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta. 2002;1581:89-99.

Zemel MB. Regulation of obesity risk by dietary calcium: Mechanisms and implications. J Am College Nutr. 2002;21:S146-S51.

Zemel MB, Miller SL. Dietary calcium and dairy modulation of adiposity and obesity risk. Nutr Rev. 2004;62:125-31.

Zemel MB, Richards J, Mathis S, Milstead A, Gebhardt L, Silva E. Dairy augmentation of total and central fat loss in obese subjects. Int J Obes. 2005;29:391-7.

Zemel MB, Thompson W, Milstead A, Morris K, Campbell P. Calcium and dairy acceleration of weight and fat loss during energy restriction in obese adults. Obes Res. 2004;12:582-90.

Zemel MB, Shi Hl, Greer B, Dirienzo D, Zemel P. Regulation of adiposity by dietary calcium. FASEB J. 2000;14:1132-8.

BIOGRAPHICAL SKETCH

YOUJIN KIM

EDUCATION

THE FLORIDA STATE UNIVERSITY — Tallahassee, FL
Doctor of Philosophy Candidate (ABD) — 2008-Present
Major: Nutritional Science (Bone Metabolism and Body Composition Laboratory)
Dissertation: Dairy-derived Bioactive α-Linolenic Acid, Conjugated Linoleic Acid, and Calcium as Modulators of ST2 Stromal, MC3T3-L1 Adipocyte-like, and MC3T3-E1 Osteoblast-like Cell Metabolism

THE FLORIDA STATE UNIVERSITY — Tallahassee, FL
Master of Science — April, 2005
Major: Nutritional Science (Molecular Nutrition Laboratory)

EWHA WOMANS UNIVERSITY — Seoul, South Korea
Master of Science — February, 2001
Major: Human Nutrition (Public Health Nutrition Laboratory)
Thesis: Assessment of Nutritional Status and Its Related Factors in Low-Income Elderly Women in South Korea

EWHA WOMANS UNIVERSITY — Seoul, South Korea
Bachelor of Science — February, 1998
Major: Food and Nutrition

TEACHING EXPERIENCE

2010-2011 **Instructor**, Science of Nutrition (HUN1201, 60-80 students/class); Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL

2003-2005 **Teaching Assistant**, Intermediary Metabolism of Nutrient (HUN3224, 60 students/class); Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL

2002-2003 **Teaching Assistant**, Food Laboratory (FOS3206L, 20-25 students/class); Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL

2001-2002 **Teaching Assistant**, Science of Nutrition (HUN1201, 100-150 students/class); Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL

2000-2001 **Teaching Assistant**, Lifestyle and Nutrition (300 students/class); Department of Food and Nutrition, College of Health Sciences, Ewha Womans University, Seoul, South Korea

RESEARCH EXPERIENCE

2009- At present	**Graduate Research Assistant**, Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL
2002-2005	**Graduate Research Assistant**, Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL

WORKING EXPERIENCE

2011	**Research Assistant,** Center on Better Health and Life for Undeserved populations (BHL Center), The Florida State University, Tallahassee, FL
2011	**Reviewer**, Textbook of "Visualizing Nutrition: Everyday Choices - Chapter 5. Lipids & Chapter 14. Feeding the world", the 2nd Edition, Mary Grosvenor and Lori Smolin, Wiley-Blackwell, NJ
2000-2001	**Laboratory Technician**, Department of Bioorganic Chemistry, College of Art and Sciences, Sajong University, Seoul, South Korea
2000	**Reviewer**, "Korean Food Composition Table", the 6th edition, The National Rural Living Science Institute (volunteer), Seoul, South Korea
1998-1999	**Office Administrator**, Department of Food and Nutrition, College of Health Sciences, Ewha Womans University, Seoul, South Korea
1998	**Researcher**, Project of "Assessment of Health and dietary Status of Disabled Adolescents in South Korea" (volunteer)
1998	**Dietetic Internship**, Ewha Womans University Medical Center, Seoul, South Korea

