2017

The Effects of Artemisia Derived Natural Products on Adipogenesis

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DOI: 10.25148/etd.FIDC001953

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THE EFFECTS OF *ARTEMISIA* DERIVED NATURAL PRODUCTS ON ADIPOGENESIS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Steven Abood

2017

To: Dean Michael R. Heithaus
College of Arts, Sciences, and Education

This dissertation, written by Steven Abood, and entitled The Effects of *Artemisia* Derived Natural Products on Adipogenesis, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2017

DEDICATION

This dissertation is dedicated with affection to my parents, Dr. Faye Abood and Maurice Abood, who journeyed from a Pennsylvania coal mining town and a Lebanese fishing village to offer their boundless love and support to a son that allowed him to embark on a journey of exploration to the deep reaches of science. You are my best friends and the paragon of compassionate human beings.

ACKNOWLEDGMENTS

A deep thanks to Dr. M. Alejandro Barbieri and Dr. Maria Luisa Veisaga for taking a chance on me and inviting me into your lab and lives. Thank you to all Barbieri Lab members for your help, friendship, humor, and patience.

My appreciation to all my original Committee Members, Dr. Javier Francisco-Ortega, Dr. Juan Liuzzi, Dr. John Makemson, and Dr. Martin Tracey, for your time, suggestions, and guidance. Thanks to Dr. Jennifer Richards for stepping in to offer your time and insight as an expert reader of this dissertation. Thank you Dr. Luiz Lopez and your entire lab for so warmly welcoming me to Mendoza, Argentina and working with me each day on the experiments. Your lab and Mendoza will always have a special place in my heart.

Thank you to all other present and past collaborators and researchers whose work made this dissertation possible.

My appreciation to the entire Florida International University (FIU) Biology Department, the International Center for Tropical Botany, and all other funding sources for providing the support to make my research possible.

Thank you to my entire family, including my grandparents, my brothers Michael and Brandon, their families, and their precious daughters Anna and Ruby, who will inherit and create a world beyond all our imaginings. Thank you again to my parents, Maurice and Faye Abood: through tireless years of work and 4 AM mornings at the bakery, through countless bookstore and museum trips, through your words of encouragement…without this I would not be who I am. I will continue to move forward to honor your sacrifices.

Thank you to all my early scientific mentors and role models, including Buckminister Fuller, who took the time to speak to my class when I was in elementary school, and my science teacher at Palmer Academy who wrote Longfellow's words in my school yearbook that "The heights by great men reached and kept were not attained by sudden flight, but they, while their companions slept, were toiling upward in the night."

Thank you to Mr. Moo H. Kim, for my early training in martial arts and the profound parables that instilled within me the importance of belief in self, hard work, and perseverance in the face of adversity.

Thank you Mr. Steven Alphabet, for your teachings and friendship. Your encouragement, selfless assistance, belief in me, and humor, helped me immensely during times of struggle. Thank you Mr. Alberto Fernandez and the Baragua Judo crew, for your teachings and good humor.

Thank you CJ Strawn for your immense friendship and humor which makes this journey so much more enjoyable. Keep your tan sao up. Thank you to old friends James Harley Bailey, Tim Sheehan, Stewart Hendricks, and all the many others. I wish I could name you all, but you know who you are. A man with good friends is truly blessed, and I am grateful for this blessing and the many others in my life.

ABSTRACT OF THE DISSERTATION

THE EFFECTS OF *ARTEMISIA* DERIVED NATURAL PRODUCTS ON ADIPOGENESIS

by

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Miami, Florida

Professor M. Alejandro Barbieri, Major Professor

For the first time in human history, more people worldwide suffer from obesity than are undernourished. Numerous health complications are associated with obesity including cardiovascular disease, Type 2 Diabetes, cancers of reproductive tissues, stroke, depression, anxiety disorders, and Alzheimer's disease. A deeper understanding of the anti-adipogenic effects and mechanism of action of sesquiterpene lactones may have pharmacological import in the continuing search for therapeutic modalities to ameliorate the effects of this global obesity epidemic.

Dehydroleucodine (DhL), 11,13-dihydro-dehydroleucodine (DH-DhL), and dehydroparashin-B (DhP), sesquiterpene lactones extracted from or derived from compounds extracted from *Artemisia douglasiana*, were investigated for their anti-adipogenic effects on 3T1-L1 preadipocytes.

Dehydroleucodine inhibited the expression of C/EBPα and PPARγ, and also strongly blocked the expression of C/EBPβ, an early stage biomarker of early adipogenesis, in a concentration-dependent manner. Dehydroleucodine arrested the cell cycle at the G_0/G_1 phase, increased p27 and decreased both cyclins A and D and their

partners (e.g., CDK2 and CDK4). Furthermore, DhL downregulated expression of histone demethylase JMJD2 as well as repressed the expression of histone methyltransferase MLL4, which in turn diminished the expression of C/EBPβ and PPARγ, respectively.

11,13-dihydro-dehydroleucodine blocked the accumulation of lipid droplets and inhibited the expression of PPARγ and C/EBPβ. Collectively, the results indicate that the inhibition of early stage preadipocyte differentiation by DH-DhL may be associated with cell cycle arrest at the G_0/G_1 phase.

Dehydroparashin-B significantly decreased the accumulation of lipid content and downregulated the expression of CEBPβ, PPARγ and CEBPα as well as FAS. Interestingly, the addition of DhP inhibited the number as well as the size of the lipid droplets during the differentiation of 3T3-L1 preadipocytes. Taken together, this data suggests that DThP has an important inhibitory effect on cellular pathways regulating adipocyte differentiation.

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LIST OF ABBREVIATIONS AND ACRONYMS

A. absinthium.....*Artemisia absinthium*

A. annua L……*Artemisia annua L*

A. capillaris……....*Artemisia capillaris*

A. douglasiana.....*Artemisia douglasiana*

A. herba-alba……...*Artemisia herba-alba*

A. judaica L……*Artemisia judaica L*

A. maritima ssp. *monogyna*.....*Artemisia maritima* ssp. *monogyna*

A. scoparia Waldst. et Kit…….*Artemisia. scoparia* Waldst. Et Kit

A. tridentata subsp. parishii……..*Artemisia tridentata subspecies parishii*

A. vulgaris……...…… *Artemisia vulgaris*

ACC……..Acetyl-coenzyme A Carboxylase

ACT……Artemisinin Combination Therapy

ACTH…….Adrenocorticotrophic Hormone

Ad-2…….Adenovirus-2

Ad-36…….Adenovirus-36

AML……..Acute Myeloid Leukemia

AMPK……...AMP-activated Protein Kinase

ANOVA……...Analysis of Variance

ANP……...Atrial Natriuretic Peptide

AP-1……Activator Protein 1

aP2……..Adipocyte Fatty Acid-binding Protein 2

ASC-2……Activating signal cointegrator-2

ASCOM……...Activating signal cointegrator-2 Complex

ATGL……..Adipose Triglyceride Lipase

ATP…….Adenosine Triphosphate

ATF5……Activating Transcription Factor 5

B. thetaiotaomicron…….……. *Bacteroides thetaiotaomicron*

BCA Assay……..Bicinchoninic Acid Assay

BDNF……Brain Derived Neurotrophic Factor

BMI……...Body Mass Index

cDNA……..Complementary DNA

C/EBPα……..CCAT/Enhancer Binding Protein α

C/EBPβ……..CCAT/Enhancer Binding Protein β

C/EBPδ……CCAT/Enhancer Binding Protein δ

CDK……...Cyclin-dependent Kinase

Cidea……Cell Death-inducing DFF45-like Effector A

CHOP……...CCAAT/Enhancer-Binding Broein Homologous Protein

Coup-TFII…….....COUP Transcription Factor 2

CREB…….....Cyclic Adenosine Monophosphate Response Element-binding Protein

CRH……..……Corticotropin-releasing Hormone

D2 Receptors……...……Dopamine Subtype 2 Receptors

DDT…….....Dichlorodiphenyltrichloroethane

DH-DhL……...11,13-Dihydro-dehydroleucodine

DHA……Dihydroartemisinin

DhL……...Dehydroleucodine

DhP……..Dehydroparishin-B

DMEM…….Dulbecco's Modified Eagle's Medium

DMSO……Demethyl Sulfoxide

DNA……...Dexyribonucleic Acid

ERα…..……Estogen Receptor Alpha

ERK…….Extracellular Signaling Regulated Kinase

ES Cells…….Embryonic Stem Cells

F1……Filial 1

F2……Filial 2

F3……Filial 3

FAD……Factor for Adipocyte Differentiation Gene

FAS……Fatty Acid Synthase

FGF……Fibroblast Growth Factor

FGF-2……..Basic Fibroblast Growth Factor

FoxO1……Forkhead Box O1

FPP……..Farnesyl Pyrophosphate

FTO……..Fat Mass and Obesity-associated Gene

G_0……...Gap 0 Phase

G_1……...Gap 1 Phase

G_2……...Gap 2 Phase

$G_i\alpha_1$……...GTP binding protein

GAP……...Guanine Triphosphate-Hydrolysis Activating Protein

GAPDH……..Glyceraldehyde 3-phosphate Dehydrogenase

GM……..Growth Media

GR……...Glucocorticoid Receptor

GSH…….……Glutathione

G. verrucosa……...*Gynoxys verrucosa*

H. pylori.....*Helicobacter pylori*

HKMT…….....Histone Methyltransferase

H3K4MT…….Histone H3 Lysine 4 Methyltransferase Complex

HPA Axis…….…….Hypothalamic-Pituitary-Adrenal Axis

HPLC……High Performance Liquid Chromatography

HSL…….Hormone-sensitive Lipase

IBMX……...3-Isobutyl-1-Methylxanthine

ICU…….Intensive Care Unit

IFNα……...….Interferon Alpha

IFNγ……..Interferon Gamma

IGF-1……Insulin-like Growth Factor 1

IL-5……Interleukin-5

IL-10……... Interleukin-10

IL-12……... Interleukin-12

IL-13……Interleukin-13

IM……Induction Media

iNOS……...Inducible Nitric Oxide Synthase

JNK…….c-Jun Amino-terminal Kinase

KLF…….....Kruppel-like Factor

LPL……..Lipoprotein Lipase

L. reuteri……...*Lactobacillus reuteri*

Lys9……..Lysine 9

M. smithii……...…*Methanobrevibacter smithii*

MACP……Mitochondrial Anion Carrier Proteins

MAPK……..Mitogen-activated Protein Kinase

MCE……..Mitotic Clonal Expansion

MEF……...Mouse Embryonic Fibroblasts

MKK6……...Mitogen-activated Protein Kinase Kinase 6

MLL3…….Mixed-lineage Leukemia Protein 3

MLL4…….Mixed-lineage Leukemia Protein 4

MMP-2……..Matrix Metalloprotease-2

mRNA……..Messenger Ribonucleic Acid

MRSA…….Methicillin Resistant *Staphylococcus aureus*

MRSE……Methicillin Resistant *Staphylococcus epidermis*

mTOR……..Mechanistic Target of Rapamycin

NF-$_K$B……..Nuclear Factor Kappa-chain-enhancer of Activated B Cells

NFAT……Nuclear Factor of Activated T Cells

NH_4.....Ammonium

NLK……..Nemo-like Kinase

NMDA Receptor……..*N*-methyl-D-aspartate Receptor

NMR……...Nuclear Magnetic Resonance

NO_3…… Nitrate

Omega-3……Omega-3 Fatty Acid

Omega-6……Omega-6 Fatty Acid

ORO……...Oil Red O

PACAP……...Pituitary Adenylate Cyclase Activating Peptide

PCNA…….Proliferating Cell Nuclear Antigen

PDE3B……Phosphodiesterase-3B

PDK1…..……3-Phosphoinositide-dependent Protein Kinase-1

PE-conjugated……Phycoerythrin-conjugated

PEDF……Pigment Epithelium-derived Factor

Peg10…….Paternally Expressed Gene 10

PI3K……....Class I Phosphoinositide 3-Kinase

PI3K/Akt Pathway……Phosphoinositide 3-kinase/Protein Kinase B Pathway

PKB……Protein Kinase B

PCR……Polymerase Chain Reaction

PTIP……...Pax Transactivation Domain-interacting Protein

PPARγ……....Peroxisome Proliferator Activated Receptor Gamma

PR Diet……Protein Restricted Diet

PREF1……Pre-adipocyte Factor 1

PBS……Phosphate-buffered Saline

rbST……Recombinant Bovine Somatotrophin

RA……Retinoic Acid

Rb……Retinoblastoma Protein

ROS……...Reactive Oxygen Species

RUNX1T1……...Runt-related transcription factor 1

RXR…….9-cis Retinoic Acid Receptor

S phase…….....Synthesis Phase

S. epidermidis……..…….*Staphylococcus epidermidis*

SDS-PAGE…….Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SREBP-1……...Sterol Regulatory Element-binding Transcription Factor 1

SEM……Standard Error of Mean

STAT……...Signal Transducers and Activators of Transcription

TGF1……Transforming Growth Factor 1

TLC……Thin-layer Chromatography

TNF-α…….Tumor Necrosis Factor Alpha

UCP……Mitochondrial Uncoupling Protein

VEGF……Vascular Endothelial Growth Factor

VIP……..Vasoactive Intestinal Peptide

VO_2 max……Maximal Oxygen Consumption

XOR……Xanthine Oxidoreductas

I. INTRODUCTION

1.1. The Epidemiology of Obesity

On the eve of the apocalypse, the German Renaissance painter Albrecht Dürer completed his magnum opus, a woodcut of the four horsemen from the Book of Revelations (Bartrum et al. 2003). It was a work which simultaneously unsettled and resonated with the populace, as the riders, avatars of famine, pestilence, war, and death, gave symbolic representation to the existential fears of both Dürer's generation and those manifested in archetypal events through the history of human evolution. Primary among these fears was that of famine, represented in Dürer's work by the rider of the black horse, who is curiously depicted as robust and healthy, perhaps because he has taken all the prosperity of the land for himself (as the scale he is holding which is tipped in his favor may indicate) (Bartrum et al. 2003). As the world failed to end and the 16th century began, the fears of a starvation seemingly inseparable from the shift to an agricultural society continued as 95 famines ravaged medieval Britain and 75 afflicted medieval France. In the 20th century, a century in which the rider of the black horse claimed 70 million lives, the fear of starvation continued (Bartholomew 2004; Devereux 2000). Famine's rampage was only to subside in recent decades with industrialization and globalization mitigating but not dissipating famine's Malthusian check on exponential population growth (Malthus 1798; Suweisa et al. 2015). Technology may have tamed Famine's reign, but the threat of his re-emergence to savage the fields once again remains in a world of fluctuating grain and synthetic nitrogen markets, climate change, water insecurity, human conflicts, and the fragilities of an increasingly interconnected world food system (Suweisa et al. 2015; Lal 2004).

There is no concomitant existential fear of obesity in the historical record. Even the Christian sin of gluttony, codified in the Seven Deadly Sins by the Desert Fathers of the 3rd century A.D., was subject to opprobrium not because of the physical danger of obesity to oneself, but because of the moral danger to one's soul from selfishly depriving the community of food and subjecting it to starvation by taking more than one's fair share (Aquinas 1981). The phenomena of food sharing is observed in nonhuman primates (de Waal 1989). The opprobium of those who refused to engage in food sharing, may have been a driver of hominid evolution through its reinforcement of a system of mutual obligation (de Waal 1989). The tribalism that both stems from and reinforces this norm, as well as punishes its breach, is linked in folklore to the existential fear of famine, often manifested by an outsider to the group. This outsider is either an avatar of famine itself or its epiphenomena of infanticide, cannibalism, disease and death, such as the wendigo of Algonquin traditions, the fear gorta of Irish mythology, the ghoul of Arabic mythology, the gashadokuro of Japanese mythology, or the zombie of modern pop culture (Yeats 2015; Haddawy 1995; Foster 2015).

And yet, while no obesity monster haunts our collective consciousness, for the first time in human history, more people worldwide suffer from obesity than are undernourished. In a recent study published in *The Lancet* that pooled data from adults in 186 countries, it was reported that there were 641 million obese people worldwide in 2014, compared to 462 million underweight people (Di Cesare et al. 2016). What is remarkable is the rapidity, too fast for a memetic shift, that the transition has occurred from a primarily undernourished to over nourished planetary population.

The *Lancet* study found that the number of obese people worldwide had risen from 105 million in 1975 to 641 million in 2014. In contrast, during this same period, the

number of underweight people rose from 330 million to 462 million. In just four decades, an incredible planetary caloric shift occurred in which the prevalence of underweight *Homo sapiens* transitioned from more than triple that of obese members of the species, to a world in which obese individuals outnumber underweight individuals by 179 million (Di Cesare et al. 2016).

1.1.1. Measuring Obesity

To understand these figures, we must understand how obesity is most commonly measured, and the validity of this measure, as well as its limitations. The most widely used criteria is body mass index (BMI), which is defined as the weight of a person in kilograms divided by the square of their height in meters (Ortega et al. 2016).

BMI is strongly associated with the proportion of body mass that consists of adipose tissue among all races and ethnic groups so far examined (Gallagher et al. 2000). However, since it remains an indirect measure of adipose tissue amount, and instead simply measures a height to weight ratio, certain mesomorphic individuals may erroneously be aggregated as false positives (Ortega et al. 2016). In these cases, BMI will not accurately reflect a concept of obesity where excessive adipose tissue accumulation is the defining metabolic characteristic (Ortega et al. 2016).

There is also some variation in the average amount of adipose tissue at a given BMI between different racial and ethnic groups. For Caucasian populations, classifications include underweight (BMI less than 18.5 kg/m2), normal weight (BMI from 18.5 to 25 kg/m2) and overweight (BMI over 25 kg/m2). Within the overweight category, there is obese (BMI over 30 kg/m2), severely obese (BMI over 35 kg/m2 but less than 40 kg/m2), morbidly or extremely obese (BMI over 40 kg/m2 but less than 50

kg/m2), and super obese (BMI over 50 kg/m2) (Ortega et al. 2016). However, people of Asian descent have higher fat mass, especially visceral fat, for any given BMI (Araneta et al. 2005). Thus, for individuals of Asian descent, BMI is categorized differently: for instance, the overweight category consists of a BMI of 25 to 27.5 kg/m2 (compared to over 25 kg/m2 but less than 30 kg/m2 in Caucasians), and obesity is defined as a BMI of greater than or equal to 27.5 kg/m2 (Araneta et al. 2005). BMI remains a useful but imperfect measure of obesity (Ortega et al. 2016)..

Another measure, abdominal adiposity, a waist circumferential indicator of visceral fat, is associated with the cardiovascular risk, and is a better predictor of insulin sensitivity in men ages 50 to 95 than oxygen consumption (VO_2 max), BMI, or percentage body fat percentage (assayed via dual-energy X-ray absorptiometry or hydrodensitometry) (Racette et al. 2006). Like BMI, abdominal adiposity also exhibits slight categorical variations among different races and ethnicities: for abdominal adiposity, increased cardiovascular risk is defined as greater than or equal to 94 cm in European men, and greater than or equal to 80 cm in European women, but greater than or equal to 90 cm in men and greater than or equal to 80 cm in women, of South Asian, Chinese, and Japanese descent (Racette et al. 2006).

1.1.2. Global Changes in Obesity Rates

Although the aforementioned survey (Di Cesare et al. 2016) and other surveys of global obesity (Ng et. al 2014) may vary in exact estimates, obesity rates have ballooned globally in the last several decades. For instance, in the study conducted by Ng et al., overweight (a BMI of 25kg/m² or higher) and obese individuals rose globally from 857 million in 1980 to 2.1 billion in 2013 (Ng et al. 2014). By most measures, over a third of

the world's population today are either overweight or obese (Di Cesare et al. 2016; Ng et al. 2014).

Morbid obesity continues to climb in economically affluent countries in both adults and children. While lower BMI categories of obesity have slightly slowed their rates of increase, obesity prevalence in developing countries continues to skyrocket. If these trends continue, by 2030 an estimated 58% of the world's adult population will be overweight (38%) or obese (20%) (Di Cesare et al. 2016).

1.1.2.1. Gender and Obesity

Globally, obesity is more prevalent in women than men, but has increased at a greater rate in men. In men, global prevalence of obesity increased from 34 million cases (3.2%) in 1975 to 266 million (10.8%) by 2014. In women, global prevalence of obesity increased from 71 million (6.4%) to 375 million women (14.9%) (Di Cesare et al. 2016).

By 2014, more than twice the number of women than men (126 million versus 58 million) were severely obese (BMI over 35 kg/m^2) (Di Cesare et al. 2016). Additionally, women are disproportionately affected by morbid obesity (BMI over 40 kg/m^2) compared to men regardless of age, race, or ethnicity (Di Cesare et al. 2016).

1.1.2.2. Regional Trends in Obesity Rates

Over half of the world's 671 million obese people live in one of 10 countries: China, the United States, India, Russia, Brazil, Mexico, Egypt, Germany, Pakistan, and Indonesia (Wise 2014).

In 2014, China overtook the United States as the world's leader in absolute numbers of obese people. Nearly a fifth of the world's obese people, 43.2 million men and 46.4 million women, live on Chinese soil. What is striking is the rapidity of the

increase: in less than two decades, between 1993 and 2009, obesity nearly quadrupled in Chinese men (from 3 to 11%), and doubled in Chinese women (from 5 to 10%). For absolute rates of severe obesity, China moved from 60th place in the world for men and 41st place for women in 1975, to 2nd for both men and women in 2014 (Di Cesare et al. 2016).

The United States remains close behind China in absolute numbers of obese individuals, with 41.7 million obese men and 46.1 million obese women. Almost 65% of US adults are overweight or obese (World Health Organization 2015; Ogden et al. 2014; Boyle et al. 2010; Wang and Beydoun 2007). Like China, obesity rates in America rose sharply in a miniscule amount of time: from only 1990 to 2000 for instance, the percentage of obese people in the United States increased from 21 percent to 33 percent, and 66 percent of Americans are now overweight or obese (Ogden et al. 2014). Some estimates postulate that if these trends continue, over 85% of adults in the United States will be overweight or obese by 2030 (Boyle et al. 2010).

Within the United States, rates of obesity in three subpopulations - adults of African ancestry (47.8%), Hispanic ancestry (42.5%), and non-Hispanic Caucasians (32.6%), range from three to five times greater than obesity rates among those of Asian ancestry (10.8%)(Di Cesare et al. 2016). In adults of African ancestry, 56.6% of women are obese compared with 37.1% of men (Ogden et al. 2013).

To the south, in Mexico, obesity rates have increased by15% since 2000, with a full 71.3% of adults now overweight or obese (38.8% overweight and 32.4% obese). Extreme obesity increased by 76.5% between 2000 and 2012 (Wijnhoven et al. 2013).

Across the Atlantic, in Europe, prevalence of obesity varies by region with Eastern Europe, southern Italy, and southern Spain exhibiting higher rates than Western and Northern Europe (Gallus et al. 2015). France exhibits the lowest European obesity rates (4.0 and 6.2% in French men and women, respectively), while the Czech Republic exhibits the highest European rates (30.0 and 32.0% in Czech men and women, respectively) (Gallus et al. 2015).

In Polynesia and Micronesia, 45% of men and 50% of women are obese (Di Cesare et al. 2016). At the other end of the spectrum, South Asia has the highest prevalence of underweight individuals, with 23.4% rates of underweight in men, and 24.0% in women, and obesity prevalence is less than 2% in Burundi, Timor-Leste, and regions of central, east, and west Africa (Di Cesare et al. 2016).

1.1.2.3. Childhood Obesity

In China, between 1993 and 2009, aggregated overweight and obesity rates rose from 6 to 13% in children 6 to 17 years of age (Liang et al. 2012). In the United States, between 2003 and 2007, obesity rates in children increased from 6 to 16% (Singh et al. 2010), and between 2000 and 2014 extreme obesity in children doubled (Di Cesare et al. 2016). The prevalence of overweight amongst children in the United States tripled between 1971 to 2004, increasing from 5% to 15% (Singh et al. 2010). In babies under the age of one in the United States, adipose tissue mass doubled between 1970 and 1990 (Ailhaud and Guesnet 2004).

1.1.3. Morbidity and Mortality of Obesity

1.1.3.1. General Health Effects

Obesity shortens life expectancy by 6.5 years for those with a BMI between 40 and 44.9 kg/m^2, which is more than normal weight participants who currently smoke

cigarettes (compared to those whom have never smoked) (Kitahara et al. 2014). For those with a BMI between 55 and 55.9 kg/m^2, it shortens life expectancy by 13.7 years (Kitahara et al. 2014).

The evidence is overwhelmingly against the concept of benign obesity for the vast majority of health conditions. To borrow a concept from virology, obesity can be viewed as an initial infection that predisposes individuals to a myriad of disease states, or secondary infections. Obesity increases the prevalence of multiple disease states including cardiovascular disease (Freedman et al. 1999), atherosclerosis (Wofford et al.1999), diabetes (Sartipy and Loskutoff 2003), hypertension (Pi-Sunyer 2002), insulin resistance (Sinha et al. 2002), dyslipidemia (Klop et al. 2013), fatty liver disease (Ray et al. 2013), gall bladder disease (Galal 2003), obstructive sleep apnea (Leon-Cabrera et al. 2015), osteoarthritis (Griffin 2008), asthma (Gennuso 1998), and cancer (Lagra et al. 2004) (see **Figure 1** below).

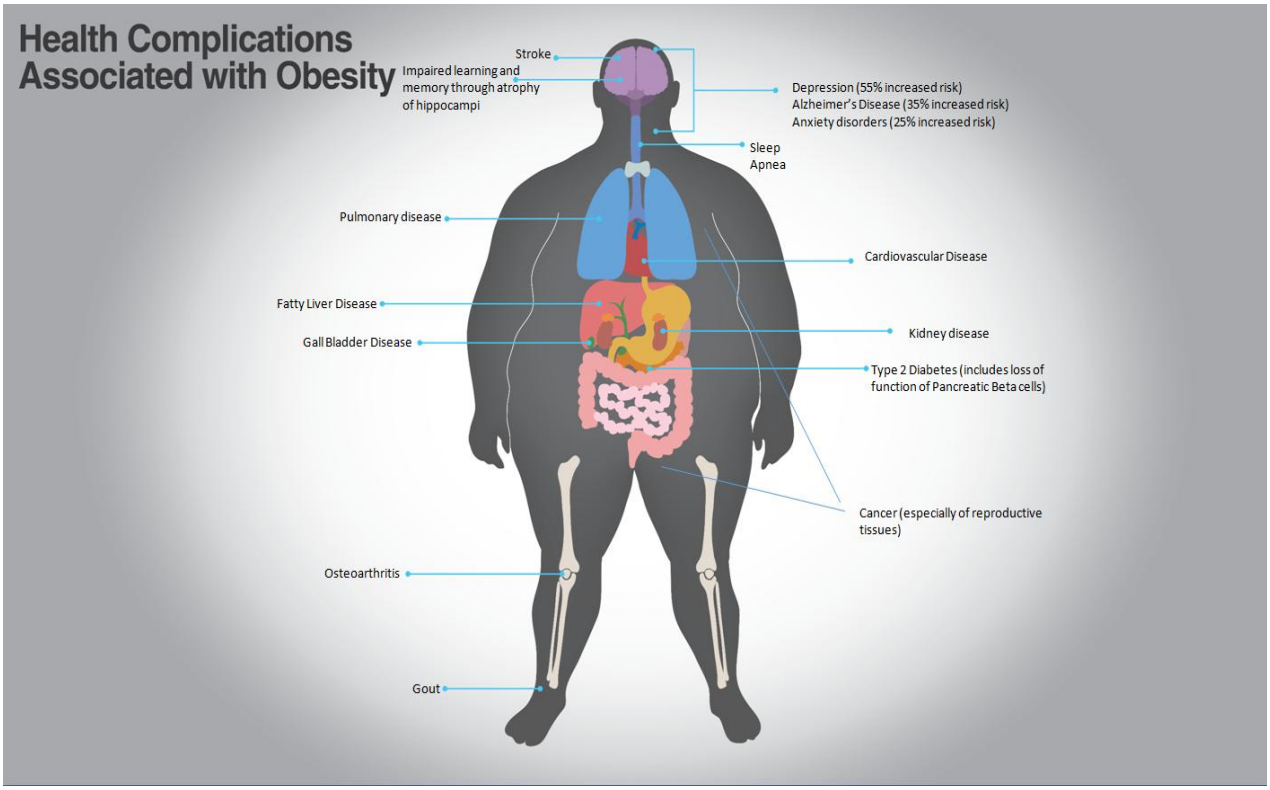

Figure 1. Health complications associated with obesity

1.1.3.2. Cardiovascular Disease

Ischemic heart disease and stroke are the leading causes of death both globally and in the United States (Lozano et al. 2010). Overweight and obesity increases risk for ischemic heart disease, stroke, hypertension, dyslipidemia, and dysglycemia (Reilly and Kelly 2011).

1.1.3.3. Diabetes

Diabetes was estimated to afflict 14% of the adult population in the United States in 2010 (Boyle et al. 2010). Various estimates predict that diabetes prevalence will rise to between 21 and 33% by 2050 (Boyle et al. 2010). Compared with normal weight individuals, being overweight increases the risk of developing type 2 diabetes by a factor

of three. Eighty percent of individuals with diabetes are overweight or obese. Persons with diabetes have 2.1 times the risk of death of those without diabetes (Abdullah et al. 2010).

Additionally, the association between obesity and diabetes may actually be underreported: one study reported that only 39% of the death certificates of individuals with diabetes who died of cardiovascular disease mentioned diabetes (McEwen et al. 2006). Thus, if indicative of wide spread practice, this misclassification could have led to an underreporting of obesity-associated deaths for diabetes.

1.1.3.4. Cancer

Six percent of all new cancers (33,966 in males and 50,535 in females) may be attributable to obesity (Polednak 2008). As discussed in the previous section, obesity is a major risk factor for diabetes. In turn, diabetes is a risk factor for most cancers (Flegal et al. 2007). Additionally, obesity is associated with both increased risk and increased mortality of leukemia and esophageal, colon, pancreatic, postmenopausal breast, endometrial, renal, gallbladder, liver, ovaries, and prostate cancer (Flegal et al. 2007).

1.1.3.5. Diminished Recovery Outcomes

A recent meta-analysis of obesity in trauma care reported that obesity was associated with a 45% increased risk of mortality (Liu et al. 2013). Additionally, an obesogenic BMI doubles the risk of major complications during trauma care (Liu et al. 2013). These poorer recovery outcomes stem from higher risk of influenza, pneumonia, bacteremia, and sepsis in obese patients (Huttunen and Syrjanen 2013).

1.1.3.6. Enhanced Pregnancy Risks

A 2011 meta-analysis reported that overweight and obese women had significantly higher miscarriage rates compared with women with a normal BMI (Rittenberg et al. 2011). In addition, obese women have overall lower pregnancy and live birth rates, compared to women with a BMI in the normal range (Rittenberg et al. 2011).

1.1.3.7. Mental Health

A meta-analysis of the correlation between obesity and depression concluded that obese persons have a 55% increased risk of developing depression over time (Luppino et al. 2010). Obesity is also associated with an increased 25% risk of developing an anxiety disorder (Simon et al. 2006).

Individuals who are overweight in midlife have a 26% increased risk of developing some form of dementia, a 33% increased risk of vascular dementia, and a 35% increased risk of Alzheimer's disease (Anstey et al. 2011).

Obese older adults exhibit higher rates of atrophy in their frontal lobes, anterior cingulate gyrus, hippocampus, and thalamus (Driscoll et al. 2012). A prospective cohort study that followed a group of normal weight and highly active individuals, and a group of overweight and inactive individuals, over the course of seven years, found that the overweight individuals presented a nearly 40% increased risk of poor mental functioning (Lindholm et al. 2013).

1.1.3.8. Health Effects of Childhood Obesity

Unfortunately, obesity in childhood either results in the development of many of these pathological conditions or leads to increased risk for them later in life. Obesity in childhood or adolescence is associated with twofold or higher risk of adult hypertension,

diabetes, asthma, coronary heart disease, and stroke (Sinha et al. 2002; Freedman et al. 1999). In addition, obesity in children and adolescents is associated with smaller orbitofrontal cortex gray matter volume, with concomitant impairment of certain domains of executive function, such as inhibitory control (Barkin et al. 2013).

1.1.4. Economic and Other Costs of Obesity

It is estimated that obesity and obesity-related conditions cost $190 billion dollars per year of additional healthcare spending, or 21% of total U.S. healthcare expenditures. Obese men are estimated to incur an additional $1,152 per year in healthcare costs, and obese women incur an additional $3,613 per year (Cawley and Meyerhoefer 2012).

Obesity also takes an economic toll on employers through the cost of absenteeism. Obesity-related absenteeism is estimated to cost employers between $3.38 billion ($79 per obese individual) and $6.38 billion ($132 per obese individual) each year in the United States (Trogdon et al. 2008).

In terms of maintaining the national defense, in 2008 it was reported that 5.7 million men and 16.5 million women who would otherwise have been eligible for military service under the U.S. Army's current active duty enlistment standards, were disqualified for exceeding weight and body fat requirements (Cawley et al. 2012). This is a dramatic shift from a century earlier, in 1901, when under nutrition and malnutrition caused one third of recruits to be either too short (under the 5 foot minimum) or too weak for military service (Cawley et al. 2012).

1.2. The Evolution of Obesity: How we got Fat

1.2.1. The Mismatch Paradigm Part 1: Primordial Food Scarcity and Caloric Expenditure

It is evident from this litany of epidemiological data that a rapid global increase in the accumulation of adipose tissue in *Homo sapiens* has occurred. Sub-Saharan Africa now remains the only region in the world where obesity is not common (Haslam and James 2005). It is also evident that this transformation from an undernourished to overnourished planet has occurred far too quickly to implicate a primarily genome-wide mechanism of change. Why the global increase in such a short time span? The short time span that led to the creation of an obese planet points towards a mismatch between our modern environment and the primordial environment in which our obesogenic genes developed as a leading explanatory model for the obesity epidemic (Philipson and Posner 2008). Environments of either food scarcity or surplus could have led to different strategic behaviors of feeding and movement to fulfill the fundamental drive of hunger and provide sufficient energy to maintain homeostatic and allostatic conditions (Eaton and Eaton 2003). A primordial environment of food scarcity could have selected for a genome biased towards adipose tissue storage (Neel 1962). If so, our obesogenic genome is therefore mismatched with the modern environment of food surplus, and the decrease in movement and caloric expenditure required to obtain this food. This basic mechanism could explain the rapid shift from a negative to positive energy balance and the resulting global incidence of excess adipose tissue storage.

One historical example of a mismatch paradigm that may illustrate the general plausibility of an environmental shift as a mechanistic construct for pathologies concerns

a shift from a vitamin C rich environment to a vitamin C deficient environment. It is hypothesized that primates, fruit bats, and guinea pigs once had the ability to synthesize vitamin C in the liver, as many organisms today can. However, as hinted at by the vitamin C rich diets of modern apes and monkeys, the primordial environment was likely rich in vitamin C and the ability to synthesize vitamin C in the liver was unessential and eventually lost (see **Figure 2** below). This new physiological construct, like the thrifty genotypic and phenotypic proclivity towards adipose tissue storage, only became maladaptive when the environment shifted: in this case, to an environment of vitamin C scarcity on sailing ships in the 1600s and 1700s resulting in the pathology of scurvy, which is characterized by vitamin C deficiency (Milton 1999).

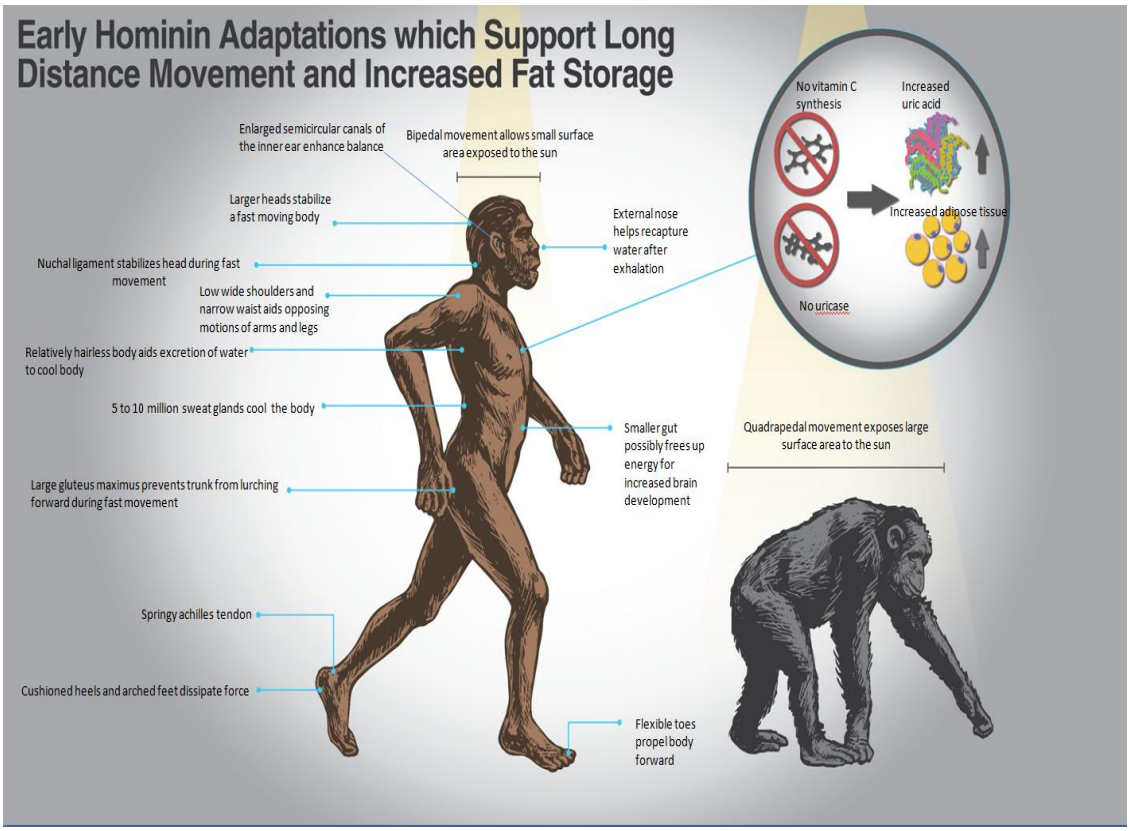

Figure 2. Early hominin adaptations which support long distance movement and increased fat storage

Why the need in *Homo sapiens* for a genotype that would support a pro-adipogenic phenotype in the first place? Why the hypothesized shift from a pregenus *Homo* diet that was likely vegetarian to one where *Homo sapiens*, like modern day chimpanzees, bonobos, and baboons, were motivated to seek out high energy density animal calories? (Milton 1999; Bunn 1981).

The selection for and maintenance of a larger body size that could support more adipose tissue may have allowed the genus *Homo* to metabolically rely on both these adipose reserves and low caloric food such as plant underground storage organs (Laden and Wrangham 2005). Such low caloric food would return too low a rate of energy for a smaller animal that could not rely on fat stores while entering into the caloric deficit necessary to seek out such foods (Laden and Wrangham 2005).

The need to rely on adipose tissue stores to survive in a food-scarce environment is a central explanatory tenet for the development of an obesogenic phenotype (Aiello and Wheeler 1995). However, a corollary hypothesis is that an increase in high energy density food acquisition, and a concomitant adipose tissue-storing thrifty phenotype, were the costs of the development of larger brains (Aiello and Wheeler 1995). The *Homo sapien* brain, a high-fat organ of approximately 33% lipid content, accounts for roughly 2% of a person's body weight, yet demands at least 20% of the body's energy consumption (Magistretti and Allaman 2015). So, to maintain the energy requirements of a larger brain necessary for a cognitive processing capacity that supports survival, especially in times of food scarcity, a genotype that favored the storage of adipose tissue reserves would be selected for (Aiello and Wheeler 1995).

In addition to maintaining the normal high metabolic energy requirements of

larger brains, a pro-adipogenic genotype and the motivational circuits to seek out higher energy density foods may have been necessary for infant brain development (Wang et al. 2002). Since the pelvic girths of species in the genus *Homo* limit the birth size of the infant brain to approximately 250 to 300 cubic centimeters, most of human brain growth occurs after birth supported by energy obtained from lactation (Martin 1981). Since the fatty acid composition of milk is affected by dietary fatty acid intake (Milligan et al. 2008), it is possible that through a kin selection mechanism through which their offspring would benefit, females were selected for with enhanced motivational circuits to seek out and store adipogenic food sources. Human babies are among the fattest of all mammal babies (Kuzawa 1998).

Why the selective pressure for larger brains? One theory postulates that in a virtuous cycle, larger brains were selected for because although they required increased energy consumption, the increased cognitive capacity increased success in acquiring high energy density foods (Aiello and Wheeler 1995). This increase in available energy consumption and the advent of cooking which decreased the energy consumption required by the gut, continued to make more energy available for the development and maintenance of larger brains (see **Figure 2** above) (Wrangham and Conklin-Brittain 2003).

This expensive tissue hypothesis of brain development postulated a shift from continuous feeding on low energy density foods (scavenger gatherers) to intermittent high energy density foods which facilitated the development of larger brains (with increased energy expenditure and metabolic demands). This led to increased success in obtaining high density energy foods through cognitively complex activities such as

hunting and the advent of cooking (Aiello and Wheeler 1995). High density foods and increased cooking allowed for more easily digestible nutrients, which may have led to a smaller gut with less metabolic requirements, saving nutrients for the development of an even larger brain in a virtuous cycle (Aiello and Wheeler 1995). Behind and resulting from this increased brain development most likely was the establishment of a motivational reward circuit to fuel the sustained search for high density foods (see **Figure 2** above) (Wang et al. 2002). The crux of the mismatch paradigm is the transition from a niche of food scarcity where this reward circuit was highly sensitive to the pursuit and acquisition of high energy density foods to the modern environment of food surplus (Power 2012).

The glycemic index is a measure of the increase in blood glucose concentration following ingestion of a food. The glycemic index of the majority of foods in this early environment was likely low, as simple sugars may have been available only seasonally from fruit or episodically as when honey from a bee hive was located (Aiello and Wheeler 1995).

How was high energy density food obtained? Through the movement required to track game, movement which required the development and maintenance of cognitive modules which increased focus, memory, and the complex thinking necessary to communicate and hunt in groups (Shafia et al. 2017). Just as factors stimulated by movement such as an increase in brain derived neurotrophic factor (BDNF) helped stimulate the growth and maintenance of hippocampal neurons necessary to support the memory needed to become better hunters, the dopaminergic motivational pleasure circuit emanating from the ventral tegmental area to the nucleus accumbens likely was co-opted

to motivate movement over vast distances to find this food (Wang et al. 2002). There may be utility in conceiving of obesity related pathologies not as the introductions of harmful vectors (such as one would think of infectious agents such as viruses, bacteria or prions) but as the removal of an evolutionary strategy that positively supported both physical and mental health as a side effect of the physiological needs selected for to optimally acquire scarce foods.

The calorie expending movement to obtain high energy density foods in this early environment may have consisted of persistence hunting, where the bipedal *Homo sapien* hunter, although slower than the prey sought over short distances, would exhaust it by tracking it through alternating periods of running and walking (Liebenberg 2008). A relatively hairless body which enhanced the ability to thermoregulate through sweating, the ability to carry water, and the ability to carry and throw projectile weapons, all likely aided this endurance based strategy (Liebenberg 2008) (see **Figure 2** above). Persistence hunting is still utilized by the San people in the central Kalahari Desert in Southern Africa and by the Raramuri people of Northwestern Mexico (McDougall 2011).

1.2.2. The Mismatch Paradigm Part 2: Modern Food Surplus and Movement Deficit

1.2.2.1. Food Ubiquity, Ease of Acquisition and Altered Composition

The ability of modern *Homo sapiens* to instantly conjure up high energy density food by reaching in his/her pocket and tapping his/her fingers a few times on a small rectangular object to order a round, caloric rich delicacy directly to him, would befuddle his primitive ancestor. The dopaminergic fueled motivational hunger drive is no longer linked to movement. The unconditioned response of movement (that the unconditioned stimuli of hunger formerly triggered) is now replaced with new conditioned behavioral

responses that use technological mechanisms to obtain food without caloric expenditure (see **Figure 3** below).

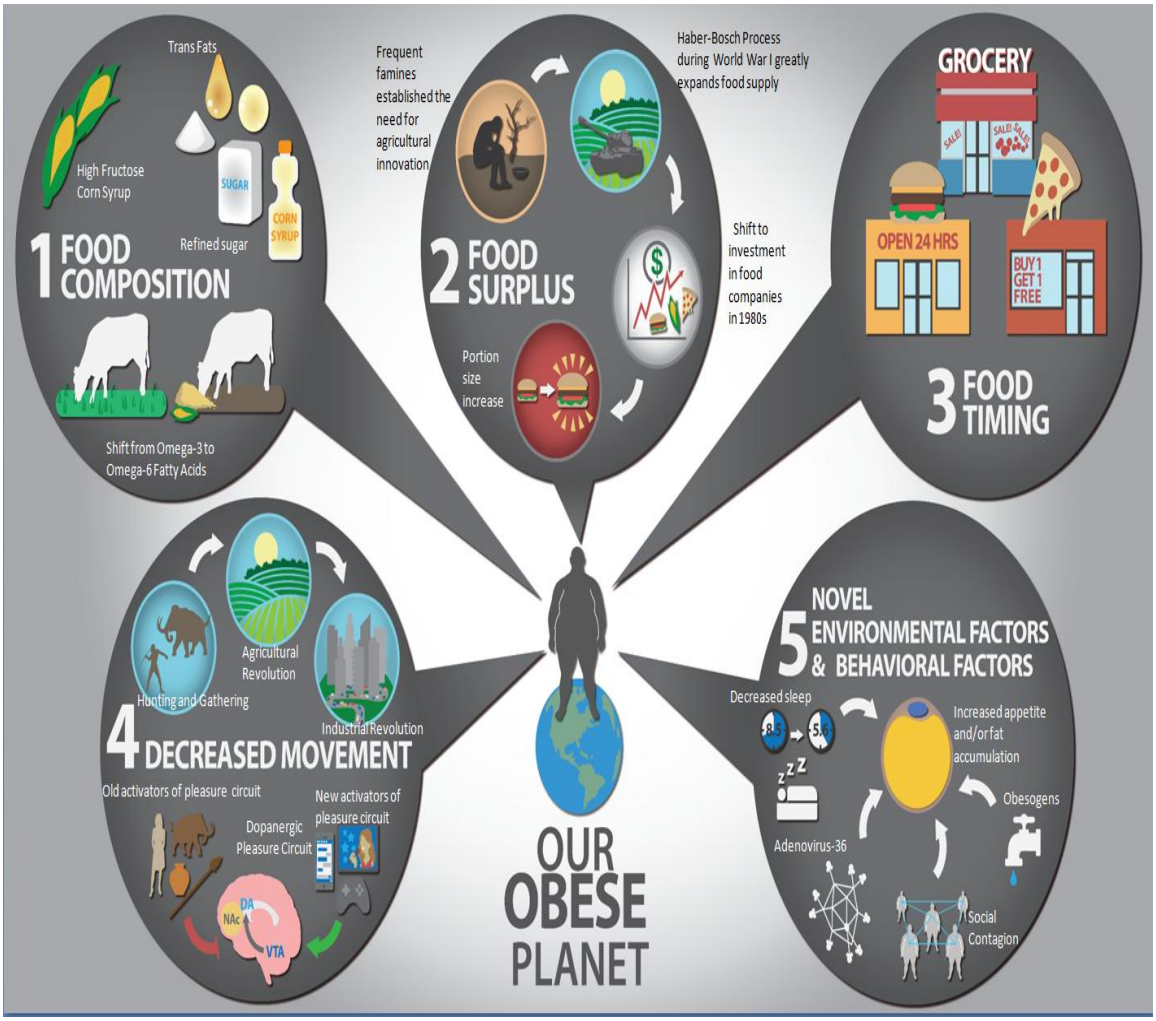

Figure 3. Our obese planet

The obesogenic results of food delivery without the caloric costs of acquiring prey and evading predators is observed in the weight gain of non-human animals taken from their natural habitats into zoological parks, homes, and labs, where food is ubiquitous and divorced from effort (Klimentidis et al. 2011; Power et al. 2001).

How did a planet historically ravaged by famine manage to transition to a food ubiquitous one? The discovery of an artificial nitrogen fixation process, the Haber-Bosch

process, by German chemists during World War I, was indispensible in the production of crops that increased the capacity of the world's food supply and resulted in a population explosion where in a little over a century, *Homo sapiens* multiplied from 1.6 billion in 1900 to over 7.4 billion today (Smil 1999). Through a process which converts atmospheric nitrogen (N_2) to ammonia (NH_3) by a reaction with hydrogen (H_2) using a metal catalyst under high temperatures and pressures ($N_2 + 3H_2 \rightarrow 2NH_3$), nitrogen is dissociated, and then bonded to hydrogen or oxygen to form inorganic compounds, primarily ammonium (NH_4) and nitrate (NO_3) (Vojvodic et al. 2014). The amount of nitrogen extracted from the atmosphere by the Haber-Bosch process applied to crops during the decade from 1980 to 1990 is greater than that from all industrial fertilizer applied previously in human history. It now produces more than 450 million tons of nitrogen fertilizer per year, and nearly 80% of the nitrogen found in human tissues originated from the Haber-Bosch process. In combination with pesticides, the Haber-Bosch process has quadrupled the productivity of agricultural land (Smil 1999; Erisman et al. 2008).