PROFESSIONAL SOCIETIES

American Society for Nutrition (ASN)
Korean Nutrition Society (KNS)

TRAINING AND CERTIFICATES

2011	Environmental Health & Safety Training - Annual Hazardous Waste Awareness Refresher Training, The Florida State University, Tallahassee, FL (Renewal)
2010	Program for Instructional Excellence (PIE) Training, The Florida State University, Tallahassee, FL
2010	Environmental Health & Safety Training - Hazardous Waste Awareness & Introduction to Laboratory Safety/Hazard Communication, The Florida State University, Tallahassee, FL (Renewal)
2010	Environmental Health & Safety Training – Biosafety Level 2 (BSL-2), The Florida State University, Tallahassee, FL
2009	Environmental Health & Safety Training - Hazardous Waste Awareness & Introduction to Laboratory Safety/Hazard Communication, The Florida State University, Tallahassee, FL

2008 Environmental Health & Safety Training - Blood-borne Pathogens, The Florida State University, Tallahassee, FL (Renewal)
2004 Environmental Health & Safety Training - Blood-borne Pathogens, The Florida State University, Tallahassee, FL
2002 Medical Monitoring Program for Vertebrate Animal Exposure, The Florida State University, Tallahassee, FL
2001 Program for Instructional Excellence (PIE) Training, The Florida State University, Tallahassee, FL

- **Certificates**

  Program for Instructional Excellence (PIE) Certificate (2011)
  Certificate in Didactic Program in Dietetics (D.P.D) (2009)
  Laboratory Animal Training Certificate Issued by the FSU Animal Care and Use Committee (2002)
  Registered Dietician in South Korea (License No. 71476) (1998)

HONORS AND AWARDS

2010 Wilson-Sitton Scholarship, College of Human Sciences, The Florida State University, Tallahassee, FL
2010 Reutlinger and Munn Scholarship, College of Human Sciences, The Florida State University, Tallahassee, FL
2009 Reutlinger and Munn Scholarship, College of Human Sciences, The Florida State University, Tallahassee, FL
2009 Research and Creativity Day Presentation Award, College of Human Sciences, The Florida State University, Tallahassee, FL
2009 Graduate Society of Advisory Council (GSAC) Travel Presentation Grant Award, The Florida State University, Tallahassee, FL
2004 Anne Marie Erdman Scholarship, Department of Nutrition, Food & Exercise Sciences, College of Human Sciences, The Florida State University, Tallahassee, FL
2004 Graduate Society of Advisory Council (GSAC) Travel Presentation Grant Award, The Florida State University, Tallahassee, FL
2003 Sigma Xi Grants-in Aid of Research Award, Sigma Xi – the Scientific Research Society, Research Triangle Park, NC
2001 Tyner Scholar Award, College of Human Sciences, The Florida State University, Tallahassee, FL

PUBLICATIONS

Kim, Y. & Ilich, J.Z. Implications of Dietary α-Linolenic Acid in Bone Health. (2011) Nutrition 27:1101-7

Kim, Y., Kelly, O.J., Ilich, J.Z. Dairy-derived Bioactive α-Linolenic Acid, Conjugated Linoleic Acid, and Calcium as Modulators of ST2 Stromal, MC3T3-L1 Adipocyte-like, and MC3T3-E1 Osteoblast-like Cell Metabolism (In Preparation)

Kelly, O.J., **Kim, Y**., Gilman, J., Ilich, J.Z. Modulation of Low-Grade Chronic Inflammation

Associated with Obesity and Osteoporosis by Polyunsaturated Fatty Acids: Evidence for a Connection. (In Preparation)

RESEARCH PRESENTATIONS

Kim, Y., Kelly, O.J., Ilich, J.Z. Role of Alpha-Linolenic Acid (ALA) in the Regulation of Differentiating Preadipocyte-like MC3T3-L1 and Preosteoblast-like MC3T3-E1 Cell Metabolism. (2010) Annual American Society for Bone and Mineral Research (ASBMR) Meeting, Toronto, Canada
► *J Bone Miner Res 25. Available at: http://www.asbmr.org/Meetings/AnnualMeeting/AbstractDetail.aspx?aid=dd614bb5-819b-4400-ae79-25521c12f34d*