Food ubiquity has also emerged globally partly because of economic trends such as a shift in the early 1980s by investors from blue chip companies to food manufacturers and distributors. This shift pressured these entities to generate short term profits by increasing output and consumption through larger portion sizes, aggressive marketing, and value added strategies such as increased food processing and the addition of sugar, salt, and fat. The cost of fat and sugar are traditionally low cost commodities (Drewnowski 2000; Drewnowski, 2007) and this low cost, as well as their ability to activate the dopaminergic motivational circuits to crave high energy density foods,

facilitates their utilization to increase perceived value. Due to the mathematical pressures of fusion and first mover advantages, increasingly oligarchic and monopolistic food conglomerates have emerged which have repeatedly selected these value-added strategies to increase market share. For instance, over 20 cents of every food dollar spent in the U.S. is spent at Walmart, and more than half US grocery market is controlled by only six retailers (Moss 2013). These economic forces favor the widespread addition of adipogenic food compositions such as sugar and fat (Moss 2013).

As a result of these forces, the United States food supply (total food produced, less exports, plus imports) now provides 500 kcal/day per capita more than in the 1970s.

This increased food availability is matched by increased consumption. Consumption of refined sugar has skyrocketed. Whereas our genes developed in a Paleolithic environment where one person likely consumed at most 2 kilograms (4 pounds) of honey a year, human sugar consumption rose to 5 kilograms (11 pounds) a year in 1830, and 70 kilograms (150 pounds) by the year 2000 (Cordain et al. 2005).

Food consumption of the high energy density foods represented 18% of the calories consumed per capita in the United States in 1977 but 27.7% of total caloric intake in 1996 (Nielsen and Popkin 2003). Between 1977 and 2002, consumption of pizza in the United States increased by 425%, and bread is now the number one source of carbohydrates in children (Subar et al. 1998).

A shift in cereal composition occurred in 1949 when Post became the first brand to add a sugary coating to their product called Sugar Crisp. Kellogg and General Mills followed suit, and sugary cereals became the norm, replacing breakfasts traditionally cooked by mothers who increasingly joined the workforce (Moss 2013). Cereal sales

rapidly increased from $660 million in 1970 to $4.4 billion by the mid-1980s, and companies aggressively marketed to promote the norm of highly processed convenience-based meals as opposed to traditional home-cooked meals (Moss 2013).

During World War II, Coca-Cola President Robert Woodruff implemented a policy that all American soldiers, regardless of where they were stationed could purchase Coke for the discounted rate of five cents a bottle, thus establishing a post-war time habit of sugary drink consumption in a generation of returning servicemen (Moss 2013). From 1970 to 1997, United States consumption of sugary soda had doubled to 54 gallons a year (Isganaitis and Lustig 2005). From 1977 to 2002, consumption of soda in children increased by 70% (Isganaitis and Lustig 2005). Soft drinks are the second leading source of carbohydrates in children (Subar et al, 1998).

Government subsidies and policies also affect food ubiquity. For instance, since the 1930s the United States government has subsidized dairy farmers (Blayney 2002). The extracted milkfat is utilized for cheese and pseudo-cheese food products. In less than four decades Americans tripled their consumption of cheese, eating on average 11 pounds of cheese a year in 1970, but 33 pounds a year by 2007 (Moss 2013).

The shift in consumption quantity matches a shift in consumption composition. It is estimated that 56 percent of our calories come from three sources that were absent when our genes were developing: refined, sugars, bleached flour, and vegetable oils (see **Figure 3** above) (Cordain et al. 2005).

The relative composition of omega-3 and omega-6 fatty acids in *Homo sapien* food sources has recently and dramatically altered. Prior to the 1950s, cows were raised in pastures and ate spring grass, a rich source of omega-3 fatty acids, which then became

concentrated in dairy products consumed by humans. However, in the 1950s, demand for dairy products and beef increased, reducing grazing areas, and pastures were replaced by battery farming which utilized corn, soy, and wheat to feed cattle, all rich in omega-6s (Weill et. al 2002; Ailhaud and Guesnet 2004). While omega-3s and omega-6s are optimally balanced in the human body in a 1 to 1 ratio, milk from corn- and soy-fed cows resulted in an imbalance in this ratio anywhere from 1:15 to 1:40 in favor of omega-6s (Weill et al. 2002). Similarly, eggs from chickens raised on grass retained a 1:1 ratio, as opposed to corn fed chickens whose eggs exhibited a 1:20 ratio in favor of omega-6s (Simopoulos and Salem 1989). Additionally, over the last 100 years, Americans have undergone an approximately thousand-fold increase in the amount of soybean in their diet, a source of omega-6s (Blasbalg et al. 2011). Omega-3 fatty acids inhibit adipogenesis and inhibit inflammation (Ailhaud and Guesnet 2004), and omega-6 fatty acids promote adipogenesis (see **Figure 3** above), as well as coagulation and inflammation (Weill et. al 2002; Ailhaud and Guesnet 2004). Since the fatty acid composition of mother's milk is determined by diet, an increase in the mother's diet of adipogenic omega-6s likely led to the doubling of obesity in children under one between 1970 and 1990 (Jensen et al. 1978; Ailhaud and Guesnet 2004; Ailhaud et al. 2006).

Trans fatty acids, omega-6 oils altered to become solid at room temperature to increase shelf life, are more inflammatory than regular omega-6s. Additionally, in a cohort of 19,934 women, it was found that those with high serum levels of phospholipids *trans*-palmitoleic and elaidic acids had a risk of breast cancer that was increased 50 percent to twofold in comparison with women with low serum levels (Chajes et al. 2008).

Apart from fatty acid composition, dairy products have also been altered since the 1993 FDA approval of recombinant bovine somatotrophin (rbST), which increases yields in cows by 10 to 20 percent. rbST increases bovine IGF-1, and high levels of IGF-1 has been linked to increased risk of prostate cancer in men and breast cancer in premenopausal women (Renehan et al. 2004). It is unclear whether any significant amount of bovine IGF-1 is absorbed in the human bloodstream (Renehan et al. 2004).

Another novel dietary component, high fructose corn syrup, emerged in the 1970s, when rising prices of cane and beets, the two main sources from which sugar was derived, triggered the search for an alternative. Economically cheap to produce due to government subsidies on corn, the liquid nature of high-fructose corn syrup enabled it to be easily added to both food and drinks (Moss 2013). Consumption of sugar-sweetened soda doubled between 1970 and 2005, and Americans now consume 40 gallons of sugary soda a year and 71 pounds of caloric sweeteners on average each year (22 teaspoons of sugar, per person, per day) (Moss 2013). In less than two decades, from 1978 to 1995, consumption of high-fructose corn syrup in the United States increased from 80 to 132 kcal/day per person (see **Figure 3** above) (Johnson et al. 2009). Fructose ingestion produces smaller increases in circulating satiety hormones compared with glucose ingestion (Page et al. 2013). Unlike glucose ingestion, fructose ingestion does not result in deactivation of the striatum, which occurs when initially hungry individuals reach satiety (Page et al. 2013; Small et al. 2001). Additionally, the hippocampus, the activation of which consolidates memories and also promotes satiety, showed reduced activity upon ingestion of fructose (Page et al. 2013; Volkow et al. 2011; Horne et al. 2010).

1.2.2.2. Change in Eating Norms

Between 1972 and 1995 the United States population increased by barely a third, but the number of restaurants nearly doubled and the number of fast food restaurants nearly tripled. During this time period, the percent of calories contributed to our diets by eating outside of the home increased from 18% to 34%. (Bowman et al. 2004; Bowman and Vinyard 2004).

This shift towards eating outside of the home was accompanied by an increase in portion sizes: an increase in daily energy intake of 179 kcal a day by United States children and adolescents, was associated with an increase from 23.4% to 33.9% of calories eaten outside the home (see **Figure 3** above) (Piernas and Popkin 2011).

A meta-analysis of 72 studies demonstrated that people consistently eat more food when offered larger portion sizes (Hollands et al. 2015). Additionally, those offered larger portion sizes do not compensate by eating less during a subsequent meal (Wansink and Park 1996; Rolls et al. 2004).

1.2.2.3. Movement Deficit

It has been estimated that our energy expenditure per unit of body mass is at less than 38 percent of that of our Paleolithic ancestors (Cordain et al. 1998). As would appear obvious, sedentary behaviors are independently and longitudinally associated with overweight and obesity (see **Figure 3** above) (Su et al. 2017).

Another momentous population shift has accompanied the transition from a famished to an obese planet: in 2009, for the first time in human history, the number of people living in urban areas (3.42 billion) surpassed the number living in rural areas (3.41 billion). This shift in activity is exacerbated by the exodus from rural to urban

communities, where individuals are far more likely to engage in occupations divorced from physical exertion (Popkin, 2001; Prentice, 2005).

This migration was accompanied by the development of transportation technology and infrastructure which increasingly made human exertion less necessary.

From1980 to 1990, widely publicized kidnapping incidents in the United States decreased the numbers of children who walked to school from over 80% to less than 33%.

The increasing sophistication of video game technology and the birth of social media and mobile electronic devices served as technological distracters of our attention. While we once satisfied dopaminergic motivational circuits through movement to find food and physical games which likely first originated to hone group hunting skills, we now receive dopaminergic stimulation through awards obtained by electronic avatars who move through vast worlds and realms, while we sit in stasis, moving only our fingers.

In 2016 a rare instance of entertainment technology increasing movement occurred that may be a harbinger of a norm of augmented and virtual reality exercise based games in the future. The augmented reality game, called Pokemon Go, increased the activity level of participants by 1473 steps a day on average, a more than 25% increase compared with prior activity level. In only 30 days, the game added a total of 144 billion steps to US physical activity levels (Althoff et al. 2016). Fittingly, the game's objective was to track and hunt virtual creatures, a calorie consuming activity more than a little reminiscent of the persistence hunting of days long past.

1.2.2.4. Overactivation of the Hypothalamic-Pituitary-Adrenal Axis

Because of cognitive modules that allow us to imagine far into the future and ruminate into the past, *Homo sapiens* now experience chronic activation of the hypothalamic-pituitary-adrenal (HPA axis) threat response system to nonphysical threats that do not engender physical defensive responses. Like our ancestors, cortisol is released which stimulates lipid and carbohydrate metabolism and leads to increased blood glucose levels, insulin, neuropeptide Y, and CRH release, which stimulate increased appetite. In the environment in which our genes evolved, this made a kind of elegant evolutionary sense: a threat engendered a physical response which triggered the hunger mechanism in a feedback loop to replace the calories expended from the physical response. However, when we react to what we perceive as stressful situations in modernity which do not physically challenge us by activating our threat response system, we trigger a hunger reflex without the accompanying caloric expenditure. The result is a tendency towards a positive energy balance and increased adipose tissue accumulation (see **Figure 3** above). In Cushing's Syndrome, the same chronic activation of the HPA axis is observed, and obesity is the most common resulting symptom.

1.2.3. The Mismatch Paradigm Part 3: Other Changed Conditions of our Modern Environment

1.2.3.1. Sleep

In 1960, sleep duration of adults in the United States averaged 8.5 hours. However, by 2008, adults were averaging 6.8 hours of sleep a night with one in three adults routinely sleeping fewer than 6 hours a night (Kripke et al. 1979; Swanson et al. 2011). Sleep restriction results in physiological dysfunctions, including those associated with obesity, such as decreased glucose tolerance, decreased insulin sensitivity, increased

evening concentrations of cortisol, increased levels of ghrelin, decreased levels of leptin and increased hunger and appetite. Sleeping less than 6 hours per night doubles the risk of developing type 2 diabetes in men (Yaggi et al. 2006).

Normally, leptin, a satiety hormone secreted by adipocytes, is mainly dependent on caloric intake, exhibits a morning minimum and increasing levels throughout the day culminating in a nocturnal maximum. Ghrelin, a hunger inducing hormone release primarily from stomach cells, is also higher during the night, but decreases during the second part of the night suggesting an inhibitory effect of sleep on hunger. During this second part of the night, leptin levels reach their maximum, also acting to inhibit hunger.

The normal circadian profile of cortisol consists of an early morning maximum, declining levels throughout the day, a minimal level during the first part of the night, and an abrupt rise during the later part of the night.

In a study of where participants were deprived of only two nights of full sleep (4 hours total sleep each night), the sleep deprived group exhibited a 18% decrease of leptin levels and a 28% of ghrelin levels compared to a non-sleep deprived group (10 hours sleep per night) (Spiegel et al. 2004; Taheri et al. 2004). Growth hormone was also elevated during waking which could have an adverse impact on glucose metabolism. Sleep loss also altered pancreatic beta cell secretion so that the cells did not respond to insulin resistance with increased insulin secretion (Schmid et al. 2007). Subjects experienced a 24% increase in hunger with a 32% increase in appetite for carbohydrates (see **Figure 3** above) (the nutrient most affected) (Spiegel et al. 2004). Reduced sleep quality, without change in sleep duration, can also have adverse effects on glucose metabolism (Tasali et al. 2008).

1.2.3.2. Social Networks

Obesity exhibits a pattern of contagion through social networks: the chance of becoming obese increases by 57% if a person has a friend who becomes obese, by 37% if he or she has an obese spouse, and 40% if a person has an obese sibling (see **Figure 3** above) (Christakis and Fowler 2007). Independent of geographical distance, up to three degrees of separation, individuals with obese friends are more likely to become obese than would be predicted by chance alone. Mechanistically this phenomena may be due to behavioral imitation (consciously or through the mirror neuronal system), or through the shifting of norms (Christakis and Fowler 2007).

Is this propensity for obesity to spread through social networks augmented by technology that serves as a memory prosthetic to increase social group size? It's hypothesized that primate brain size correlates with long term memory capacity and that this serves as an upper limit on the size of a social group that a particular species can comfortably maintain (Dunbar 1992). Dunbar's number postulates this number to be approximately 150 for *Homo sapien*s (Dunbar 1992), while the Bernard Killworth estimate is 231 (Bernard et al. 1987). If the significant cognitive module is long term memory capacity, then memory prosthetics such as social media services should augment the number and thus exacerbate the three degrees of separation effect by increasing the overall size of the network. Preliminary research has not demonstrated such a group size extension (Goncalves et al. 2011), but it seems plausible that memory prosthetic technology that current in widespread use such as online social networks, as well as emerging technology such as brain-computer interfaces will increase interconnectivity and make individuals more susceptible to norms, including obesogenic ones.

The combustible mixture of modern feeding trends combined with a more sedentary world, and the technological and social forces that cement these two primary drivers together, are spreading to the developing world. Currently, 62% of the world's obese population live in developing countries (Ng et al. 2014).

1.2.4. How Environmental Mismatch Affects Health

1.2.4.1. Hypothesized Thrifty Gene Categories

What functions could genes in this hypothesized thrifty genotype affect? Bouchard suggested five categories of thrifty genes: those associated with appetite, metabolism or thermogenesis, predisposition for physical activity, adipocyte lipid storage capacity, and lipid oxidation rate (Bouchard 2007). Additionally, genes that are associated with adult BMI have been linked with infant growth, pubertal timing and insulin metabolism (Elks et al. 2010, Hattersley et al. 1998). These genetic transitions are not detrimental to health in isolation, but as the mismatch theory postulates, when combined with our modern day environmental niche, increase risk for a multitude of pathogenic conditions.

1.2.4.2. Physical Conditions: Diabetes, Cancer, Surgical Risk

Tumor metabolism is dependent on glucose consumption: rapid consumption of glucose is characteristic of multiple kinds of neoplasms (Coller 2014). In fact, excess glucose consumption is so indicative of tumors, that primary and metastatic lesions can be identified with a specificity and sensitivity near 90% by using the imaging agent 2-[18F]fluoro-2-deoxy-d-glucose, coupled with positron emission tomography (PET) (Czernin and Phelps 2002).

The global increase in consumption of high glycemic index foods increases the population wide propensity for rapid release of insulin and insulin-like growth factor (IGF), which stimulate cell growth, promote inflammatory factors, and increase the capacity of tumors to invade neighboring tissue (Grothey et al. 1999; Long et al. 1998). When BALB/C mice were injected with an aggressive mammary tumor and placed on three different dietary regimens, mortalities 70 days after injection were 16 of 24 for the hyperglycemic diet, 8 of 24 for the normoglycemic diet, and 1 of 20 for the hypoglycemic diet (Santisteban et al. 1985). In prospective studies with humans, high glycemic diets were associated with cancers of the pancreas, colon, and ovaries (Michaud et al. 2005; Augustin et al. 2003). These results seem to mirror epidemiologic cancer statistics where those who eat low sugar Asian diets tend to have five to ten times fewer hormonally driven cancers than those eating western diets with high glycemic index foods (Parkin et al. 2005). In women under fifty, those with the highest levels of IGF were approximately seven times more likely to develop breast cancer than those with the lowest (Hankinson et al. 1998), and the risk for men with the highest levels of IGF were approximately nine times of developing prostate cancer (Chan et al. 2002).

In a massive study of over 161,801 healthy postmenopausal women, women with the higher insulin levels (who were not diabetic or taking hormone replacement therapy), had almost twice the risk of developing breast cancer within the six year study period compared to those with lower levels (Gunter MJ et al. 2008). These results mirror the finding that those with diabetes, which is characterized by high glucose and (if pancreatic beta cells remain functional) high insulin levels, have increased risk for cancer (Weiderpass et al. 1997). Additionally, obese individuals have significantly elevated serum concentrations of pro-inflammatory cytokines IL-5, IL-10, IL-12, IL-13, IFNγ and

TNF-α (Schmidt et al. 2015). Inflammation depressed immune response may be the mechanism behind lower vaccine efficacy: for instance, there is an approximately eightfold increase in the odds of non-responsiveness to hepatitis-B vaccination in obese versus normal-weight women (Young et al. 2013)

1.2.4.3. Mental Health Conditions

A meta-analysis of studies which included 55,387 individuals, found significant bidirectional associations between depression obesity (Luppino et al. 2010). Obese persons had a 55% increased risk of developing depression over time, whereas depressed persons had a 58% increased risk of becoming obese (see **Figure 1** above) (Luppino et al. 2010).

In addition to social and psychological bidirectional factors, physiological dsyregulation resulting from the obesogenic phenotype, such as increased inflammation, may have a causal relationship with depression onset. Obese individuals have elevated levels of pro-inflammatory cytokines (Schmidt et al. 2015) and patients with inflammatory medical conditions have elevated rates of depression (Shelton and Miller 2010). Patients with inflammatory conditions who are depressed also have upregulated inflammatory markers compared with patients with these conditions who are not depressed (Shelton and Miller 2010). Additionally, when the inflammatory cytokine IFNα is administered to treat diseases such as hepatitis C and malignant melanoma, it induces depressive symptoms in previously asymptomatic individuals (Loftis et al. 2004). When TFNα is inhibited, symptoms of depression are reduced in those with inflammatory disease (Shelton and Miller 2010). Mechanisms involved in a pro-inflammatory response

such as serotonin depletion, dopamine depletion, and NMDA receptor activation, also mirror hypothesized mechanistic models for depression(Shelton and Miller 2010).

Other obesogenic mechanisms may also contribute to bidirectional depression causality. Depression is associated with over activation of the HPA axis. This increases levels of cortisol, which in the presence of insulin, inhibits lipid-mobilizing enzymes. This chronic HPA activation could exacerbate lipid storage, especially intra-abdominal visceral fat, and contribute to the obesogenic phenotype (Björntorp 2001).

As exercise increases pro-tropic brain factors such as FGF-2, VEGF and BDNF, as well as atrial natriuretic peptide (ANP) which inhibits the HPA axis (as does the exercise induced increase in the resting tension of muscle spindles), a mechanistic relationship between consistent movement and the diminution of depression and other mental health symptoms seems evident (Shafia et al. 2017; Krzeminski 2016; Zhao et al. 2016).

1.3. The Neuroscience of Obesity: How we got Addicted

Hunger is regulated by a motivational reward system which includes dopaminergic projections emanating from the ventral tegmental area to the nucleus accumbens (Erlanson-Albertson 2005; Kelley and Berridge 2002; Koob and Le Moal 2005). The hypothalamus, striatum, orbitofrontal cortex, amygdala, and insula are also implicated in this system (Morton et al. 2006). Endorcrine satiety signals such as high levels of leptin and low levels of ghrelin can be over ridden due to the palatability of available food (Pelchat 2002).

Pain and an elevated HPA response to stress can increase perceptions of hunger and caloric intake, perhaps acting as evolutionary heralds for the possibility of imminent

periods of food shortage and the resulting need to obtain rapid nutrients to use for defensive threat responses or to increase adipose stores. Participants in one experiment ate more sweet-tasting food following a painful experience than they did after a non-painful experience (Darbor et al. 2016). And in a study of 9 year old children, children who were more stressed by laboratory challenges, also increased consumption of sweet-tasting foods (Roemmich et al. 2002).

If some are more likely to turn to eating in times of pain and stress, can over eating be considered an addictive behavior? The evidence is mixed and the conception of eating as an addiction is complicated by the fact that unlike other common addictive behaviors like gambling and drug use, continual satisfaction of the hunger urge is necessary for survival.

Still, substantial hallmarks of an addictive paradigm are evident: as in other addictions, anticipation for the reward of food is modulated through the dopaminergic nucleus accumbens-ventral tegmental axis, and dopamine release results in downstream subjective feelings of pleasure that are rewarding and reinforcing (Abizaid et al. 2006; Hyman et al. 2006; Wise and Bozarth 1985). Blocking dopamine receptors increases appetite and causes weight gain, which suggests that overeating may be a means of compensation of the blunting of pleasurable responses (Del Parigi et al. 2003).

The molecular evidence reveals that obese individuals have fewer dopamine subtype 2 (D2) receptors (Wang et al. 2001). A chicken and egg quandary emerges here as at least two causal possibilities emerge. One possibility is that normal weight individuals initially possess fewer D2 receptors and eat more to achieve baseline levels of pleasure from food resulting in obese phenotypes (Wang et al. 2001; Stice et al. 2008).

Another alternative is that normal weight individuals originally had normal levels of D2 receptors but upon chronic overeating, overstimulate their D2 receptors which lead to their downregulation (Wang et al. 2004). It is noteworthy that within obese individuals, the number of D2 receptors is inversely correlated with increasing BMI (Wang et al. 2001). Additionally, D2 receptor density is significantly decreased in chronic drug and alcohol users (Volkow and Fowler 2000). Future research could begin to resolve these issues by measuring D2 receptor density in a normal weight cohort, following the cohort over time, and comparing D2 levels in those that became obese versus those who remained normal weight.

Evidence also points to the possibility that many obese individuals utilize food consumption as a substitute for substance addiction. There is an extremely high lifetime history of any substance use disorder (32.6%, which is over twice that in the general population) in currently obese patients who are candidates for bariatric surgery (Kalarchian et al. 2007; Kessler et al. 2005). However, only 1.7% of these currently obese patients report a current substance abuse disorder (Kalarchian et al. 2007). . It is plausible that some normal weight individuals who were substance abusers, switched to food addiction and dropped their substance addiction as they remained obese and continued their habits of over consumption (Kalarchian et al. 2007).

Obese people also have higher metabolic activity in the bilateral parietal somatosensory cortex in the regions where sensation to the mouth, lips and tongue are located (Wang et al. 2002). This raises similar questions to the D2 issue since it remains to be determined whether this increased somatosensory sensitivity is a cause, effect, or mere correlate of obesity.

1.4. The Environment of Obesity: Obesogens, Viruses, Bacteria, and Epigenetics

1.4.1. Obesogens

Obesogens, chemicals in the environment that inappropriately stimulate adipogenesis and/or triglyceride storage into existing adipocytes (see **Figure 3** above), have been found to have transgenerational effects. Multigenerational phenotypic effects are effects observed in F1 and F2 offspring, and may be due to in utero exposure to a chemical. However, with transgenerational (F1 to F3 and beyond) effects, the F3 organism was never exposed to the chemical. Thus an in utero mechanism is impossible. As the Sherlock Holmes adage states "Once you eliminate the impossible, whatever remains, no matter how improbable, must be the truth" (Doyle 1890). Having eliminated the in utero mechanism, what remains is an epigenetic mechanism of transmission (Grens 2015; Stel 2015).

Transgenerational effects of obesogens in rats have been observed with diethylhexyl phthalate and DDT. These and many other obesogens work by acting as high affinity ligands for

for two nuclear receptors critical for adipocyte development: the 9-cis retinoic acid receptor (RXR) and peroxisome proliferator activated receptor gamma (PPARγ) (Holtcamp 2012).

Expression of PPARγ commits cells to the adipogenic lineage whereas Wnt signaling inhibits PPARγ expression and diverts mesenchymal stem cells toward the osteogenic lineage. Tributyltin, another obesogen with transgenerational effects persisting at least to the F3 generation, acts upon this PPARγ pathway, and suppresses Wnt signaling to divert stem cells to an adipogenic fate (Holtcamp 2012).

What is the precise mechanism for these obesogenic epigenetic changes? The scientist that coined the word "obesogen", Dr. Bruce Blumberg, wrote recently that "nothing is currently known about how obesogen exposure causes heritable changes in the genome that alter mesenchymal stem cell fate" (Grens 2015).

However, we can speculate from the evidence available to us. In addition to DNA methylation (5-meC), 5′ hydroxymethylation at cytosines (5-hmeC) plays a crucial role in stem cell differentiation. During the adipocytic differentiation of 3T3-L1 preadipocytes, PPARγ recruits Tet enzymes to its binding sites in adipogenic gene promoters causing local demethylation, decreased 5-meC and increased 5-hmC (Stel et al 2015). It is therefore likely that these two methylation processes are implicated in these observed obesogenic transgenerational effects. As endocrine disrupting chemicals have also altered epigenomes through histone tail modifications (Li et al. 2010), this may also prove a fruitful area of obesogenic epigenomic transgenerational research.

Additionally, recently it has been found that many epigenetic changes are harmful only in the present of concomitant nucleotide base mutations in functionally relevant genes (Janesick 2014). Therefore, any future attempts at elucidating a mechanism would be well advised to include the interplay between both the genome and epigenome.

1.4.2. Viruses

In one study, adenovirus-36 (Ad-36) antibodies were found in 30% of obese versus 11% of nonobese humans (see **Figure 3** above) (Atkinson et al. 2005). BMI amongst both obese and nonobese participants was significantly and directly related to levels of Ad-36 antibodies (Atkinson et al. 2005). If individuals become obese first and then acquire Ad-36, one would expect lower antibody levels than those in the nonobese

population, since obese individuals are often immune suppressed (obese people vaccinated with hepatitis B virus have a reduced prevalence of antibodies) (Atkinson et al. 2005). In an in vitro study utilizing 3T1-L1 preadipoyctes, cells infected with Ad-36 differentiated much faster and accumulated triglycerides more rapidly than those infected with a control virus, Ad-2 (Vangipuram et al. 2004). In a study utilizing male Wistar rats, inoculation with Ad-36 significantly increased body weight and foot pad weight compared to controls, and increased expression of pro-adipogenic markers PPAR gamma, and C/EBPα and β (Pasarica et al. 2006). The Ad-36 viral E4 orf-1 gene stimulates pro-adipogenic differentiation in both 3T3-L1 and human adipose stem cells (Rogers et al. 2008).

1.4.3. Bacteria

Administration of *Lactobacillus reuteri* ATCC PTA 4659 was associated with weight decrease in mice, whereas the administration of *L. reuteri* L6798 was associated with weight gain (see **Figure 3** above) (Fak and Backhed 2012).

Staphylococcus aureus in feces was found to increase obesity risk during development and pregnant overweight women have higher concentrations of this bacteria. This may increase the risk of obesity in these women's offspring due to a vertical transfer of bacterial populations during birth or rearing (Kalliomaki et al. 2008).

Mice deficient in G-protein-coupled receptors were more likely to have higher concentrations of *B. thetaiotaomicron* and *M. smithii* and are leaner than those with lower concentrations of these bacteria. (Samuel et al. 2008; De Silva and Bloom 2012).

The presence of *Akkermansia muciniphila* is also associated with leanness in mice (Dao et al. 2016; Chakraborti CK 2015).

1.4.4. Epigenetics

The powerful historical example of the Dutch Hunger Winter of 1944-1945, the German blockade of the West Netherlands until May 1945, illustrates the important role of maternal nutrition in offspring adult-onset obesity (Ravelli et al. 1998). Similar effects were observed in Mao Tse Tung's "secret famine" of 1958-1962 (Li et al. 2010). In the Netherlands example, approximately 20,000 individuals starved to death and some mothers were rationed down to 30% of their normal daily caloric intake (Ravelli et al. 1998; Li et al. 2010).

Babies whose mothers were malnourished during the first three months of pregnancy had a dramatic increase in adult obesity and a decrease in mental functioning with increased prevalence of schizophrenia, a disorder with an amalgam of symptoms often characterized by paranoia and auditory hallucinations with a .5-1% global prevalence with over 50 times the likelihood of attempted suicide. Schizophrenia has a 50% concordance in monozygotic twins (almost three times higher than the 17% concordance in fraternal twins, illustrating the significance of the epigenome) (Brown and Susser 2008).

In a way, maternal nutritive epigenomic effects mirror the effects of the mismatch hypothesis of obesity albeit on a much shorter time scale and via a different mechanism (genetic versus epigenetic changes). The mismatch paradigm states that our metabolic and motivational circuits evolved in a niche of food scarcity with intermittent access to high density foods that could be obtained only through high calorie expending activities (Neel 1962). The transition from a niche of food scarcity where this reward circuit was highly sensitive to the pursuit and acquisition of high energy density foods to the modern

environment of food surplus is the crux of the mismatch paradigm (Neel 1962). Likewise, an early fetal environment of scarcity mismatched with a post-natal environment of surplus.

The best studied animal model of maternal epigenetic transmission of obesity due to nutritional deprivation is feeding pregnant rats a protein restricted (PR) diet. Feeding a protein restricted diet to pregnant rats (F1 generation) increased glucocorticoid receptor (GR) expression and reduced expression of 11b-hydroxysteroid dehydrogenase type II, the enzyme that inactivates corticosteroids, in the liver, lung, kidney and brain of the offspring (F2 generation) (Bertram et al. 2001). Increased GR activity in the liver up-regulates phosphoenolpyruvate carboxykinase expression and activity and so increases capacity for gluconeogenesis which may contribute to the induction of insulin resistance, with resulting risk for obesity (Burns et al. 1997).

There is also evidence that feeding a PR diet to pregnant rats up-regulates glucokinase expression in the liver of the F2 generation, increasing capacity for glucose uptake (Bertram et al. 2001).

PPARα expression has also been reported to be increased in the liver of the offspring of rats fed a PR diet during pregnancy and was accompanied by up-regulation of its target gene acyl-CoA oxidase (Burdge et al. 2007). This is particularly interesting because PPARα ligands can also bind to PPARγ which, and if such binding occurred during a critical developmental window, could trigger mesenchymal stem cells to favor an adipogenic as opposed to an osteogenic lineage (Burdge et al. 2007).

The major epigenetic processes that effect this expression or inhibition of genes are DNA methylation, histone modification and microRNA (Carey 2013). What is the

evidence for these mechanisms in maternal transgenerational transmission? It has been reported that feeding pregnant rats a PR diet-induced hypomethylation of the GR and PPARα promoters in the livers of juvenile and adult offspring, which was accompanied by increased mRNA expression of these genes (Slater-Jefferies et al. 2011). This was associated with an increase in histone modifications at the GR promoter that facilitate transcription including acetylation of histones H3 and H4 and methylation of histone H3 at Lys4, while those that suppress gene expression were reduced or unchanged (Slater-Jefferies et al. 2011). The altered methylation pattern of the PPARα promoter in the liver was due to hypomethylation of four specific CpG dinucleotides, two of which predicted the level of the mRNA transcript, in F2 generation and which persisted through adulthood (Slater-Jefferies et al. 2011; Okamura et al. 2010).

In human subjects, hypomethylation of the imprinted insulin-like growth factor-2 gene in genomic DNA isolated from whole blood from individuals who were exposed to famine in utero during the Dutch Hunger Winter compared to unexposed same-sex siblings (Heijmans et al. 2008). Additionally, insulin-like growth factor promoter was hypomethylated in individuals whose mothers were periconceptually exposed to famine, while IL-10, leptin, ATP binding cassette A1 and the guanine nucleotide-binding protein were hypermethylated (Tobi et al. 2009).

Histone methylation, catalyzed by histone methyltransferases and reversed by histone demethylases, has a crucial role in promoting or inhibiting adipogenesis and obesity (Takeda et al. 2009).

The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR-gamma) triggers mesenchymal stem cells to favor an adipogenic as opposed to an osteogenic

lineage. It is crucial for adipocyte differentiation and is expressed approximately 48 hours post induction of preadipocytes with differentiation factors such as IBMX, dexamethasone and insulin. Histone methyltransferases are implicated in PPARγ gene expression and subsequent adipogenesis. In addition, demethylation of histone H3 at lys9 is associated with resistance to obesity (Okamura et al. 2010).

H3K9 methylation by histone methyltransferase (HKMTs) has been shown to trigger heterochromatin formation and transcriptionally silence euchromatic regions. Two HKMTs, Setdb1 and Setd8 are regulated by PPARγ and that their expression leads to adipocyte differentiation through chromatin modification (Okamura et al. 2010). Setdb1 tri-methylates histone H3K9 while Setd8 mono-methylates histone H4K20. Knockdown studies show that Setdb1 acts as an anti-adipogenic factor and Setd8 acts as pro-adipogenic factor. It appears that PPARγ downregulates Setdb1 and upregulates Setd8 (Okamura et al. 2010). In contrast to PPARγ's effect on Setdb1, noncanoical Wnt5a activates Setdb1 which can drive mesenchymal stem cells down an osteogenic, as opposed to adipogenic lineage (Takada et al. 2009; Takada et al. 2012).

Transcription factors and chromatin modifying enzymes such as MLL3/4, PTIP, Wnt/β-catenin signal are associated with altered H3K4 methylation states in the promoters of PPARγ, CEBPα (Lee et al. 2003). Like PPARγ, CCAAT/enhancer-binding Proteins (C/EBPs) also play an important role in adipocyte differentiation. CEBPα in particular, is a key early regulator of differentiation. Activating signal cointegrator-2 (ASC-2), a component of a Set1-like complex named ASCOM (for ASC-2 complex) which possesses H3K4MT activity, exerts pro-adipogenic function by acting as a coactivator of both C/EBPα and PPARγ (Viswakarma et al. 2010).

Two other H3K4MTs, MLL3 and MLL4, are recruited by the PPAR-gamma activated gene aP2 (along with ASC-2 and ASCOM) (Lee et al. 2003). Additionally, the histone methylation regulator PTIP (Pax transactivation domain-interacting protein), a component of the histone lysine methyltransferase (HKMT), that also contains the aforementioned histone H3K4 methyltransferases MLL3 and MLL4, is also required for the expression of both PPARγ and C/EBPα (Yongson and Morris 2013).

Canonical Wnt/β-catenin signaling prevents activation of PPARγ and CEBPα by activating the expression of the nuclear receptor Coup-TFII which in turn recruits the SMRT co-repressor complex to the first introns located downstream from the first exons of both PPARγ1 and γ2 mRNAs, maintaining the local chromatin in a hypoacetylated state accompanied by the repression of H3K4me3 and thereby represses expression of PPARγ (Jeong et al. 2014). COUP-TFII also inhibits the C/EBPα promoter (Jeong et al. 2014).

The histone demethylase (H3K9 demethylase) JHDM2a has protective effects against obesity and directly targets PPAR-alpha and Uncoupling protein 1 (Ucp1) (in skeletal muscle and brown adipose tissue), crucial genes in metabolism, and may downregulate β-adrenergic signaling (Tateishi K et al. 2009).

1.5. The Fundamentals of Adipogenesis

1.5.1. Types of Adipocytes

The result of these ecological, societal, and psychological changes in food consumption and movement frequency is the accumulation of adipocytes, the lipid cells which comprise the majority of adipose tissue. The two primary classifications of adipose tissue are white adipose tissue, and brown adipose tissue, which have different origins,

structures, and functions. Other classifications of adipose tissue include beige adipocytes and pink adipocytes. The origins of beige adipocytes, (where white adipocytes take on some of the characteristics of brown adipoyctes such as multiocularism) are controversial, with the two competing theories of origin consisting of the existence of precursors in white adipose tissue (Wu et al. 2013) and transdifferentiation (a process whereby one mature somatic cells transforms into another mature somatic cell without undergoing an intermediate pluripotent state) (Wu et al. 2013). Pink adipocytes have been recently been characterized in mouse subcutaneous fat depots during pregnancy and lactation and consist of mammary gland alveolar epithelial cells (Giordano et al. 2014).

White adipose tissue consists of the majority of lipids present in adult humans and is utilized as an energy depot (Farmer et al. 2008), whereas brown adipose tissue primarily functions to produce heat through nonshivering thermogenesis. The differing processes by which white and brown adipocytes store and metabolize lipids, reflect these differing functions. White adipocytes accumulate triglycerides then release them via lypolysis during periods of caloric deficit (Farmer et al. 2008). Brown adipocytes oxidize their lipid stores in a heat-producing pathway utilizing the mitochondrial uncoupling protein (UCP), UCP1 (Golozoubova et al. 2001). Mitochondrial uncoupling proteins are members of the family of mitochondrial anion carrier proteins (MACP) and facilitate the transfer of anions from the inner to the outer mitochondrial membrane and the return of proteins from the outer to the inner mitochondrial membrane. UCP1, highly expressed in brown adipocytes, mediates the ability of brown adipose tissue to produce adaptive heat: UCP-1 deficient mice were unable to survive long-term exposure to cold temperatures in

contrast to controls (Golozoubova et al. 2001). In contrast to brown adipocytes, white adipocytes do not express UCP (Rajan et al. 2014).

It has been hypothesized that the primary role of brown adipose tissue is to maintain body temperature equilibrium upon birth (Frontini and Cinti 2010). The developmental biology of each primary kind of adipose tissue would support this: while white adipose tissue begins to develop in late gestation, brown adipose tissue proliferates primarily in utero. White adipocytes are larger than brown adipocytes and can range from 10 μm to 100 μm in size (Cinti 2005). White adipocytes increase with increased age of the organism, while brown adipocytes decrease with increased age (Rajan et al. 2014).

Brown and white adipocytes contain a number of other opposing structural characteristics: white adipocytes contain unilocular lipid droplets while brown adipocytes contain multilocular lipid droplets (Rajan et al. 2014). White adipocytes possess lower iron, mitochondria, and β3 adrenergic receptor concentrations than brown adipoyctes, and are less sensitive to sympathetic nervous system innervations (Rajan et al. 2014). Comparatively, white adipocytes have poorly developed capillaries and vasculature, and area found in subcutaneous, visceral, and epididymal fat pads, while brown adipocytes are found in the neck, supraclavicular, cervical, and paravertebral regions (Rajan et al. 2014).

Both brown and white adipocytes require key transcription factors to differentiate successfully into mature adipocytes. For instance, adipocytes require the transcriptional factor peroxisome proliferator-activated receptor γ (PPARγ) and factors from other transcription factor families, including CCAAT/enhancer-binding proteins (C/EBPs), signal transducers and activators of transcription (STATs) (a family comprised of seven

proteins, when in response to stimulation primarily on cytokine receptors, and are phosphorylated on tyrosine residues which initiates their migration into the nucleus), and Kruppel-like factor (KLF) proteins (especially KLF15), which can induce adipogenesis in non-precursor cells (Mori et al. 2005). Most of these regulators have been discovered in vitro, utilizing the 3T3-L1 or 3T3-F442A murine preadipocyte cell lines.

Additional transcriptional families important for adipogenesis include SREBP-1, and AP-1, a large group of transcription factors that include v-Jun, c-Jun, JunB, JunD, v-Fos, c-Fos, FosB, Fra1, Fra2, ATF2, ATF3/LRF1, and B-ATF (Kim and Spiegelman 1996).

Some transcription factors are particular to white adipose tissue while others aid in differentiation of brown adipose tissue. For instance, the fibroblast growth factor family members (which consist of herapin binding proteins) FGF 16 and 19 are predominantly expressed in brown adipose tissue and are hypothesized to play a central role in embryonic development of brown adipose tissue (Miyake 1998). Mice over expressing FGF19 had increased brown adipose tissue mass and gain less white adipose tissue after caloric excess when compared to controls. In contrast, FGF10 and FGF21 are primarily expressed in white adipose tissue (Sakaue et al. 2002; Hata et al. 2003).

1.5.2. Stages of Adiopogenesis

While adipogenesis occurs primarily during childhood and adolescence, new adipocytes are formed during the entire lifespan to replace dying adipocytes or to increase the storage capacity of adipose tissue that results from an energy surplus (Gavin et al. 2006). Obesity results from a significant expansion of white adipose tissue because of adipogenesis resulting in hyperplasia (increased cell number). Additionally, the storage

capacity of adipose tissue is also increased through adipocyte hypertrophy (increased cell size) (Sun et al. 2011). In wild type mice, high fat diet feeding initially results in hypertrophy during the first month, after which hyperplasia becomes the predominant means of white adipose tissue expansion. Approximately 10% of adipoyctes are turned over annually in white adipose tissue (Spalding et al. 2008).

While the majority of white adipocytes are thought to be derived from mesenchymal progenitor cells, bone marrow derived progenitor cells can accumulate in adipose tissue and differentiate into adipocytes in adults (Rodeheffler and Horowitz 2016). Initially, embryonic stem cells or mesenchymal stem cells from adipose tissue, commit to become adipocyte precursor cells, and then differentiate into pre-adipocytes. Cultured preadipocytes undergo proliferation, then express early markers of differentiation (Cawthorn et al. 2012). Cell-to-cell contact may play a role in activating mechanisms that induce early differentiation markers (Tong and Hotamisligil 2001).

The differentiation program in 3T3-L1 preadipoyctes is induced over the course of approximately one week by the inducers dexamethasone (responsible for the expression of C/EBPδ), isobutylmethylxanthin (IBMX) (responsible for the expression of C/EBPβ), and insulin (stimulates the cells to take up glucose, which is stored in the form of triacylglycerol) (Tang et al. 2003) (see **Figure 4** below). Although C/EBPβ is expressed within two to four hours after hormonal induction, it initially lacks the ability to bind DNA until 10 to 16 hours after induction (corresponding to progression from G_1 to S phase, synchronous reentry into the cell cycle, and the beginning of mitotic clonal expansion) (see **Figure 4** below) (Lo et al. 2013). This progression into S phase requires down-regulation of p27/kip1 in 3T3-L1 preadipocytes and the activation of cdk2 by

cyclins E and A (Patel and Lane 2000). Various transcription factors regulate this early growth arrested stage of adipogenesis, including Kruppel like factor 4 (KLF4) (expressed with the first 30 minutes post-induction, and peaking at two hours post induction). Knockdown of KLF4 downregulates C/EBPβ and inhibits adipogenesis (Birsoy et al. 2008). KLF5, KLF9 and KLF15 are also expressed early in the differentiation program. It has been reported that over-expression of the dominant-negative KLF5 inhibits adipocyte differentiation while over-expression of wild type KLF5 induces adipocyte differentiation even in the absence of hormonal stimulation (Oishi et al. 2005; Pei et al. 2011; Mori et al. 2005).

Both dexasmethasone and insulin stimulate the expression of the cyclic AMP response Element-binding protein (CREB) (Tang et al. 2003). It has been reported that expression of the active form of CREB in 3T3-L1 preadipocytes is sufficient to induce adipogenesis as seen by accumulation of triacylglycerols and expression of two adipocyte marker genes, PPARγ and fatty acid binding protein (Reusch et al. 2000). Additionally, transfection of 3T3-L1 preadipocytes with a dominant-negative form of CREB blocks adipogenic differentiation (Reusch et al. 2000).

After growth arrest, adipocytes enter the mitotic clonal expansion stage, which will be delineated in more detail in later sections of this review (Tang et al. 2003). Next, adipocytes undergo terminal differentiation, where they express genes specific to the mature adipogenic phenotype, become spherical, and accumulate lipid droplets, the morphological hallmarks of mature adipogenic cells (see **Figure 4** below) (Fei et al. 2011).

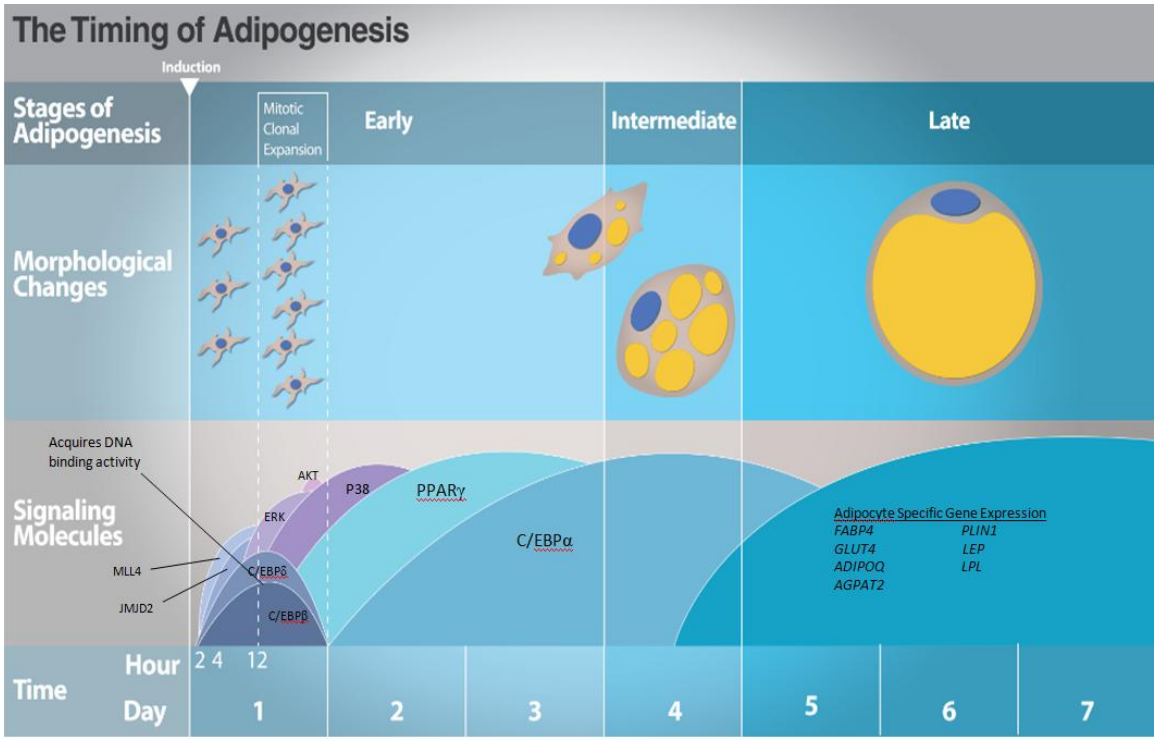

Figure 4. The timing of adipogenesis

1.5.3. MAPK Signaling

1.5.3.1. Overview of MAPK Signaling

Adipogenic transcription factors perform their roles through the various acts of adipogenesis within the context of the intricate signaling networks that are responsible for equilibrium, adaptation, and transformation. The philosopher Jacques Derrida wrote that "there is no meaning without context", and cells may interpret and process the multitude of signals in the extracellular environment in different ways, depending on context. Highly complex and tightly regulated signaling cascades have thus evolved to respond to the information transmitted from the extracellular environment in the appropriate manner (Tang et al. 2003). Activation of particular pathways generates a variety of responses; short-term responses result in cytoplasmic changes, such as redistribution of proteins or

cytoskeletal modifications, while long-term responses alter gene expression. Signaling networks are composed of numerous proteins, which provide multiple levels of control for the cell to regulate their response. One of the key players that has been found to play a role in multiple signaling networks, including adipogenesis, are the family of mitogen-activated protein kinases (MAPKs). These proteins are involved in a diverse variety of cellular processes that control cell proliferation, differentiation, motility, and survival (Cargnello and Roux 2011). More than a dozen of these serine/threonine protein kinases have been described in mammals and include extracellular signaling regulated kinases 1 and 2 (ERK1/2), p38 MAPK (p38) and c-Jun amino-terminal kinases 1,2 and 3 (JNKs); ERK3/4, ERK5, ERK7/8 and Nemo-like kinase (NLK) (Cargnello and Roux 2011; Pearson et al. 2001).

The MAPK family of pathways is composed of a set of three evolutionary conserved protein kinases that are sequentially activated by phosphorylation. Once active, the proteins interact with the proper substrate, each in turn, eliciting a response that comprises a link in the chain of the entire cascade. Various stimuli can induce the activation of different MAPK cascades. For instance, ERK1/2 and ERK5 are usually activated by growth factors, while p38 and JNK respond to stress cues, such as osmotic shock, cytokine stimulation or ionizing radiation. The specificity of the response will be determined by the interaction with various scaffolding proteins, subcellular location and substrate availability, which then direct the response in the appropriate direction (Roux and Blenis 2004; Ohara and Nakahata 2009).

MAPK is involved in a wide variety of cellular processes. Depending on the pathway, type of the stimuli and the duration of the stimulus itself, cells might be directed

into activation of different cellular programs. Extracellular Signaling Regulated Kinase is mostly involved in cell proliferation, cell cycle regulation and modulation of apoptotic molecules. This array of functions correlates with the preferential activation of the aforementioned pathway by growth factors (Chang et al. 2003). Activation of p38 occurs as response to stress stimuli, which involves triggering mechanisms of cell cycle control, DNA repair, chromatin remodeling, mRNA stability, protein degradation and apoptosis. p38 can also be involved in endocytosis, cytoskeleton dynamics and cell migration (Cuadrado and Nebreda 2010). In a similar manner, JNKs also respond to stress stimuli, promoting regulation of altered gene expression, apoptosis, or cell survival (Manning and Davis 2003).