Kim, Y., Kelly, O.J., Ilich, J.Z. The Synergistic Effect of Calcium (Ca), Alpha-Linolenic Acid (ALA), and Conjugated-Linoleic Acid (CLA) on Osteoblastogenesis and Adipogenesis. (2010) Annual Experimental Biology Meeting, Anaheim, CA
► *FASEB J, 24:939.11, 2010.*

Kelly, O.J., **Kim, Y.,** Ilich, J.Z. The Effect of Various Concentrations of Long-chain n-3 PUFA on Osteoblast and Adipocyte Proliferation. (2009) Annual American Society for Bone and Mineral Research (ASBMR) Meeting, Denver, CO
► *J Bone Miner Res 24. Available at: http://www.asbmr.org/Meetings/AnnualMeeting/AbstractDetail.aspx?aid=39d262aa-a962-411a-9f01-64f5f3ecaca5*

Kelly, O.J., **Kim, Y.,** Ilich, J.Z. Alpha-Linolenic Acid (ALA) as a Potential Inhibitor of Adipocyte Proliferation. (2009) New Frontiers in Skeletal Research: Bone, Fat and Brain Connections, Bethesda, MD
► *American Society for Bone and Mineral Research, Abstract Book, T70, pg. 54, 2009.*

Kelly, O.J., **Kim, Y.,** Ilich, J.Z. Role of Conjugated Linoleic Acid (CLA) in Osteoblast, Adipocyte and Bone Marrow Stem Cell Proliferation. (2009) New Frontiers in Skeletal Research: Bone, Fat and Brain Connections, Bethesda, MD
► *American Society for Bone and Mineral Research, Abstract Book, M69, pg. 36, 2009.*

Kim, Y., Kelly, O.J., Ilich, J.Z. The Effects of Oil Supplements on the Proliferation of Bone Cells. (2009) Annual Experimental Biology Meeting, New Orleans, LA
► *FASEB J, 23:543.14, 2009.*

Kelly, O.J., **Kim, Y.,** Liu, P., Shin, H., Douglas, C.C., Ilich, J.Z. The Effect of Resveratrol and Flax Oil on MC3T3-L1 Pre-adipocyte, MC3T3-E1 Pre-osteoblast, and ST2 Bone Marrow Stromal Cell Proliferation and Differentiation. (2008) Annual ASBMR Meeting, Montreal, Quebec, Canada
► *J Bone Miner Res 23. Available at: Available at:* http://www.abstractsonline.com/viewer/viewAbstractPrintFriendly.asp?CKey={7494655-6367-4285-873A-13A031D634AB}&SKey={9943B7B4-7685-4FDE-89BD-C21716CE1B77}&MKey={DCB70C83-5B38-431A-B0E1-9221D66718D0}&AKey={D0C01D4F-E23B-45E2-ACD4-0AF8AC866B8B}

Kim, Y., Mistry, A.M. The Orexigenic Effect of Agrp is Mediated by a Gut-related Factor. (2005) Annual Experimental Biology Meeting, San Diego, CA

Kim, Y., Mistry, A.M. Glucocorticoids and Leptin Modulate the Effect of Agrp on Energy Balance. (2004) Annual Experimental Biology Meeting, Washington, DC

Kim, Y., Santillo C, Sathe, S.K., Mistry, A.M. Dietary Almond Lipids Increase Plasma Total HDL, HDL_2, and HDL_3 Cholesterols in Rats. (2004) Annual Experimental Biology Meeting, Washington, DC

Kim, Y., Mistry, A.M., Sathe, S.K. Dietary Almonds Elevate Plasma HDL-cholesterol and Reduce Plasma LDL-cholesterol in Rats. (2003) Annual Experimental Biology Meeting, San Diego, CA