Several members of the MAPK family play important roles in processes like osteogenesis, neurogenesis, and adipogenesis (Change et al. 2003). The process of adipogenesis is highly complex and requires multiple sequential steps and coordination between the different players involved. MAPK is illustrative of this complexity, and modulation of MAPK's activity can often produce opposite effects throughout the process (Chang et al. 2003). Through a variety of approaches, the involvement of MAPK in the adipogenic process has been thoroughly studied. These include the use of chemical inhibitors, cell lines, over-expression of dominant negative proteins, cellular models and MAPK knockouts.

1.5.3.2. The ERK Pathway

The story of ERK is that of needing different things at different times: at some stages of adiopogenesis it is necessary to activate ERK and at others it is necessary to inactivate it. The ERK pathway is one of the most extensively researched, as a result of

its involvement in cell proliferation and activation by insulin, one of the major adipogenic stimuli. In the early stages of ERK research, contradictory results were found regarding ERK's role in pre-adipocyte differentiation. However, recent studies have shown multiple functions for ERK in adipogenesis. The first potential link between ERK and adipogenesis was a study showing the overexpression of Ras (a strong activator of ERK) promoted adipocyte differentiation (Benito et al. 1991). Moreover, it was also shown that ERK is required for adipogenesis, since its depletion inhibited the differentiation of preadipocytes into mature adipocytes (Sale et al. 1995).

However, contradictory results were found when studies involving the transcription factor Peroxisome Proliferator-Activated Receptor (PPARγ), indicated that phosphorylation by ERK reduced the adipogenic capacity of cells, while nonphosphorylatable mutations of PPARγ were more adipogenic (Camp and Tafuri 1997; Adams et al. 1997). Several studies investigated these contradictory findings, and finally established that ERK's contribution to adipogenesis must be timely regulated (Liao et al. 2008; Wang et al. 2008). Compounds which inhibited and activated ERK were utilized to elucidate the role of the ERK pathway in adipogenesis. Through the utilization of these inhibitors, when ERK was inactivated during the early stages of differentiation, adipogenesis was inhibited, while inactivation at the later stages exhibited no apparent effect. Compounds that promote a sustained activation of ERK also have an inhibitory effect on adipogenesis (Liao et al. 2008; Wang et al. 2008). Other experiments demonstrated time periods where activation of ERK was required for adipogenesis. For instance, during the early stages of adipogenesis there is a proliferative phase, also known as mitotic clonal expansion (MCE), which is required for pre-adipocytes to reach full

maturity. Activation of ERK is required during this step (see **Figure 5** below). In contrast, during the terminal differentiation phase at the later stages of adipogenesis, inactivation of ERK is necessary to prevent PPARγ phosphorylation and therefore allow the adipogenesis process to be complete (Rosen and Macdougald 2006; Qian et al. 2010; Tang et al. 2003). Studies have also shown that certain adipokines, such as pigment epithelium-derived factor (PEDF), exert an inhibitory effect on adipogenesis by inactivating the ERK pathway and its effect on MCE (Tang et al. 2003). Lack of activation of ERK prevents the phosphorylation of the CCAAT/enhancer-binding protein C/EBPβ transcription factor, which is required primarily for MCE and later for activation of C/EBPα and PPARγ (that are required to enter the final steps of differentiation) (Lee et al. 2009; Wang et al. 2009; Tang et al. 2003; Tang et al. 2005).

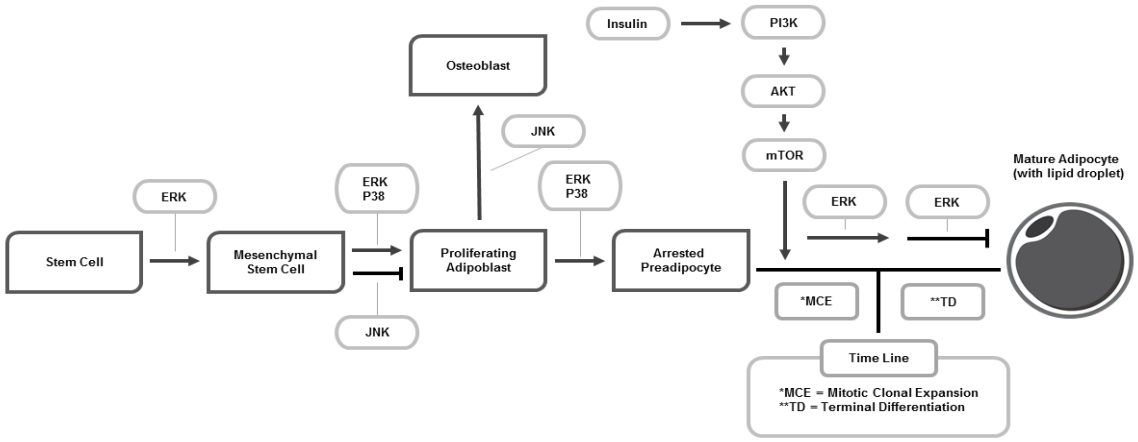

Figure 5. MAPK pathways and adipocyte differentiation

Most studies involving ERK signaling were conducted with pre-adipocytes cell lines to elucidate differentiation into adipocytes. ERK also plays a role in differentiation of embryonic stem (ES) cells. A series of experiments have shown that activation of ERK after treatment with retinoic acid (RA) is required for ES cells to commit to pre-adipocyte

lineage. Additionally, inhibition of ERK during the ES cell differentiation results in a strong inhibition of adipocyte formation, while having no effect on neurogenesis (Bost et al. 2002; Binétruy et al. 2007). Extraceullar Signaling Regulated Kinase also plays a role during development to maintain proliferating cells in an undifferentiated state. To accomplish this, pre-adipocyte factor 1 (PREF-1) induces the phosphorylation of ERK1/2, causing the activation of Sox9 which inhibits adipogenesis. Sox9, a high-mobility-group-box DNA-binding transcription factor, binds to the promoter region of C/EBPβ and C/EBPδ, and in doing so prevents the later activation of C/EBPα and PPARγ. Therefore, in order for cells to undergo adipogenesis, Sox9 must be downregulated (Wang et al. 2010).

1.5.3.3. The p38 pathway

Like ERK, research describing the role of p38 in adipogenesis has also shown mixed results. The first studies that showed involvement of p38 in adipogenesis were done using inhibitors of this kinase. They showed that activation of p38 was required for adipocyte differentiation, suggesting C/EBPβ as a possible downstream target of the kinase (Aouadi et al. 2007; Engelman et al. 1998). Another study showed that activation of p38 by the constitutively active form of MKK6, an upstream activator of p38, induced adipogenesis. However prolonged activation also induced cell death (Engelman et al. 1999). Later studies demonstrated that bone morphogenic protein-2, induces adipogenesis by up-regulating PPARγ activity as a result of p38 activation. On the other hand, treatment of pre-adipocytes with genistein, which reduced phosphorylation of p38, also reduced differentiation into adipocytes (Zhang et al. 2009; Hata et al. 2003). Similarly, treatment of pre-adipocytes with pseudoprotodiocsin reduces their adipogenic capacity.

These cells also show diminished p38 phosphorylation, which might be alter the expression pattern of the estrogen receptor alpha (ERα) isoform (Xiao et al. 2010). In contrast with previous results, others have found that activation of p38 can inhibit adipogenesis. Since active p38 phosphorylates the nuclear protein CHOP, it can then interact with transcription factors from the C/EBP family, preventing them from interacting with their classical binding sites and therefore inhibit differentiation (Batchvarova et al. 1995; Wang and Ron 1996). In a similar way, p38 phosphorylates the nuclear factor of activated T cells (NFAT), which is a protein involved in adipocyte differentiation and dephosphorylation that allows nuclear translocation and transcriptional activation to induce adipogenesis (Yang et al. 2002). Hence phosphorylation of NFAT by p38 prevents it from reaching the nucleus and activating PPARγ (Yang et al. 2002; Ho et al. 1998). Recent studies have shown that p38 plays a role in differentiation through the epidermal growth factor receptor and the Erb2 receptor (EGFR/EB2R) when cells are stimulated with low concentrations of EGF. Inhibition of the receptors, or knockdown by siRNA, has shown a reduced activation of p38 and expression of PPARγ (Harrington et al. 2007). Furthermore, p38 is a part of the melanocortin system, where stimulation with adrenocorticotrophic hormone (ACTH) significantly increases phosphorylation of p38, which mediates and in turn increases uncoupling protein-1 (UCP)-1, which is a marker for brown adipose tissue (Iwen et al. 2008).

1.5.3.4. The JNK pathway

There is extensive evidence showing the involvement of JNK pathway in insulin signaling and the development of obesity (Jaeschke et al. 2004), however little was

known about the possible role of this pathway in adipogenesis. One experiment showed that activation of JNK and NF-KappaB by TNF-alpha inhibits adiopocyte differentiation (Chae and Kwak 2003). Studies performed with human mesenchymal stem cells demonstrated that repression of adipogenesis by JNK is in part due to inhibition of the transactivating function of CRE-binding protein. Inhibition of JNK promoted adipogenesis and increased adipogenic transcription factors like C/EBPβ and C/EBPδ as well as PPARγ (Tominaga et al. 2005). c-Jun Amino-terminal Kinase signaling is suppressed in adipogenesis under normal conditions where activation of the JNK pathway by transforming growth factor 1 (TGF1) disrupts PPARγ dependent expression of aP2, an adipocyte-specific gene (Hong et al. 2007). Moreover, activation of iNOS by JNK also reduces expression of PPARγ affecting adipogenesis (Lee et al. 2010). Studies performed in mesenchymal stem cells and fibrocytes indicate that JNK is involved in determining the lineage commitment of these cells (Kilian et al. 2010). Activation of JNK promotes commitment to osteoblast and myofibroblast, while inhibiting commitment to and adipogenic lineage (see **Figure 5** above) (Kilian et al. 2010).

1.6. Mitotic Clonal Expansion (MCE)

1.6.1. The Timing of Mitotic Clonal Expansion

After induction and growth arrest, preadipoyctes enter a proliferative phase called mitotic clonal expansion (MCE) where cells restart the cell cycle to increase cell numbers two to four fold (Tang et al. 2003). The first round of mitosis is completed 24 to 36 hours after induction, and the second round is completed 48 to 60 hours after induction. After 60 hours, in experiments where only two rounds of mitosis have been observed, the cell

number remained constant and by day 8, virtually every cell underwent adipogenesis (see **Figure 4** above) (Tang et al. 2003).

1.6.2. ERK and MCE

It has been reported that activation of ERK is required for MCE (Tang et al. 2003). Extracellular Signaling Regulated Kinase is necessary for the phosphorylation of C/EBPβ, a critical step in facilitating MCE (see **Figure 4** above). Tang et al. 2003 showed that the MEK-inhibitor U0126 at 40 μM, blocked ERK phosphorylation, adipocyte differentiation (as indicated by failure to accumulate cytoplasmic triacylglycerol), expression of cyclin A and cdk2 and completely blocked MCE (Tang et al. 2003). It has also been reported that ERK activity is necessary for the expression of the crucial adipogenic regulators C/EBPα, δ and PPARγ (Prusty et al. 2002). Inhibition of adipocyte differentiation by chemical blockage of mitotic clonal expansion was circumvented by the addition of a PPARγ agonist suggesting that the importance of mitotic clonal expansion is linked to a concomitant production of PPARγ agonists necessary for the initiation of adipocyte differentiation. Addition of U0126 after MCE had been completed had no effect on the expression of adipocyte markers C/EBPα, PPARγ, and 422/aP2 or the accumulation of cytoplasmic triacylglycerol (Tang et al. 2003). In sum, these results indicate that the adipogenic differentiation program depends upon MCE.

As the cells cross the G_1/S checkpoint, C/EBPβ acquires DNA-binding activity, initiating a cascade of transcriptional activation (Tang and Lane 1996). Preadipocytes traverse the G_1/S checkpoint synchronously as evidenced by the expression/activation of cdk2-cyclin-E/A, turnover of p27/kip1, hyperphosphorylation of Rb, translocation of

cyclin D_1 from nuclei to cytoplasm and GSK-3β from cytoplasm to nuclei, and incorporation of [^{3}H]thymidine into DNA (Patel and Lane 2000). The transcriptional activation initiated by C/EBPβ culminates in the expression of the adipocyte phenotype (see **Figure 4** above) (Patel and Lane 2000).

It has been reported that C/EBPβ acts through induction of the expression of the enzyme

xanthine oxidoreductase (XOR) (Hallenborg et al. 2014)It has been proposed that lipid autooxidation by ROS generated by XOR then results in the production of substrates for eLOX3 which then converts the peroxidized lipids into hepoxilin-like products that function as endogenous PPARγ agonists (Hallenborg et al. 2014). An eLOX3 inhibitor, baicalein, decreases PPARγ ligand generation (Madsen at al. 2003).

A number of compounds, some extracted from medicinal plants, inhibit mitotic clonal expansion. For instance, dioscin, a steroidal saponin present in a number of medicinal plants that has been shown to exert anticancer, antifungal and antiviral effects, inhibits mitotic clonal expansion of cells by inducing G_0/G_1 phase arrest (Poudel et al. 2014). By applying these compounds at various time points in the adipogenic program, and then assaying for molecular markers at these time points, mechanistic aspects of adiopogenesis and mitotic clonal expansion can be elucidated. For instance, western blot analysis revealed that dioscin inhibited the phosphorylation of the ERK and p38 pathways, but did not inhibit JNK. The inhibition of MCE by dioscin is accompanied by an inhibition of the phosphorylation of the MAPKs, ERK1/2 and p38 (Poudel et al. 2014).

Another compound, sulforaphane, similarly arrested the cell cycle at the G0/G1 phase by decreasing expression of cyclin D1, CDK4, cyclin A, CDK2, and phosphorylated Rb protein, but not cyclin E (Choi et al. 2012). Vitisin A, a resveratrol tetramer, also inhibits adipogenesis through G_1arrest by increased p21 expression, and decreased cyclin A and B, CDK2 expression, but not cyclin E (Kim et al. 2008). The expression of the CDK inhibitor p27, an upstream effector of cell cycle regulatory proteins, was downregulated during adipogenesis, but sulforaphane restored the expression suggesting that sulforaphane may induce cell cycle arrest at the G_0/G_1 phase through the upregulation of the cell cycle inhibitor p27 in 3T3–L1 cells. Synthesis-phase kinase-associated protein (Skp2) targets p27 for degradation by the 26S proteasome during MCE. Sulforaphane decreased the phosphorylation of ERK1/2 and Akt (which are stimulated by the adipogenic inducers in 3T3–L1 preadipocytes), Epigallocatechin gallate and matrine also inhibit adipocyte differentiation by downregulating the ERK pathway (Choi et al. 2012).

While invalidation of the ERK2 isoform is embroyonic lethal (Hatano et al. 2003), ERK1 deficient mice have defective thymocyte maturation, enhanced long-term memory, and impaired adipogenesis (Mazzucchelli et al. 2002). The $ERK1^{-/-}$ mice challenged with high fat diet are resistant to obesity and protected from insulin resistance (perhaps as a result of serine phosphorylation of the insulin receptor substrate-1 protein, a downstream effector of the insulin receptor) (Zick 2001). It has been hypothesized that since ERK phosphorylation of C/EBPβ activates its transcriptional activity and knockout of this gene leads to defective adipocyte differentiation, C/EBPβ represents a target that could account for the ERK effect on both 3T3-L1 adipocyte differentiation and the

phenotype of ERK1$^{-/-}$mice (Hu et al. 2001; Tanaka et al. 1997). However, this hypothesis is contradictory to recent results showing that C/EBPβ is a preferential target of ERK2 and not ERK1 (Hanlon et al. 2001). Further studies are necessary to resolve these seemingly conflicting lines of evidence.

Pituitary adenylate cyclase activating peptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family which targets three different receptor subtypes expressed in differentiating 3T3-L1 cells: PAC1, VPAC1 and VPAC2. PACAP increased cAMP production within 15 minutes of stimulation and targeted the expression and phosphorylation of MAPK (ERK1/2) (Arsenijevic et al. 2013). Phosphorylation on Thr188 that occurs in G_1 phase by MAPK primes C/EBPβ for subsequent phosphorylation (Boucher et al. 2014). Cdk2/cyclinA maintains this phosphorylated state throughout S phase and MCE, until the subsequent phosphorylation by GSK3b induces DNA binding and transactivation activity of C/EBPβ. Since PACAP is involved in direct activation of ERK1/2 early during MCE via induction of cAMP, it has been hypothesized that PACAP may be necessary for MCE and adiopogenesis (Arsenijevic et al. 2013).

1.6.3. Akt and MCE

Upon stimulation by the adipogenic inducer insulin, the insulin receptor in preadipocytes is activated, insulin receptor substrates are phosphorylated, and the class I phosphoinositide 3-kinase (PI3K) signaling pathway is activated (see **Figure 5** above) (Boucher et al. 2014). Activation of class I PI3K generates the polyphosphoinositide lipid product phosphatidylinositol(3,4,5) triphosphate (PtdIns(3,4,5)P_3), which is responsible for the activation of protein kinase B (PKB)/Akt in 3T3-L1 cells (Gagnon et al. 1999).

When Akt is activated by phosphorylation on Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (Bunney and Katan 2010), it is followed by phosphorylation on Ser473 by rapamycin (mTOR) complex 2 (Salamon and Backer 2013).

Activated Akt can then translocate to the nucleus and phosphorylate and inactivate the transcription factor forkhead box O1 (FoxO1) by nuclear exclusion, thus permitting adipogenic gene transcription (Tzivion et al. 2011). Inhibiting the PI3K-PKB/Akt pathway inhibits adiopogenesis and MCE (see **Figure 5** above) (Xia and Serrero 1999).

The promotion of MCE is dependent on the ability of Akt to activate mTORC1 and its downstream effector eIF4E, which are required for Skp2 mRNA translation during MCE. S-phase kinase associated protein 2 (Skp2) promotes MCE largely through the degradation of p21 and p27 proteins. In the absence of Skp2, preadipocytes arrest in the G_2 phase of the cell cycle and fail to undergo MCE (Nogueira et al. 2012)

Both Akt1 and Akt2 are able to mediate insulin regulation of the transcription factor forkhead box O1 (FoxO1) and the glucose transporter 4 (GLUT4). It is reported that Akt1 plays the predominant role in MCE of preadipocytes (Kajno et al. 2015). Protein kinase B (Akt2) mediates glucose transport into fat and muscle cells by inhibiting the Rab GAP (GTP-hydrolysis activating protein) AS160, an Akt substrate, facilitates the plasma membrane translocation of GLUT4 (glucose transporter 4) glucose transporters. Additionally, Akt2 regulates glucose levels by decreasing hepatic gluconeogenesis through inactivation of the transcription factor FoxO1. Deletion of both Akt1 and Akt2 is required to completely block the formation of adipose tissue in mice (Kajno et al. 2015).

It has been recently reported that caffeine treatment inhibits MCE by acting on this pathway by increasing the expression of p21 and p27, the major regulators arresting cell cycle progression at the G_1/S checkpoint, and downregulating cdk2, an essential factor for G1/S transition (Kim et al. 2016). Unlike most cell types that express cdk2 throughout the cell cycle including G_1, 3T3-L1 preadipocytes do not express cdk2 in the G_1 phase of MCE (Montagnoli et al. 1999). Instead, expression of cdk2 occurs as the preadipocytes synchronously enter S phase. Cdk2 reaches a maximum level of expression concomitant with the incorporation of [^{3}H]thymidine into DNA, the expression of cyclin A, and the acquisition of DNA-binding activity by C/EBPβ (Kim et al. 2016). Additionally, mouse embryo fibroblasts (MEFs) from C/EBPβ(−/−) mice neither undergo MCE or differentiation into adipocytes but MCE and adipogenesis by C/EBPβ(−/−) MEFs could be rescued by enforced expression of WT C/EBPβ (Tang et al. 2003). The cyclin-dependent kinase inhibitor roscovitine also blocks MCE, the expression of cell cycle and adipocyte markers, and adipogenesis and roscovitine-treated preadipocytes fail to enter the S phase, as indicated by the complete inhibition of [^{3}H]thymidine incorporation into DNA and failure to proliferate (Hallenborg et al. 2014). These findings support the notion that MCE is a prerequisite for adipogenesis.

1.6.4. p38 and MCE

Activation of p38 is also required to upregulate C/EBPβ phosphorylation during MCE (see **Figure 5** above). This is shown by both by the decrease of C/EBPβ phosphorylation through the use of pyrindinyl imidazole inhibitors such as SB203580 to inhibit P38 activation, and through the use of upstream activators of p38 (such as MKK6) resulting in adipoycte differentiation (Takenouchi et al. 2004). Hata et al. 2003

demonstrated that bone morphogenic protein-2 (BMP-2) oriented pluripotent mesenchymal cell lines C3H10T1/2 into the adipocyte lineage through activation of p38. Other studies suggest the opposite result, that p38 inhibits differentiation, and that CHOP phosphorylation by p38 is required for its complete inhibitory effect (Wang and Ron 1996). Supporting an anti-adipogenic effect of p38, Yang et al. showed that once a transcription factor, nuclear factor of activated T cells (NFATc4), is dephosphorylated, it is translocated into the nucleus, increases PPARγ expression (not detected during MCE but subsequently), and thus, adipogenesis (Yang et al. 2002). NFAT4c is phosphorylated by p38 and this phosphorylation inhibits adipose cell formation in NIH3T3 (Yang et al. 2002). It is plausible that activation of p38 at different time points during the adipogenic process has different effects which could resolve the apparent discrepancies in these lines of evidence. Additionally, studying the effects of the different p38 isoforms could also help elucidate this mechanism.

Carlson et al. 2003 showed that p38 activity participates in downregulation of GLUT4 expression and is significantly higher in adipocytes isolated from type-2 diabetes patients than in nondiabetics. Several studies show that inhibition of p38 improves glucose transport in L6 and 3T3-L1 cell lines (Furtado et al. 2002, Furtado et al. 2003).

It is noteworthy that beyond the key role that p38 plays in C/EBPβ phosphorylation during MCE, other co-factors are necessary for the process resulting in MCE and in C/EBPβ then transcriptionally activating C/EBPα and PPARγ genes. Other factors such as TC10-like/TC10βLong (TCL/TC10βL), regulators of G protein signaling 2 (RGS2), factor for adipocyte differentiation 104 (fad104), fad24, and paternally expressed gene 10 (peg10), are also all required for MCE (Kawaji et al. 2010; Jomura et

al. 2008; Hishida et al. 2007). Knockdown of their expression impairs MCE (Kawaji et al. 2010; Jomura et al. 2008; Hishida et al. 2007). Additionally, it has been reported that activating transcription factor 5 (ATF5) can bind to the promoter of C/EBPα via its direct interaction with C/EBPβ (which is mediated via the p300-dependent acetylation of ATF5), leading to enhanced C/EBPβ transactivation of C/EBPα (Zhao et al. 2014). Additionally, p300 facilitates the interaction of ATF5 with C/EBPβ as well as for the binding activity of this complex on the C/EBPα promoter (Zhao et al. 2014).

It has also been reported that Topo IIα (a DNA topoisomerase that is expressed in dividing and pluripotent cells, resolve DNA topological features such as catenation and supercoiling, arising during DNA replication, transcription and sister chromatid entanglement during mitosis, and catalyzes double strand breaks) expression peaks during MCE in adipocytes in a PI3K/mTOR-dependent manner and is essential for adipogenesis (Jacobsen et al. 2016). Topoisomerase IIα expression is also involved in the replication of the heterochromatin regions, and in chromatin remodeling (Jacobsen et al. 2016).

1.6.5. Fat Mass and Obesity Associated Gene (FTO), Factor for Adipocyte Differentiation (FAD) Genes and MCE

Merkestein et al. 2016 recently reported that the fat mass and obesity-associated (FTO) gene influences adipogenesis by regulating MCE. Single-nucleotide polymorphisms in the first intron of the FTO gene were shown to increase susceptibility to obesity in genome wide association studies first in Caucasians, and then in multiple human populations and different ethnic groups (Yeo et al. 2014). Primary adipocytes and mouse embryonic fibroblasts (MEFs) derived from FTO overexpression (FTO-4) mice

exhibit increased potential for adipogenic differentiation, while MEFs derived from FTO knockout (FTO-KO) mice show reduced adipogenesis (Yeo et al. 2014).

Fat Mass and Obesity-associted Gene is a nucleic acid demethylase that removes methyl groups from both DNA and RNA. It demethylates N6methyladenosine (m6A)19, which affects the processing, stability and alternative splicing of mRNAs. In 3T3-L1 cells, FTO controls mRNA splicing by regulating the ability of the splicing factor SRSF2 to bind mRNA in an m6A-dependent way. One of the targets of SRSF2 is Runt-related transcription factor 1 (RUNX1T1), which exists in two splice variants, a long (L) and a short (S) isoform. Overexpression of the S isoform of RUNX1T1 in 3T3-L1 cells enhances adipogenesis, while overexpression of the L isoform of RUNX1T1 impairs adipogenesis (Zhao et al. 2014). The L isoform of RUNXT1 is found at much higher levels than the S isoform in 3T3-L1 cells. The effect of RUNX1T1 on adipocyte differentiation and proliferation thus depends on the balance between the L and S isoforms of RUNX1T1 (Yeo et al. 2014).

Fat Mass and Obesity-associted Gene acts during MCE to enhance adipocyte number through expression of RUNX1T1, which enhanced adipocyte proliferation, and is increased in FTO-wt MEFs and reduced in FTO-KO MEFs. Expression of RUNX1T1-S was reduced in MEFs from FTO-KO mice and was increased in MEFs from mice overexpressing FTO. Furthermore, knockdown of RUNX1T1 in WT MEFs reduced cell proliferation and expression of cell cycle genes during MCE (Yeo et al. 2014).

Fat Mass and Obesity-associted Gene overexpression also leads to expression of PPARγ and C/EBPα, as would be predicted from its inducement of MCE. In humans, a

mutation in the catalytic domain of FTO (R316Q) results in a severe phenotype accompanied by growth retardation (Boissel et al. 2009).

The mechanism by which the S isoform of RUNX1T1 stimulates the adipogenic cascade has not been fully elucidated, but it has been hypothesized that it binds to, and activates C/EBPβ (Merkestein et al. 2015). FTO has been shown to act as a transcriptional coactivator of C/EBPβ (Wu et al. 2010).

Two other genes, factor for adipocyte differentiation (fad) gene-24 (fad24), and factor for adipocyte differentiation (fad) gene-102 (fad102), were amongst 102 genes found to be expressed at the beginning of the differentiation of 3T3-L1 cells (Imagawa et al. 2016). Factor for adipocyte differentiation 23 and Fad102 are pro-adipogenic with Fad23 knockdown inhibiting MCE. FAD24 regulates DNA replication by recruiting histone acetyltransferase binding to ORC1 (HBO1) to DNA replication origins (Imagawa et al. 2016).

1.6.6. Amino Acids, Polyamines, Reactive Oxygen Species, and MCE

Polyamine levels increase during adipogenesis, and their depletion inhibits adipogenesis, MCE, and the activation of C/EBPβ and MCE (Brenner et al. 2015).

Catabolism of branched-chain amino acids contributes to both adipogenesis and lipid accumulation. However, several proteolytic pathways are active during adipogenesis, including autophagy, proteasomal degradation, and matrix metalloproteinase-mediated proteolysis (Kirkwood et al. 2016). Amino acids increase at both 24 and 48 hours post differentiation with the exception of proline and glutamate, which were decreased at 24 and 48 hours compared to earlier time points(Kirkwood et al. 2016). Proline is a precursor to ornithine, the immediate precursor for the biosynthesis of

polyamines. In addition, proline oxidation by the mitochondrial enzyme proline dehydrogenase can produce reactive oxygen species (ROS) (Kirkwood et al. 2016).

Several studies report that ROS promote adipogenesis by accelerating MCE (Vigilanza et al. 2011, Lee et al. 2009). It is hypothesized that diminished proline levels during adipogenesis results from the need to supply it as a precursor to both polyamine biosynthesis, and also to produce pro-adipogenic ROS, in addition to the ROS from complex III and NADPH oxidase (Vigilanza et al. 2011, Lee et al. 2009).

Glutathione, a tripeptide antioxidant exists in oxidized (GSSG) and reduced (GSH) forms and during adipogenesis, the ratio of GSSG to GSH increases. Antioxidants that prevent the oxidative conversion of GSH into GSSG inhibit adipogenesis, and inhibitors of GSH synthesis stimulate adipogenesis (Vigilanza et al. 2011, Lee et al. 2009).

1.6.7. Sirtuin 1 (SIRT1) and MCE

The NAD(+)-dependent protein deacetylase sirtuin 1 (SIRT1) is reported to have an anti-adiopogenic effect. SIRT1-silenced mouse 3T3-L1 preadipocytes differentiate into hyperplasic adipocytes and exhibit a phenotype associated with dysregulated adipocyte metabolism and enhanced inflammation (Abdesselem et al. 2016).

Differentiating SIRT1-silenced preadipocytes exhibit enhanced mitotic clonal expansion accompanied by reduced levels of p27 as well as elevated levels of C/EBPβ. c-Myc levels are also elevated, c-Myc is hyperacetylated, levels of p27 are reduced, and cyclin-dependent kinase 2 (CDK2) is activated upon SIRT1 reduction. c-Myc hyperacetylation leads to higher preadipocyte proliferation potential and enhanced

adipocyte MCE during differentiation, which ultimately results in dysfunctional hyperplasic adipocytes (Abdesselem et al. 2016).

An important oncogene, c-Myc, is among the earliest expressed genes during MCE of differentiating preadipocytes, and targets the key cell cycle regulator p27 and has a repressive effect upon it. Additionally, C/EBPβ was up-regulated during MCE and late differentiation in SIRT1-silenced adipocytes, suggesting a key role of these targets in the regulation of MCE by SIRT1 (Abdesselem et al. 2016).

1.6.8. G9a and MCE

Peroxisome Proliferator Activated Receptor Gamma and C/EBPα are antimitotic factors and thus their delayed expression ensures the successful progression of MCE (Tang et al. 2012). During the time lag before expression of PPARγ and C/EBPα, C/EBPβ is sequentially phosphorylated, first by MAPK/cyclin A/cdk2 on Thr^{188} and subsequently by GSK3β on Ser^{184} or Thr^{179}, which is critical for C/EBPβ to acquire its ability to bind DNA and to prevent its calpain-dependent degradation (Li et al. 2007; Zhang et al. 2012).

However, C/EBPβ phosphorylation cannot explain the time lag in the expression of PPARγ and C/EBPα (increased expression at approximately 48 hours post induction), since during MCE C/EBPβ already has DNA binding activity and transactivates several other cell cycle genes (Guo et al. 2012). It has been hypothesized that epigenetic events could account for this time lag (Li et al. 2013).

Euchromatic histone-lysine N-methyltransferase 2 (G9a) is one of the major euchromatic methyltransferases involved in the methylation of histone H3 (K9 and K27) and histone H1b (K26). Euchromatic histone-lysine N-methyltransferase 2 (G9a) plays

roles in embryonic development, genomic imprinting, lymphocyte and neuronal function, tumorigenesis, and metastasis (Li et al. 2007). Additionally, it has recently been reported in 3T3-L1 cells that C/EBPβ transactivates G9a expression during MCE (Li et al.2007). Additionally, G9a inhibits the expression of PPARγ and C/EBPα through H3K9 dimethylation of their promoters which may account for the observed time lag in their expression (Li et al.2007).

It is noteworthy that PPARγ and C/EBPα expression increased 48 hours post induction concomitant with G9a downregulation (Li et al.2007).

Abnormally early expression (that results from early downregulation of G9a) of the two antimitotic factors PPARγ and C/EBPα interrupted MCE (Li et al. 2007).

Considerable evidence has suggested other epigenetic mechanisms, including histone methylation, are critical for adipogenic differentiation. The histone H4 Lys-20 (H4K20) monomethyltransferase PR-Set7/Setd8 gene was upregulated by PPARγ during adipogenesis (Wakabayashi et al. 2009). It has also been reported that SHARP1 inhibited adipogenesis by targeting C/EBPβ activity, which is necessary to replace the corepressors HDAC1 and G9a from the promoters of their transcriptional targets (C/EBPα and PPARγ2) during differentiation (Wakabayashi et al. 2009).

1.6.9. miR-363 and MCE

Micro Ribonucleic Acid-363 was reported to be one of the most significantly downregulated miRNAs during adipogenesis. Overexpression of miR-363 in adipocytes inhibited MCE and terminal differentiation (Fajas et al. 2002). Ectopic introduction of miR-363 into ADSCs significantly reduced the levels of E2F3, a key transcription factor that regulates growth and proliferation during MCE (Fajas et al. 2002; Chen et al. 2014).

Transcription factor E2F3 is the effector of the retinoblastoma protein pRB-E2F pathway, which controls the G1/S transition during the cell cycle and regulates cell proliferation and differentiation and is also a key transcription factor during MCE. E2F3 is a substrate of the cyclin-dependent kinases and directly effects *cyclin E* transcription, which plays an important role in DNA synthesis and induces the G_1/S transition (MCE) (Chen et al.2014).

During the MCE, DNA replication and changes in the chromatin structure aid in triggering the expression of C/EBPα, which transactivates genes resulting in terminal differentiation. C/EBPα also blocks E2F3 expression by upregulating the CDK inhibitor p21. Thus, cells exit MCE and enter a growth-arrested stage before terminally differentiating (Chen et al.2014).

It has been reported that EGFP/RFP reporter assay, miR-363 can directly target the 3'UTR of E2F3 (Fajas et al. 2002). During normal early stage of adipogenesis, *E2F3* is expressed and miR-363 is downregulated. Downregulation of miR-363 allows this transitional switch from MCE to terminal differentiation to occur by reducing the inhibition of E2F3. miR-143 expression was transiently decreased after adipogenic induction while increased from day 3 and peaked on day 7 after induction (Fajas et al. 2002).

It has recently been reported that miR-143 directly represses MAP2K5, a key member of the MAPKK family in the MAPK signaling pathway (Chen et al. 2014)

1.6.10. Proliferating Cell Nuclear Antigen (PCNA) and MCE

It has been reported that Proliferating Cell Nuclear Antigen (PCNA) is subject to phosphorylation at the highly conserved tyrosine residue 114 (Y114). Mice challenged

with a high fat diet who are genetically modified so as to replace the tyrosine residue 114 with phenylalanine (which is structurally similar to tyrosine but cannot be phosphorylated) are resistant to obesity. Phosphorylation at Y114 is necessary for MCE (Lo et al. 2013).

1.6.11. KCNK10 and MCE

Potassium channel, subfamily K, member 10 (KCNK10) expression is strongly induced by 3-isobutyl-1- methylxanthine (IBMX), a potent inducer of adipocyte differentiation. KCNK10, a member of tandem pore domain potassium channel family is elevated during the early stage of adipogenesis of 3T3-L1 cells and that reduction of KCNK10 expression inhibits adipocyte differentiation (Sato et al. 2010). It has recently been reported that KCNK10 contributes to the regulation of MCE through the control of C/EBPβ and C/EBPδ expression and insulin signaling (Nishizuka et al. 2014). The reduction of KCNK10 expression suppressed the expression of C/EBPβ and C/EBPδ, the phosphorylation level of Akt during the early phase of adipogenesis, and insulin-induced Akt phosphorylation (Nishizuka et al. 2014).

In summary (see citations throughout above), the different MAPK pathways are able to regulate several steps of adipogenesis (see **Figure 5** above). Timely activation and tight control of specific proteins is an essential step in the successful differentiation of pre-adipocytes into mature adipocytes. Through the extensive study of the ERK signaling pathway, it is now understood that activation of this pathway is required at the early stages of adipogenesis, however, later inactivation is also necessary to achieve full differentiation. In a similar way, the contribution of p38 to adipogenesis seems to overlap with ERK's contribution. p38 is also required at the beginning of adipogenesis,

when cells are undergoing MCE, with later inactivation being required to guarantee fully mature adipocytes. The JNK pathway is not yet fully understood, and there is little information about the role it plays in adipogenesis, however it has been shown to determine lineage commitment of mesenchymal stem cells, favoring the osteogenic instead of the adipogenic lineage. Future research including experiments utilizing natural products to inhibit adipogenesis may lead to therapeutic strategies in the treatment or prevention of obesity and its associated disorders.The MAPK family plays an integral role in the initiation of mitotic clonal expansion (MCE), a necessary step for adipogenesis. For instance, it has been reported that MCE requires ERK and p38 activation to facilitate the phosphorylation of C/EBPβ, as well as a G9a mediated time lag between this phosphorylation and expression of PPARγ and C/EBPα. In addition other pathways and a chorus of other factors are also necessary for MCE to proceed, such as the ability of Akt to activate mTORC1 and its downstream effector eIF4E, which are required for Skp2 mRNA translation, which then degrades p21 and p27 proteins. The Fat Mass and Obesity Associated Gene (FTO), Factor for Adipocyte Differentiation (FAD) Gene expression are necessary for MCE, as are specific interplays between amino acids, polyamines, reactive oxygen species, and MCE. Downregulation of miR-363 increases the levels of E2F3, a key transcription factor that regulates growth and proliferation during MCE. Differentiating SIRT1-silenced preadipocytes exhibit enhanced MCE accompanied by reduced levels of p27 as well as elevated levels of C/EBPβ. Phosphorylation of the Proliferating Cell Nuclear Antigen (PCNA) Y114 is necessary for MCE. IBMX induced KCNK10 expression contributes to the regulation of MCE through the control of C/EBPβ and C/EBPδ expression, the phosphorylation level of Akt during

the early phase of adipogenesis, and insulin-induced Akt phosphorylation. The interplay of these elements illustrates the complexity of molecular actors involved in the crucial adipogenic step of MCE and the need for further studies to further delineate the relationships between them.

1.7. Anti-adipogenic Compounds derived from Artemisia

1.7.1. History of Artemisia

Asteraceae is a family of flowering plants (Angiospermae), etymologically derived from "aster", the Greek word for "star" (Barnhart 1995 et al.), presumably due to the morphology of their emanating inflorescence resembling the projecting radiant light from a star. *Artemisia* is a genus in the Asteraceae family, and numerous species within this genus possess sesquiterpene lactones as active constituents (Giordano et al. 1990). Long before these sesquiterpene lactones were extracted and isolated in the modern era, traditional cultures recognized these species for their therapeutic and other properties (Giordano et al. 1990).

One property of *Artemisia* that characterized conceptions of the plant by early cultures is its bitterness due to its thujone content (DeBaggio and Tucker 2009). Thujones are ketones and monoterpenes which naturally occur in two diasteromeric forms: (−)-α-thujone and (+)-β-thujone (Oppolzer et al. 1997) and act upon GABA and 5-HT3 receptors in brain tissue (Deiml et al. 2007).

A common name for various species of *Artemisia* is wormwood (Chialva et al. 1983). Wormwood (as translated into English from the Hebrew word la'anah, which means "curse" in both Hebrew and Arabic) is mentioned eight times in the Old Testament of the Bible and possibly refers to either *A. judaica* L. or *A. herba-alba* (DeBaggio and

Tucker 2009) since both these species are present in Middle Eastern regions where the books of the Bible were written and compiled.

Artemisia judaica is a perennial fragment shrub that is abundant in North Africa and Middle Eastern countries (Alzweiri et al. 2014) and has been used in traditional Egyptian medicine for the treatment of gastrointestinal disorders (Liu et al. 2004). Isolated compounds from *A. judaica* have exhibited antibacterial, antifungal, and cytotoxic effects (Alzweiri et al. 2014; Abdelgaleil et al. 2008, Bos and Sharma 2011; Hashem 2011). The composition of *A. judaica* includes artemisinic acid, methyl wormwood, artemisinic alcohol, eucalyptol, *Artemisia* ketone, camphor, caryophyllene, and piperitone (Charchari 2002; El-Sharabasy 2010). Gas Chromatography-Mass Spectrometry and GC-FID results demonstrate that the isolated oil contains octenyl acetate (2E) (26.6%), *p*-cymen-8-ol (26.6%), *p*-menth-3-en-8-ol (21.6%), pentylcyclohexa-1,3-diene (6.6%), verbenyl acetate (4.4%), isophorone (3.5%), and *Artemisia* ketone (3.5%) (Alzweiri et al. 2014). Some studies showed the presence of 1,8-cineole, α-thujone, camphor, thymol, lavandulol, and santolina triene (Dob and Chelghoum 2006), while others did not (Alzweiri et al. 2014). It has been proposed that the oxygenated terpenes octenyl acetate and *p*-menth-3-en-8-ol are responsible for *A. judaica*'s carminative effect due to the hydrophilic oxygenated part and the hydrophobic terpene part's optimal surface activity effect against interfacial tension (Alzweiri et al. 2014).

Artemisia herba-alba, the other candidate for the Biblical wormwood (DeBaggio and Tucker 2009), is commonly found on the dry steppes of the Mediterranean regions in northern Africa (Saharan Maghreb), western Asia (Arabian Peninsula), and southwestern

Europe (Al-Waili 1986). In Iraqi folk medicine, *A. herba-alba* based teas were used for the treatment of diabetes mellitus (Al-Waili 1986). The Bedouins in the Negev desert in Israel utilize *A. herba-alba* as a traditional remedy for enteritis, and various gastrointestinal disturbances (Friedman et al. 1986). In herbal medicine, *A. herba-alba* is widely used for its antiseptic, vermifuge, and antispasmodic properties (Yashphe et al. 1987). Essential oils from *A. herba-alba* have been found to have cytotoxic effects on the P815 mastocytoma and BSR kidney carcinoma cell lines, but not against peripheral blood mononuclear cells PBMCs (Tilaoui et al. 2015). Gas Chromatrography and GC-MS analysis demonstrated that the isolated oil from the leaves of *A. herba-alba* contained oxygenated sesquiterpenes (39.9%), sesquiterpenes (29.0%), esters (24.0%). monoterpenes with an α-thujone skeleton (1.24%), and monoterpenes with a pinane skeleton (4.48%) (Tilaoui et al. 2015**)**. Thujone was found to comprise 64% of the essential oil of *A. herba-alba* collected from Tunisia, but was absent from samples collected from Spain (Feuerstein et al. 1988; Salido et al. 2004). The proportion of thujone varies by chemotype (34 to 94%), and seven chemotypes were identified from samples collected from various Moroccan regions (Benjilali and Richard 1985; Benjilali and Sarris 1982) Differential percentages of α- versus β- thujone is likely due to varying geographical growth sites and climatic conditions (Ouyahya et al. 1990).

In the Old Testament of the Bible, wormwood (presumably *A. judaica* and/or *A. herba-alba*) (DeBaggio and Tucker 2009) is used metaphorically to represent the bitterness concomitant with troubled times. Wormwood, in accordance with its literal Hebrew and Arabic meaning of "curse", is also utilized to represent sorrow, suffering,

and misfortune. This is perhaps due to assumptions made by traditional cultures regarding the nexus between bitter taste and toxicity (Johnson 1891).

If the prevailing scholarly opinion that the books of the Old Testament were written between the 8th and 5th centuries B.C. (Rahimi et al. 2010) is correct, and if the Biblical wormwood does indeed refer to *Artemisia*, then it is plausible that traditional cultures in Middle East regions where *Artemisia* was prevalent during this time period recognized the plant for its bitterness and thought of this property as a defining characteristic.

At some point in early Middle Eastern history, the potentialities of *Artemisia* beyond its bitter taste were recognized. For instance, in traditional Iranian medicine, a condition known as "zahir", which seems to parallel irritable bowel syndrome, has long been treated by *Artemisia* and other local flora (Rahimi et al. 2010). These natural products have also been utilized for their anti-inflammatory, anti-ulcer, wound healing, and anti-diarrheal effects (Rahimi et al. 2010).

The origins of the word "artemisia" may derive from the Greek goddess Artemis (the Roman Diana) (Rose 1959), the goddess of the hunt and the moon (DeBaggio and Tucker 2009). It may also derive from historical individuals whose names were derived from the goddess (DeBaggio and Tucker 2009). Two possible candidates are Artemisia I and Artemisia II (DeBaggio and Tucker 2009). Artemisia I was the commander who fought as an ally of Xerxes I and warned him before the Battle of Salamis in 480 B.C. not to enter the narrow straits against the Greeks; an admonition that was ignored with dire consequences for the Persians (Herodotus 450 B.C.). Artemisia II was a botanist who died in 350 B.C. and her profession has been attributed to her association with Artemisia

(DeBaggio and Tucker 2009). However, a bizarre part of her biography reveals that she married her own brother and after his death drank his ashes (Boccaccio 1990). Since Artemisia was associated in those times with bitterness (DeBaggio and Tucker 2009), it may be that this ingestion of her brother-husband's ashes (often memorialized in medieval art) was the precipitating etymological event behind the plant's namesake.

Beyond the Middle East, *Artemisia* has also been long known and utilized in traditional cultures of the Far East. *A. annua* L. (quinghao, which translates to "green herb) grows naturally as a part of steppe vegetation in northern parts of Chatar and Suiyan province in China at 1,000–1,500 m above sea level and has been used in traditional Chinese medicine since at least the second century B.C. A piece of silk unearthed from the Mawangdui Han Dynasty tombs (168 BC) described *A. annua* as a treatment for hemorrhoids. From as early as the medical writings of Ge Hong (283–343 A.D.) during the Jin Dynasty, *A. annua* was described as a remedy for fever. Rural areas used the herb to treat malaria even before the initiation of a secret research project in 1963, called Project 523 (Cui and Su 2009).

Project 523 was initiated in a clandestine meeting held on May 23, 1967 (hence the name Project 523, for 23rd of the fifth month) during the Vietnam War between Ho Chi Minh and Mao Zedong. Ho Chi Minh was in desperate need of Mao Zedong's aid since more North Vietnamese soldiers were dying from malaria than from armed conflicts. The Chinese launched a program to find anti-malarial compounds both for the North Vietnamese and for Chinese citizens (Graziose et al. 2010) and in 1972 artemisinin, a highly oxygenated sesquiterpene, containing a unique 1,2,4-trioxane ring structure, was extracted and identified as an efficacious anti-malarial compound from *A.*

annua (Tu 1999). The effectiveness of artemisinin was initially underestimated in the Western world (Attaran et al. 2004) but has demonstrated efficacy (along with its two main derivatives artemether and artesunate) against both malaria and schistosomiasis (Zhang et al. 2014).

After the initial isolation, extraction, and testing, several other *A. annua* anti-malarial derivatives were subsequently produced including artemether and artesunate in 1987, and dihydroartemisinin (DHA) in 1992 (Cui and Su 2009; Liu et al. 2014). Artemisinin derivatives have also proved efficacious against *Schistosomiasis japonica* in China (Liu et al. 2014). As malaria still afflicts 5% of the world's population, artemisinin combination therapy (ACT) to attempt to circumvent drug resistance is an essential anti-malarial strategy (Bosman and Mendis 2007). Pyronaridine, lumefantrine (benflumetol), and naphthoquine, which were synthesized in 1973, 1976, and 1986 respectively, are all utilized in ACT (Cui and Su 2009). In the United States, *A. annua* was introduced in the nineteenth century, nicknamed "Sweet Annie", and used as a preservative, for the flavoring of vermouth, and as an addition to potpourri (Dhingra et al. 2000). Due to its now known efficacy in treating chloroquine-resistant and cerebral malarias, *A. annua* is currently cropped on a large scale in China, Vietnam, Turkey, Iran, Afghanistan, and Australia. In India, it is cultivated on an experimental basis in the Himalayan regions, as well as temperate and subtropical conditions (Bhakuni et al. 2001). Other traditional uses of *Artemisia* in China include moxibustion, the burning of a roll of specially prepared herbs containing *A. vulgaris* on acupuncture points (Hitosugi et al. 2001; Kobayashi 1988).

Another traditional Chinese remedy is Yin Chen Hao Yin Chen, consisting of *A. scoparia* Waldst. et Kit. and *A. capillaris* and used to treat liver and choleretic disorders. This traditional remedy was found to exhibit a hepatoprotective effect by ameliorating murine concanavalin A-induced hepatitis via suppression of interferon gamma (IFNγ) and interleukin-12 (IL-12) production (Mase et al. 2010).

Other Asian countries, apart from China, also have a tradition of *Artemisia* use. For instance, *A. scoparia* Waldst. et Kit. is native to Japan, Korea, and Mongolia. The aerial part of this plant is used in traditional medicine as an antiphlogistic, diuretic, for the treatment of hepatitis and urticaria, and as an antimold agent. Phytochemical investigations of the aerial part of *A. scoparia* resulted in the isolation of flavonoids, coumarins, and essential oils. Recently, *A. scoparia* has been shown to inhibit triglyceride accumulation in 3T3-L1 preadipocytes (Yahagi et al. 2014).

In some cases, the history of extraction of a therapeutic compound from *Artemisia* in a country consists of initial import followed by the establishment of domestic industrial production. For instance, prior to 1919, Japan imported santonin, an anthelmintic agent, entirely from Russia. In 1919 domestic production of santonin was initiated by Nippon Shinyaku Co., Ltd. In 1927, *A. maritima* ssp. *monogyna*, which contains santonin, was introduced from Europe and through cross breeding, additional plant taxa were developed to produce santonin on Japanese soil (Yamaura 2011). In Europe, *Artemisia* is also abundant in regions favorable to its growth. For instance, in Spain, seven chemotypes of *A. absinthium* populate certain mountainous regions of the Iberian Peninsula (Arinoa et al. 1999). Also, in the Americas, traditional cultures have long utilized the therapeutic properties of *Artemisia* in native medical practices. For

instance, various Native American tribes utilized *Artemisia* to treat gastrointestinal tract conditions, gynecological disorders, malaria, as a vermifuge, and for a variety of other conditions (DeBaggio and Tucker 2009). In various regions of South America, including Southern Ecuador, Columbia and Peru, indigenous populations utilize the shrub *Gynoxys verrucosa* in traditional medical practices. *G. verrucosa* is grown in the provinces of Loja and Zamora-Chinchipe. The aerial parts of this plant are known as "guángalo" or "congona" and the plant belongs to the Senecionea tribe of the Asteraceae family. It has numerous therapeutic uses including direct application to the skin for the treatment of skin infections and wound healing (Tene et al. 2007). One of the secondary metabolites isolated from this species is the sesquiterpene lactone dehydroleucodine which possesses numerous therapeutic properties (Tene et al. 2007).

In Argentina, dehydroleucodine has also long been used in traditional medicine as an infusion of leaves of *A. douglasiana*, popularly known as 'matico'. This traditional preparation of boiled leaves was used to treat conditions such as gastric ulcers, external treatment of skin injury, and dermal ulcers (Giordano et al. 1990). The first report of *A. douglasiana* in Argentina was in 1967, in San Juan and Mendoza provinces (Snowden 2008), which led to the isolation and identification of dehydroleucodine, a sesquiterpene lactone of the guaianolide type (Giordano et al. 1990). Dehydroleucodine was then extensively studied by a number of Argentinean and other scientists for its therapeutic action on a multitude of pathogenic conditions including peptic ulcer, gastric and duodenal lesions concomitant with collitis, *Allium cepa* L. root growth, cancer, and adipogenesis associated with obesity (Matsueda and Geissman 1967; Piezzi 1992; Guardia et al. 1994; Sartor et al. 2001; Galvis et al. 2011; Costantino et al. 2013). Its

absolute structure was elucidated by X-Ray analysis (Priestap et al. 2011), which was consistent with previous NMR studies (Bohlmann and Zdero 1972).

1.7.2. Chemical Structure of Sesquiterpene Lactones

Sesquiterpene lactones are a large and structurally diverse group of plant secondary metabolites (Heinrich et al. 1998). They are found in various plant families, most ubiquitiously in Asteraceae (formerly Compositae) (Ivanescu et al. 2015). Sesquiterpene lactones are sesquiterpenoids that contain a lactone ring (Chadwick et al. 2013). Lactones are cyclic esters of hydroxycarboxylic acids where an alkoxy group replaces at least one hydroxyl group (Xavier et al. 2010).

Biosynthesis of sesquiterpene lactones occurs via farnesyl pyrophosphate (FPP), in the endoplasmic reticulum of plants (Yu and Utsumi 2009). Three isoprene units are condensed to synthesize a 15 carbon compound (Ren et al. 2016). Next, cyclization and oxidative transformation occurs to create a fused lactone ring (Ramirez et al. 2016). The lactonization process results from the oxidation of C12 to form the carbonyl function, followed by hydroxylation at C6 or C8 (Chadwick et al. 2013).The resulting γ-lactone ring is either cis or trans-fused (Ivanescu et al. 2015), and usually contains an α-methylene group, a group implicated mechanistically for various therapeutic effects of sesquiterpene lactones (Arantes et al. 2011). This α-methylene-γ-lactone group acts as a Michael acceptor and irreversibly alkylates nucleophiles (sulfhydryl or amino groups) in enzymes, transcription factors, and other proteins (Schmidt 2006). The cytotoxic action of sesquiterpene lactones likely derives from this irreversable alkylation, which results in chemical and steric disruption of the moieties the α-methylene-γ-lactone group acts upon (Kupchan et al. 1971). Sesquiterpene lactones with both α-methylene-γ-lactone groups

and α,β-unsaturated ketones were found to be the most cytotoxic, but generally it is believed that the *α*-methylene-*γ*-lactone group is the most responsible entity (Chadwick et al. 2013). Sesquiterpene lactones with an OH or O-acyl group neighboring the *α*-methylene-*γ*-lactone are believed to have greater potency (Chadwick et al. 2013).

Other significant functional moieties in sesquiterpene lactones can include hydroxyls, esterified hydroxyls, epoxide groups, halogen and sulfur atoms (Chaturvedi 2011).

While the alkylating effect of the *α*-methylene-*γ*-lactone group is implicated as the primary mode of action of sesquiterpene lactones, other factors which confer biological effects include the number of alkylating groups, lipophilicity, molecular geometry and size, the target sulfhydryl, and functional groups in close proximity to the *α*-methylene-*γ*-lactone group (Chadwick et al. 2013; Chaturvedi 2011).

Since a single synthase may produce differential structures and post-sesquiterpene synthesis modifications such as oxidation and glycosylation may take place (Lange et al. 1987; Little and Croteau 2002), sesquiterpene lactones possess significant structural diversity with over 5000 reported chemical structures (Wedge et al. 2000). All structures contain a fused 5-membered lactone group (γ lactone) with a carbonyl moiety at the alpha position (Chadwick et al. 2013). However, numerous categories exist: germancranolides (the most prevalent category and the precursor of the majority of sesquiterpene lactones) possess a 10 membered ring; eudesmanolides have two fused 6 membered rings; guaianolides have a 7 membered and a 5 membered ring, and a methyl group at C-4; and pseudoguaianolides have a 7-membered and a 5-membered ring and a methyl group at C-5. Oxidation of the 3C side chain of germacranolides is the genesis of the lactone ring in

this category of sesquiterpene lactones, and guaianolides and eudesmanolides are further derived from this precursor (Seaman 1982) (see **Figure 6** below).

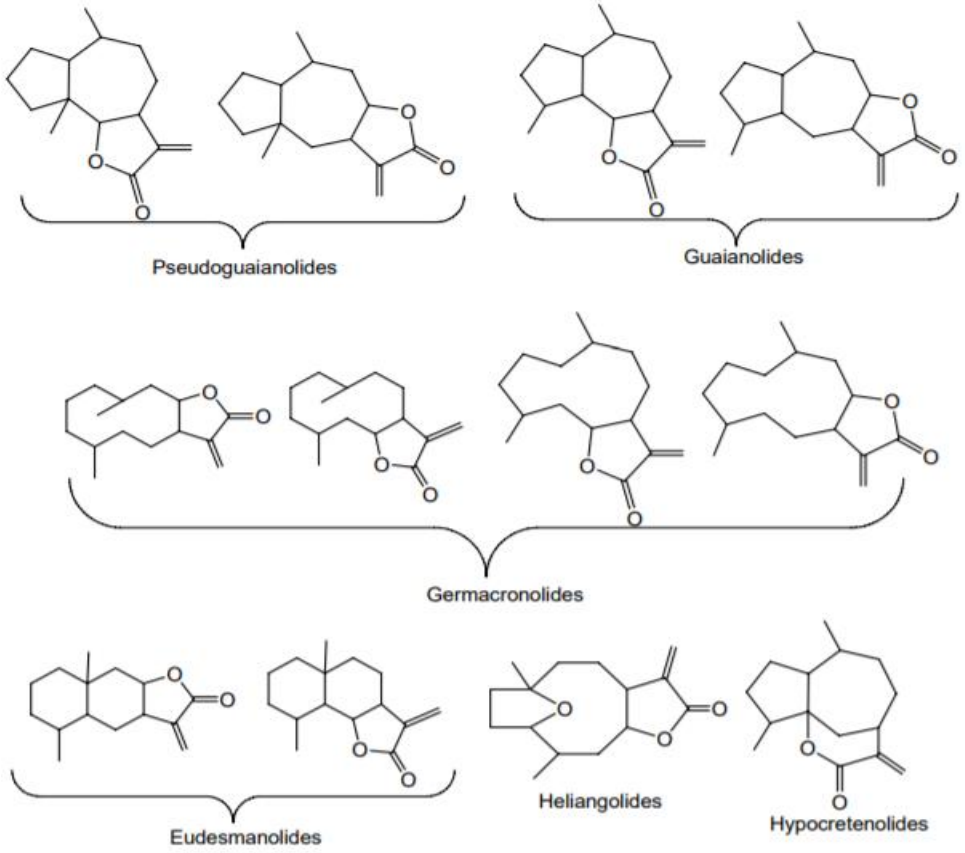

Figure 6. **Sesquiterpene lactone structures** (Chaturvedi 2011)

The chemical bonds of some sesquiterpene lactones are thought to possess the source of their biological effects: for instance, in artemisinin, the cleavage of the peroxide bond in vivo is thought to precipitate the release of reactive oxygen species which neutralize the *Plasmodium* parasite which causes malaria (Ding et al. 2011) (see **Figure 7** below). The chemical synthesis of artemisinin originated from a secret research project in 1963, called Project 523 (Cui and Su 2009) to find anti-malarial compounds both for the North Vietnamese and for Chinese citizens (Graziose et al. 2010). In 1972, artemisinin, a highly oxygenated sesquiterpene, containing a unique 1,2,4-trioxane ring structure, was

extracted and identified as an efficacious anti-malarial compound from *A. annua* (Tu 1999). The effectiveness of artemisinin was initially underestimated in the Western world (Attaran et al. 2004) but has demonstrated efficacy (along with its two main derivatives artemether and artesunate) against both malaria and schistosomiasis (Zhang et al. 2014).

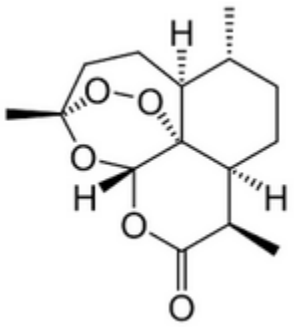

Figure 7. Artemisinin ((Ding et al. 2011)

Another chemical moiety in artemisinin, its endoperoxide bridge, is thought to account for its anti-tumor effects. The iron in cancer cells cleaves the endoperoxide bridge, and forms free radicals, which in turn, facilitate anti-cancer effects including apoptosis, and inhibition of angiogenesis, invasion, and metastasis (Crespo-Ortiz et al. 2012).

Another sesquiterpene lactone, parthenolide, appears to work via a similar mechanism as artemisinin on cancer cells: upon contact with Fe (II) in cancer cells, parthenolide is cleaved to highly oxidizing cytotoxic metabolites (see **Figure 8** below).

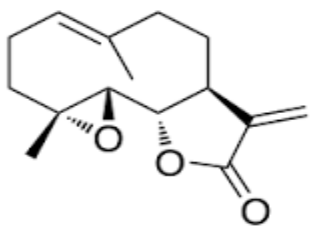

Figure 8. Parthenolide (Crespo-Ortiz et al. 2012)

Additionally, parthenolide sensitizes cancer cells to other anti-cancer drugs such as tamoxifen, by forming adducts with glutathione via cysteine bonding. Glutathione functioning is subsequently impaired which leads to impaired intercellular redox balance and a pro-apoptotic effect on the tumor cells (Guzman et al. 2005; Chadwick et al. 2013).

1.7.3. The Effect of Dehydroleucodine on the Proliferation and Migration of Tumor Cells

Dehydroleucodine is a sesquiterpene lactone of the guaianolide group (Chaturvedi 2011), which also contains the α-methylene-γ-lactone ring (see **Figure 9** below). Dehydroleucodine was first isolated from *Lidbeckia pectinata* (Bohlman and Zdero 1972), but has also been reported from the aerial parts of *A. douglasiana* (Giordano et al. 1990).

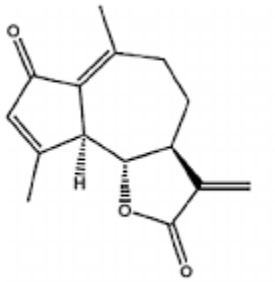

Figure 9. Dehydroleucodine (Chaturvedi 2011)

Genotoxins that are used for cancer treatment usually affect cellular proliferation by increasing replication stress (Jackson and Bartek 2009). Alterations in the coordinated replication process typically result in the accumulation of stalled, asymmetric, or broken replication forks (Gottifredi and Prives 2005). The defective activation of pathways that repair DNA lesions generally trigger cell death programs (e.g., apoptosis), permanent cell cycle withdrawal, or senescence (Bartek and Lukas 2007).

Costantino et al. (Costantino et al. 2013) also showed the antiproliferative effect of dehydroleucodine in human cancer cells. Analysis of the accumulation of DNA damage markers revealed a striking correlation between the extent of DNA damage and the activation of senescence and apoptosis programs, which were selectively stimulated by lower and higher DhL concentrations, respectively. Clonogenic assays revealed the very effective depletion of proliferating cells by dehydroleucodine-induced apoptotic and senescence programs.

Further analysis of the novel role of dehydroleucodine in cellular senescence showed that the antiproliferative process was associated with a delay in the progression through the G_2 phase that preceded an arrest in the following G_1 phase. This phenomenon was accompanied by reduced levels of cyclin B1 and higher p53 levels suggesting that p53 has the ability to promote cell cycle withdrawal (Costantino et al. 2013). Transient dehydroleucodine treatment (8 h) was equally as effective as continuous dehydroleucodine treatment in terms of the numbers of cells that displayed premature senescence. Our findings and others (Bailon-Moscoso et al. 2015) indicate that dehydroleucodine activates different antiproliferative programs depending on the time frame and on the concentration delivered to cells. Future studies on the effects of dehydroleucodine in three-dimensional cultures and in solid tumors may lead to improvements in the delivery efficiency of anticancer agents to regions of tumors that are the most difficult to reach. Finally, it was demonstrated that the novel compound dehydroparishin-B blocks cell proliferation (Priestap et al. 2012). Future work will be required to address whether dehydroleucodine and dehydroparishin-B have additive effects on cell cycle progression.

It has been reported (Priestap et al. 2012) that dehydroparishin-B (DhP) inhibits migration of B16 melanoma cells in a dose dependent manner (IC_{50} = 72.2 mM). In addition, treatment of B16 melanoma cells with 5 mM (or higher) dehydroleucodine significantly inhibits cell migration after 16 h exposure (IC_{50} = 5.4 μM). These observations indicate an increased sensitivity of B16 melanoma cells to dehydroleucodine as compared with dehydroparishin-B. The migratory inhibitory properties of dehydroleucodine and dehydroparishin-B on this cell line are comparable with those of staurosporine, a potent apoptotic agent (Wang et al. 2009). Consistent with these observations, dehydroleucodine and dehydroparishin-B inhibit secretion of matrix metalloprotease-2 (MMP-2), which is a known protease involved in migration and invasion of B16 cells (Hofmann et al. 2000). In these assays, dehydroleucodine was 10 times more active than dehydroparishin-B, as well as 11,13-dihydro-dehydroleucodine. Thus, it was hypothesized that the presence of the methylene γ-lactone moiety in the molecule would be required for optimal activity.

1.7.4. The Effect of Dehydroleucodine on Bacterial Growth

The biomass of bacteria on Earth, comprising approximately 5×10^{30} cells, exceeds that of all plants and animals (Whitman et al. 1998). Bacterial cells, which exceed human cells in the human body by a ratio of ten to one (Sears et al. 2005), were here before us and will most likely be here after us. With such a gargantuan presence in our bodies and biosphere,the implications of bacteria on human health cannot be underestimated. Parasitic bacteria are linked to approximately 20% of all human cancers (Zur Hausen et al. 2009). *Helicobacter pylori* alone, which infects approximately half of all humans, is linked to 5.5% of all cancers worldwide (Parkin 2006). *Helicobacter pylori* serotypes

cause inflammation resulting in aberrant methylation in the CpG islands of multiple promoters, including those of several tumor-suppressor genes, as well as general hypomethylation, which is linked to the epigenetic loss of gene function, genomic instability, and increased cancer risk (Niwa and Ushijima 2010).

Compounding the infectious disease problem is the emergence of a hyper connected post-industrial world where abundant host-to-host interactions increase the presence and proliferation of parasites (Okeke and Edelman 2001). This mass prokaryotic exodus has resulted in the parasitic colonization of vulnerable hosts. Sepsis infection, caused primarily by Gram-positive bacterial infection (Martin 2012), which can trigger an inflammatory response leading to multiple organ failure, is characteristic of this trend. While sepsis occurs in sterile and less crowded ICUs at a rate of 6%, it can occur at a rate of up to 30% in more crowded ICUs (Vincent et al. 2006). Sepsis rates overall are increasing and over 200,000 people die each year of sepsis in the United States alone; a far greater number than mortality from HIV, breast cancer, or stroke (Martin 2012).

Increasing rates of antibiotic resistance are also alarming and add to the overall magnitude of the infectious disease conundrum. It has been proposed that in Gram-negative bacteria the outer cell membrane (diderms) evolved as a protective mechanism against antibiotic selection pressure (Gupta 2011). Due to this state of affairs, it is a finding of significant importance that certain sesquiterpene lactones have been found to exhibit significant antibacterial activity against both Gram-negative and Gram-positive species (Vega et al. 2009).

Dehydroleucodine has been shown to be effective against several bacterial species, including *Bacillus cereus, Staphylococcus aureus, S. epidermis, Escherichia coli*

and *Klebsiella pneumoniae* (Vega et al. 2009). In addition, it has also been shown to be active against *H. pylori* strains (Vega et al. 2009). Recent work has also shown dehydroleucodine to be active against methicillin resistant *S. aureus* (MRSA) and *S. epidermis* (MRSE), respectively (Ordonez et al. 2011). These observations show that both the α-methylene-γ-lactone group and α-β-unsaturated moieties are structurally necessary for the observed therapeutic effects although other structural moieties may also be required. However, no mechanism of action has been proposed. More lately, we have found that dehydroleucodine also inhibits growth as well as the secretion of several toxins of *Pseudomonas aeruginosa* (i.e., ExoS and elastase A) (Mustafi et al. 2015). Thus, sesquiterpene lactones have the potential to be utilized more specifically by targeting a resistant mechanism, such as the inhibition of a multiple drug resistance efflux pump and/or altering an unknown mechanism.

1.7.5. Additional Therapeutic Effects of Dehydroleucodine

Peptic ulcer disease is characterized by chronic inflammation of the stomach or duodenum that affects as many as 10% of the population at some time in their lives (Penissi et al. 2003). Traditional cultures in Argentina utilize *A. douglasiana* for treatment of peptic ulcer, which led to cytoprotective studies of extracted dehyrdroleucodine from *A. douglasiana*. These studies revealed that dehydroleucodine pretreatment prevents gastrointestinal damage in response to necrosis-inducing agents such as absolute ethanol in a dose-dependent manner (Giordano et al. 1990; Penissi et al. 2003).

Additionally, these studies revealed that the exocyclic methylene group conjugated to a γ-lactone is required for dehyroleucodine's cytoprotective activity, but the

presence of the β-substituted or α-β-unsubstituted cyclopentenone ring is not required, although it is implicated in additional dehydroleucodine activity, including anti-tumor, anti-microbial and anti-feedant properties (Giordano et al. 1990; (Penissi et al. 1998; Penissi and Piezzi 1999), and in inhibiting pro-inflammatory mediator release from mast cells (Penissi et al. 2003).

Given these structural and functional components of dehydroleucodine and its cytoprotective effect, it is perhaps unsurprising that dehydroleucodine has also been found to have antidiarrheal effects. In a study where it was suggested that the α2-adrenergic receptors mediate the effect of dehydroleucodine on intestinal motility, dehydroleucodine inhibited castor oil-induced diarrhea in mice and also reduced intraluminal accumulation of fluid (Wendel et al. 2008).

Dehydroleucodine has various other therapeutic efficacies beyond its anti-adipogenic, cytoprotective, and anti-diarrheal effects. For instance, it has been found to have an anti-parasitic effect on Chagas disease, a tropical disease of the cardiac and neurological system found primarily in endemic areas of 21 Latin American countries through infection by the parasite *Trypanosoma cruzi*. Only two drugs, Benzindazol and Nifurtimox, are accepted for treatment of Chagas disease, yet both cause severe side effects and are of controversial efficacy. Dehydroleucodine was found to induce apoptosis in the replicative epimastigote form, and the infective trypomastigote form, whereas Benzindazol and Nifurtimox do not (Jimenez et al. 2014). Trypanocidal activity of dehydroleucodine may be mainly dependent on the covalent bond formation between the γ-lactone moieties and -SH group of trypanothione and trypanothione-dependent enzymes affecting these parasite oxidative stress conditions (Krauth-Siegel et al. 2003).

Thus, dehydroleucodine shows an adequate selectivity index comparable with trypanocidal drugs.

Dehydroleucodine also inhibits the *in vitro* growth of *Leishmania mexicana* (Barrera et al. 2008), an obligate intracellular protozoan parasite that causes the one form of leishmaniasis which can present with ulcers of the skin, mouth, and nose (Barrett and Croft 2012). This currently affects 12 million people worldwide, and causes between 20 to 50 thousand deaths each year (Lozano 2010). With two million new cases each year (Lozano 2010), dehydroleucodine 's IC_{50} of 2–4 μM on *Leishmania mexicana*, which constitutes a higher efficacy than ketoconazole, appears to be a much needed and promising therapeutic modality.

Additional anti-microbial activity was found with *in vitro* treatment with dehydroleucodine of *Staphylococcus aureus* and *S. epidermidis* (Ordonez et al. 2011). The lactone ring of dehydroleucodine was found to be essential for this antimicrobial activity (Ordonez et al. 2011). As *S. aureus* is the microbe implicated in methicillin resistant *S. aureus* (MRSA), which has resulted in deaths that exceed the mortality rates for HIV/AIDS in the United States, dehydroleucodine's effect on these microorganisms is an important avenue for therapeutic investigation (Klevens et al. 2007). It is generally accepted that the α-methylene-γ-lactone moiety is the most important part of these compounds to confer biological activity. However, other sesquiterpene lactones containing neither the α-methylene-γ-lactone moiety nor chlorine, bromine, and hydroyxl groups (groups possessed by other sesquiterpene lactones which exhibit activity) in their structure are active compounds (Ozcelik et al. 2009). Therefore, it is possible to

hypothesize that these structural requirements are important, but not essential for a potent antimicrobial activity.

1.8. Research Questions

The 3T3-L1 murine preadipocyte cell line is a common experimental model system for adipogenesis research which allows for the observable accumulation of triglycerides in this system upon differentiating in culture (Rosen and Spiegelman 2000). Adipocyte differentiation is induced by the expression and/or phosphorylation of specific genes such as AMPKα (Kemp et al. 2003), Akt1 (Rosen and Spiegelman 2000), Erk1/2 (Rosen and Spiegelman 2000), transcriptional factor peroxisome proliferator-activated receptor γ (PPARγ) (Spiegelman et al. 1993) and factors from other transcription factor families, including CCAAT/enhancer-binding proteins (C/EBPs) (Bost et al. 1990), signal transducers and activators of transcription (STATs), and Kruppel-like factor (KLF) proteins (Mori et al. 2005). Most of these regulators have been discovered *in vitro*, utilizing the 3T3-L1 or 3T3-F442A murine preadipocyte cell lines (Rosen and Spiegelman 2000).

After preadipoycte proliferation to confluence, an induction media (IM) cocktail of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) initiates the differentiation program (Okamura et al. 2010). Growth arrest of confluent preadipoyctes is followed by rapid expression of C/EBPβ but not its activation (Choi et al. 2013). Other early adipogenic transcription factors include C/EBPδ , and Kruppel-like factors 4 and 5. The process of mitotic clonal expansion (MCE), which coincides with the activation of C/EBPβ, then follows, where growth arrested preadipocytes exit the cell cycle and undergo two to four rounds of expansion (Tang et al. 2003). CCAAT/enhancer-binding

proteins Beta is activated via sequentially phosphorylation, followed by dimerization of the phosphorylated C/EBPβ, which enables it to bind its DNA binding element with the help of reactive oxygen species (Tang et al. 2005; Tang et al. 1999; Kim et al. 2007; Lee et al. 2009). The activation of ERK and p38 are necessary for this process and MCE is required for the activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway (Rosen and Macdougald 2006; Zhang et al. 2009).

Indicators of whether preadipocytes are undergoing MCE include assaying cyclin and cyclin-dependent kinase (CDKs) cell cycle progression markers. The cell cycle progression or its arrest is evidenced by either the initiation or downregulation of these markers which correspond to different stages of the cell cycle. For instance, during G1 phase, D-type cyclins and cyclin E are activated. Next, activated cyclin D assembles with CDK4 or CDK6 and cyclin E binds to CDK2 (Choi et al. 2012). Cyclins A and B are then recruited to CDK2 and CDK1, respectively, and the resulting binding induces cell cycle progression through S phase and mitosis. (Choi et al. 2012).

The aforementioned early adipogenic regulators such as C/EBPβ induce PPARα and C/EBPγ expression approximately 36 to 48 hours after induction. Peroxisome Proliferator Activated Receptor Alpha and C/EBPγ then stimulates the expression of triglyceride synthesis genes, such as the genes for adipocyte fatty acid-binding protein 2 (aP2), fatty acid synthase (FAS), acetyl-coenzyme A carboxylase (ACC), which result in the concentrated lipid droplets indicative of the mature adipoycte phenotype (Moseti et al. 2016).

Using 3T3-L1 preadipocytes, an *in vitro* system for adipocyte differentiation, it has been demonstrated that dehydroleucodine significantly inhibited the differentiation of

murine preadipocytes and also resulted in a significant decrease in the accumulation of lipid content by a dramatic downregulation of the expression of adipogenic-specific transcription factors PPARγ and C/EBPα (Galvis et al. 2011).

Since the level of expression of PRARγ and C/EBPα is undetectable in preadipocytes, but increases two days after induction of differentiation into mature adipocytes, and are maximally expressed five days after the induction of differentiation (Rosen et al. 2000), it would appear that the selective inhibition of PRARγ and C/EBPα by DhL is responsible for its observable anti-adipogenic effects, which include the inhibition of differentiation, and decreased triglyceride accumulation (Galvis et al. 2011).

However, numerous questions arise from these evidentiary foundations. To what extent does DhL affect the process of preadipoycte proliferation which is a perquisite for the subsequent process of mitotic clonal expansion? How early does DhL inhibit the progression of the 3T3-L1 preadipocyte through the differentiation program? Does DhL inhibit cell cycle regulators cyclin A, cyclin D, CDK2 and CDK6? If so, what is the timing of this inhibition? By evaluating these queries and determining what genes may be altered at what time points by DhL, a greater understanding of the precise role of the mechanism through which DhL arrests adipogenesis will be achieved. Therefore, I propose the following Specific Aims:

1.8.1. First Aim: Determine the role of dehydroleucodine during the early steps of in vitro 3T3-L1 preadipocytes differentiation

I will examine the effect of dehydroleucodine on the expression of specific genes involved in the two early and essential steps of adipogenesis: 3T3-L1 preadipocyte proliferation and cloning expansion.

1.8.2. Second Aim: Elucidate how 11,13-dihydro-dehydroleucodine derivatives affect different steps of in vitro 3T3-L1 preadipocytes differentiation

Utilizing a variety of methods, I aim to study the anti-adipogenic effects of 11,13-dihydro-dehydroleucodine derivatives on preadipoycte proliferation, and the molecular timing of its mode of action in relation to PPARγ and mitotic clonal expansion, including western blotting for signaling markers indicative of different temporal stages of these processes, and ORO assaying of triglyceride accumulation after treatment subsequent to the application of a PPARγ agonist.

1.8.3. Third Aim: **Identify the additional compound(s) accountable for inhibition of *in vitro* 3T3-L1 preadipocyte differentiation**

As preliminary results showed that structures closely related to dehydroleucodine may be responsible, compound(s) will be also tested for anti-adipogenic effects.

1.9. References

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II. DEHYDROLEUCODINE INHIBITS MITOTIC CLONAL EXPANSION DURING ADIPOGENESIS THROUGH CELL CYCLE ARREST

2.1. Introduction

Dehydroleucodine (DhL), a sesquiterpene lactone of the guaianolide group, which contains an α-methylene-γ-lactone ring, has been isolated from the aerial parts of *Artemisia douglasiana* (Chaturvedi 2011; Giordano et al. 1990). Although DhL significantly inhibited the differentiation of 3T3-L1 preadipocytes in a time and concentration dependent manner by blocking the expression of PPARγ and C/EBPα (Galvis et al. 2011), the exact cellular and molecular mechanism by which DhL affected differentiation of 3T3-L1 preadipocytes is still not well understood. Interestingly, DhL inhibited proliferation and altered the cell cycle of cancer cells (Costantino et al, 2013). Therefore, the inquiry behind this line of experiments was founded on the hypothesis and possibility that DhL affects the early stage of the differentiation of 3T3-L1 preadipocytes (e.g., mitotic clonal expansion). The objective of this line of experiments was to explore the effect of DhL on MCE during differentiation of 3T3-L1 preadipocytes, as well as elucidate aspects of the mechanism by which these effects occur.

The effects of DhL were investigated on adiopgenic processes modeled *in vitro* by utilizing a 3T3-L1 murine preadipoycte model system (Ruiz-Ojeda et al. 2016; Green and Kehinde 1975), where cells are differentiated into mature adipocytes over the course of approximately 9 days through stimulation with an induction media (IM) cocktail consisting of growth media (GM) with dexamethasone, isobutylmethylxanthin (IBMX), and insulin, in the first phase of differentiation, and GM with insulin alone in the second phase of differentiation. The transition to a mature adipogenic phenotype is evidenced by

the accumulation of triglycerides (Kassotis et al. 2017; Rosen and Spiegelman 2000), as well as the expression and/or phosphorylation of numerous genes including Akt1, Erk1/2, (Jeong et al. 2015), the transcriptional factor peroxisome proliferator-activated receptor γ (PPARγ), and CCAAT/enhancer-binding proteins (C/EBPs) (Tao et al. 2016).

CCAAT/enhancer-binding Protein Beta is expressed within two to four hours of treatment with IM, and crucially acquires DNA binding activity at 12 hours post induction, which coincides with the essential process of mitotic clonal expansion (MCE). Mitotic clonal expansion, where cells undergo growth arrest followed by two to four rounds of mitosis, is a necessary phase for the progression and completion of the adipocyte differentiation program (Tang et al. 2003). CCAAT/enhancer-binding Protein Beta's DNA binding functionality is aided by retinoblastoma protein (Rb) (Chen et al. 1996). In contrast to Rb's role in negatively regulating transcription factor E2F-1 to prevent quiescent cells from passing a restriction point in G_1, Rb binds with and activates C/EBPβ and does so specifically during the adipogenic differentiation process (Chen et al. 1996). Mitotic clonal expansion also requires a synchronized activation of the ERK and p38 signaling pathways (Aouadi et al. 2007; Tang et al. 2003; Yang et al. 2002). Progression from G_1 to S requires downregulation of p27 and activation of CDK2 by cyclins E and A (Patel and Lane 2000; Sherr et al. 1999). Cyclin D1 and cyclin E are activated during the G_1 phase of the cell cycle (Choi et al. 2012). Once activated, cyclin D1 assembles with CDK4 or CDK6, or both, and cyclin E binds to CDK2 (Choi et al. 2012). Cyclin A and B are then recruited to CDK2 and CDK1, respectively; associations necessary for the inducement of cell cycle progression through S phase and mitosis (Choi et al. 2012). Activated C/EBPβ, along with C/EBPδ, triggers the expression of C/EBPα,

which leads to the expression of PPARγ (Farmer et al. 2006). Once C/EBPβ is activated during MCE, it targets the promoter of the chromatin regulator gene *Kdm4b*, whereas C/EBPβ as a co-factor targets the promoters of cell cycle genes, including *Cdc45l* (cell division cycle 45 homolog), and demethylates H3K9me3 in their regulatory regions, which in turn activates their transcription (Guo et al. 2012). Cyclin dependent kinase 451 is a component of the CMG complex, which is required for the initiation and elongation steps of eukaryotic chromosomal DNA replication (Tercero et al. 2000). Histone demethylase CDC25A is required for progression from G_1 to the S phase of the cell cycle, and acts by activating the pro-mitotic Cdc2 (CDK1) and also by activating G_1/S cyclin-dependent kinases CDK4 and CDK2 by removing inhibitory phosphate groups from adjacent tyrosine and threonine residues (Goloudina et al. 2003). CCAAT/enhancer-binding Protein Beta also stimulates expression of histone H3K9 demethylase JMJD2B, which removes H3K9me3 and H3K9me2 on the promoters of PPARγ and C/EBPα, which stimulates their expression (Guo et al. 2012; Jang et al. 2017). Myeloid/lymphoid or mixed-lineage leukemia protein 3 and MLL4, H3K4me1/2 methyltransferases that become part of the activating signal cointegrator-2-containing complex (ASCOM), both redundantly co-activate PPARγ and C/EBPα (Lee et al. 2008; Qi et al. 2003; Kuang et al. 2002; Hong et al. 2001). In opposition to these effects, histone methyltransferase G9a represses *PPARγ* expression by adding H3K9me2 to the entire *PPARγ* gene locus and promotes *Wnt10a* expression independent of its methyltransferase activity, therefore repressing adipogenesis (Wang et al. 2013).

Therefore, to investigate the role of DhL during the early steps of *in vitro* 3T3-L1 preadipocytes differentiation, the aforementioned 3T3-L1 experimental system was

utilized and 3T3-L1 preadipocytes were incubated in IM with wells of 3T3-L1 preadipoyctes in growth media (without dexamethasone, IMBX, or insulin) as controls. *Artemesia douglasiana* was obtained from Mendoza, Argentina, and DhL was extracted, purified and identified. Treatments of varying concentrations of dehydroleucodine for varying time points were then applied. To examine whether DhL causes stage-dependent inhibition of adipocyte differentiation, 3T3-L1 cells were treated with 9 μM DhL at several time points after induction with IM. The Oil Red O assay, in which lipid droplets can be quantified (see Materials and Methods below) was utilized to determine whether and when DhL inhibited differentiation.

Next, the effect of DhL on the expression of CBEPβ in 3T3-L1 cells, an early marker of adipogenesis, was investigated via western blotting to gather additional evidence regarding when in the molecular cascade DhL exerts its effects. Early stage markers further down the cascade, PPARγ and C/EBPα, were also investigated in a similar fashion utilizing western blot.

The effects of DhL on proliferation of 3T3-L1 preadipocytes after induction of differentiation with IM were investigated via a cell proliferation assay. Additionally, the effects of DhL on cell cycle progression were investigated via flow cytometry and via western blotting of cyclin D1, cyclin A, and CDK2.

Since several histone methyltransferases and demethylases have been reported to be involved in adipogenesis, the expression of JMJD2B and the cell cycle genes CDC25A and CDC451 in DhL-treated 3T3-L1 cells during the MCE process to gain further mechanistic clues, was investigated.

Lastly, to examine whether whether DhL affects the expression of PPARγ as well as methytransferases involved in its expression during adipogenesis, qPCR was utilized to gage mRNA levels of PPARγ, MLL3, MLL4 and G9a in DhL–treated 3T3-L1 cells.

2.2. Materials and Methods

2.2.1. Reagents

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). The Dulbecco's Modified Eagle's Medium (DMEM), bovine calf serum, and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). All reagents were obtained from Sigma-Aldrich unless otherwise stated. Antibodies against C/EBPβ, PPARγ, FAS, phospho(p) AMPK, C/EBPα, cyclin A, cyclin D1, CDK2, CDK4, p-Rb, p27, total(t)-Erk1/2, phospho(p)-Erk1/2, t-Akt, and p-Akt, were obtained from Cell Signaling Technology (Beverly, MA). The dilution was 1 uL in 10 mL of tris-buffered saline (TBS) and Polysorbate 20 (TBST) (1:10,000 dilution). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All chemicals were of analytical grade. A Triglyceride Quantification Kit was purchased from BioVision Research Products (Mountain View, CA).

2.2.2. Cell Culture and Differentiation

Reagents used to culture these cells in growth media (GM) include DMEM, 1% penicillin/streptomycin and 1% L-glutamine, all of which were purchased from Mediatech, Inc. (Manassas, VA), and 10% fetal bovine serum purchased from Invitrogen (Carlsbad, CA). Two different recipes for the induction media (IM) utilized to trigger adipoycte differentiation were employed during different times in the process. For the IM used during day 1 and day 3 of differentiation, GM was supplemented with 670 nM

insulin (IS), 65 nM dexamethasone (DEX) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Induction media applied to the 3T3-L1 cells during days 5, 7, and 9 of differentiation consisted of GM supplemented with 670 nM of insulin. Cells of passage three or below were grown to confluence in a humidified atmosphere of a 5% CO2 incubator at 37°C.

2.2.3. Treatments

Dehydroleucodine (DhL) and dehydroparishin-B (DhP) was isolated as previously described (Giordano et al. 1990; Priestap et al. 1990) from *Artemisia douglasiana* collected in Mendoza, Argentina, voucher #37546 (**Figure 10**). Dehydroleucodine was gently reduced through the utilization of sodium borohydride to obtain the corresponding 11,13DH-[11R]-DhL epimer (DH-DhL), which was purified as previously described (Galvis et al. 2011). 3T3-L1 cells were exposed to 11,13DH-[11R]-DhL throughout the entire differentiation process, unless otherwise indicated.

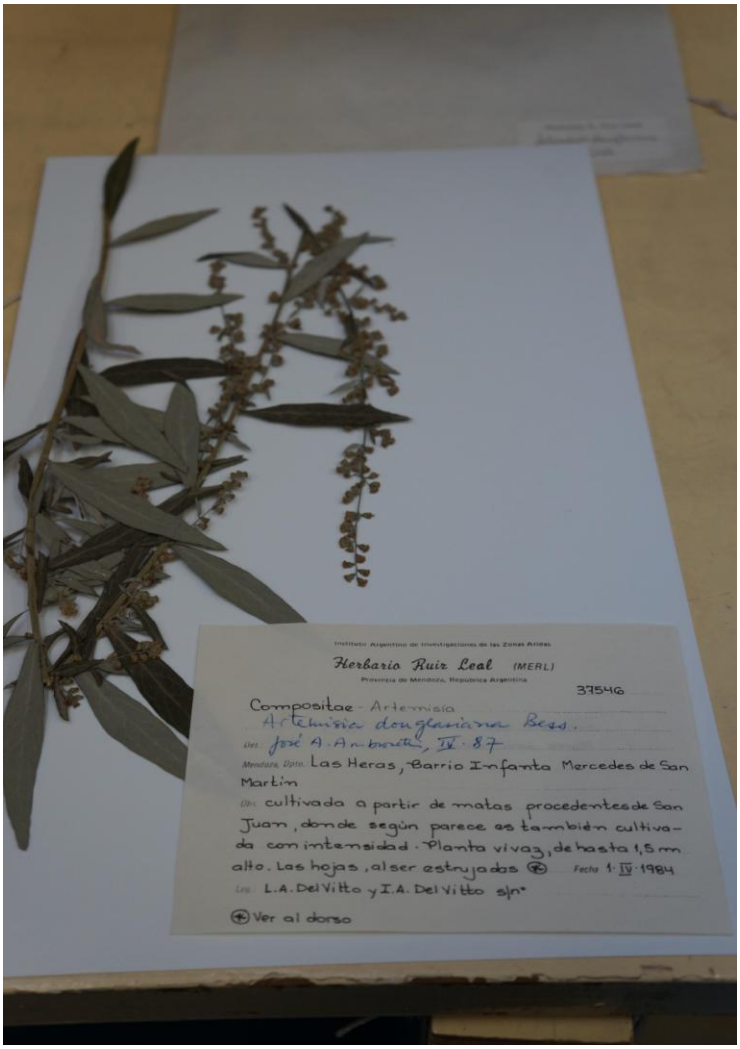

Figure 10. ***Artemisia douglasiana*** **Voucher**

2.2.4. Cell viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Thermo Fisher Scientific. Inc., Pittsburgh, PA) colorimetric assay was utilized to quantify cellular viability. Cells (1 x 10^6 cells/mL) were incubated at 37°C and 5% CO_2 in the absence or presence of different concentrations of 11,13DH[11R]-DhL for various periods of time. After this initial incubation, cells were incubated with MTT reagent at a final concentration of 10µl/ml, and further incubated at 37°C and 5% CO_2. Next, the

formazan crystals were dissolved in a detergent reagent of sodium dodecyl sulfate in diluted hydrochloric acid, and each condition was then quantified via spectrophotometry (Ultrospec 2100 Pro UV/Visible Spectrophotometer) at an optical density of 570 nm (OD_{570}). The trypan blue dye exclusion assay was also utilized for the DhP series of experiments. Cells (1x105) were plated in 6-well plates in DMEM +10% FBS, serum-starved for 12 h, and then stimulated with 10% serum for 24 h in the absence or in the presence of different concentration of dehydroparishin-B (1 to 9 μM). After incubation, the cells were removed from the 6-well plate by treatment with trypsin. Cell suspensions were aspirated, centrifuged at 600 rpm for 5 minutes and washed twice with sterile PBS (pH 7.4). The cell suspension was then treated with trypan blue dye (0.4% solution) at a ratio of cell suspension:dye 4:1 and placed in hemocytometer. Live (unstained) and dead (blue stained) cells were counted using a 63x magnification in phase-contrast inverted microscope. The untreated cells (control) were also processed simultaneously under the identical conditions.

2.2.5. Oil Red O staining, microscopy, and spectrophotometry

On the tenth day of the differentiation program, the adipocytes were fixed with 10% formalin (Thermo Fisher Scientific. Inc., Pittsburgh, PA) solution in phosphate buffer saline (PBS) for 1 hour at 4°C. After fixing, cells were washed with 60% isopropanol, and then stained with 0.3% Oil Red O solution for 45 minutes at room temperature. Cells were washed twice with double distilled water. The stained lipid droplets were visualized by light microscopy and photographed with a digital Leica DC 500 camera at 100x magnification. Images from 250 3T3-L1-control cells and 250 11,13DH[11R]-DhL treated cells were examined. The size distribution of lipids droplets

was analyzed by the NIH Image J software, which can be accessed at the web site (http://rsb.info.nih.gov/nih-image/). For quantification of the lipid-content, Oil Red O was eluted from cells by adding 100% isopropanol for 20 minutes, and quantified by spectrophotometry at $OD_{540.}$ Cells were treated for all days of the experiment with the appropriate sesquiterpene lactone, unless otherwise indicated.

2.2.6. Triglyceride assay

The lipid droplets in the cellular suspension were analyzed for total concentration of triglyceride in the following manner: cells were washed with PBS, scraped, centrifuged, and lysed with 5% Triton X-100. Cell lysates were then centrifuged at 10,000 x g for 20 minutes at 4°C. Then, cell supernatants were used to measure the conversion of triglycerides to fatty acids and glycerol. The supernatants were then oxidized and quantified at OD540 based on a triglyceride standard curve (Cayman Chemical, Arbor, MI).

2.2.7. Cell proliferation assay

The 3T3-L1 cells (5×10^4 cells/mL) were cultured in appropriate plates and then incubated with GM. When 3T3-L1 cells reached confluence, cells were incubated in a humidified atmosphere of a 5% CO_2 incubator at 37°C with IM in the absence or in the presence of various concentrations of 11,13 DH[11R]-DhL up to 48 hours. Cells were then harvested and quantified using an automated cell counter machine from Bio-Rad (Hercules, CA).

2.2.8. Western blot analysis

Cell lystate preparation was conducted via the washing of cell monolayers with a mixture of ice-cold lysis buffer (20mM Tris-HCL pH7.5, 150mM NaCl, 1% NP40, 1mM

Na2 EDTA, and 0.1% Na Deoxycholate), protease inhibitors, and phosphatase inhibitors. After centrifugation, protein concentrations were quantified through the use of the BCA protein assay. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Thermo Fisher Scientific. Inc., Pittsburgh, PA), blocked in 5% bovine serum albumin overnight at 4°C, and probed with the appropriate primary antibodies. Membranes were next incubated with horseradish peroxidase-conjugated secondary antibodies overnight at 4°C. Bands were visualized with enhanced chemiluminescence and the intensities of the bands were quantified in WCIF Image J for Windows (University Health Network Research, Toronto, Canada) and relative levels of proteins were determined by densitometric analysis.

2.2.9. Flow Cytometry Analysis

Cell cycle progression was measured by flow cytometric analysis after propidium iodide staining. Post confluence 3T3-L1 preadipocytes were incubated with IM in the absence or presence of 11,13DH[11R]-DhL for 24 hours. A suspension of cells were fixed with 70% ethanol at 4°C and then incubated with propidium iodide staining buffer for one hour. Cells were analyzed with an Acurri-C6 Flow Cytometer (BD Biosciences, San Diego, CA) and cell cycle progression was measured with the Modfit LT program (Verity Software House, Topsham, ME).

Caspase-3 was analyzed by flow cytometry using PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences, USA). Cells ($1x10^6$ cells/0.5 mL) were collected, washed once with cold PBS and then washed twice with a washing buffer, before PE-conjugated monoclonal anti-active caspase-3 antibodies (BD Biosciences, 1:500) were added. After washing, cells were incubated for 30 minutes at

room temperature. The marked samples were analyzed by flow cytometry as described above.

2.2.10. High-performance liquid chromatography

The high-performance liquid chromatography (HPLC) equipment consisted of a SpectraSystem SMC1000 solvent delivery system, vacuum membrane degasser, P4000 gradient pumps and AS3000 autosampler (Thermo Electro Corporation, San Jose, CA). Column effluent was monitored at 254 nm with Spectra System UV6000LP variable wavelength PDA detector and ChromQuest 4.1 software. DhL and DhP were separated using a C18 YMC column (A-302, 150 Å~ 4.3 mm i.d., S-5 µ m, 12 nm; Waters) and the following solvents: A. acetonitrile; B. 0.1 % TFA in water. System 1: linear gradient 10% to 100% A in 120 min; flow rate 1 ml/min. Preparative HPLC was performed in the above equipment with a XTerra Prep MS C18 OBD column, 15 µ m, 19 Å~ 50 mm (Waters) and the solvent system 25% acetonitrile -75 % 0.1 % TFA in water (isocratic); flow rate 2.5 ml/min.

2.2.11 Compound Extraction, Purification and Identification

The dried crushed plant material (100 g, dry weight) was exhaustedly extracted with boiling methanol. The methanol extracts were extracted with hexane ethyl acetate, acetone, and aqueous 5% acetic acid and 5% $NaCO_3H$, respectively. The organic phases were set-aside and evaporated. The aqueous $NaCO_3H$ phases containing dehydroparishin-B were acidified with 0.1N HCl and extracted with ethyl acetate. The organic and water phases were evaporated to dryness to give an oily residue (280 mg), which was subjected to column chromatography on silica gel (60, H70-230, Merck 7734). The column was eluted with hexane containing increasing proportions of ethyl acetate (0 to 40 %), and

fractions were monitored by TLC. Fractions containing the Rf 0.29 compound were joined and re-chromatographed in a similar way to give pure dehydroparishin-B (3.2 mg).

(7R)-2-Oxo-guaia-1(10),3(4),5(6),11(13)-tetraen-12-oic acid, dehydroparishin-B (**1**): White needles (ethyl acetate); HPLC, Rt 14.92 min (system 1); UV/PDA, λ_{max} 239, 256, 317 nm; GC/MS, Rt 18.74 min; MS (EI), m/z (rel. int.): 244 (100) $[M]^+$, 198 (35.1) $[M\text{-}CO_2\text{-}H_2]^+$, 183 (63.3), 159 (46.6), 155 (46.9), 141 (34.1), 129 (50.6), 128 (48.5), 115 (64.0), 91 (48.1), 77 (38.2).^{1}H NMR and ^{13}C NMR, see Table 1. ESI-TOF-MS m/z 245.1162 $[M + H]^+$ (calcd for $C_{15}H_{17}O_3$, 245.1172), 267.0997 $[M + Na]^+$ ($C_{15}H_{16}O_3Na$, 267.0992), 289.0817 $[M – H + 2Na]^+$ ($C_{15}H_{15}O_3Na_2$, 289.0811).

2.2.12. Real Time Quantitative PCR

After treatment of 3T3-L1 cells with DhL, RNA was extracted from 3T3-L1 cells by using RNeasy Plus Mini Kit (QIAGEN, USA) and a cDNA library was synthesized by using iScript cDNA synthesis kit (BIO-RAD, USA) according to manufacturer's suggestion. Reverse transcription was performed with 200 ng of total RNA-sample, 1X iScript reaction mix and 1XiScript reverse transcriptase. Quantitative analysis of cDNA was performed using CFX96 Touch^{TM} real time PCR detection system (BIO-RAD, USA) and SsoAdvancedTM 227 universal probes supermix (BIO-RAD, USA) according to manufacturer's instruction. Briefly, amplification of the target cDNA was carried out with each 10 µL PCR mixtures containing 1 µL cDNA, 5 µL SSO probes, 0.5 µL primers and 3.5 µL nuclease free water. Target cDNA was amplified by using verified commercially available primer pairs (Target cDNA was amplified by using verified commercially available primer pairs (Mm01205647_g1 for Beta actin, NM_007658.3 for CDC25A, NM_003504 for CDC451, NM_172132.2d for JMJD2B, NM_001081383.1 for

MLL3, NM_001290573.1 for MLL4, NM_001289413.1 for G9A, NM_002046.5 for GAPDH, Mm00440940_m1 for PPARγ and Mm00514283_s1 for C/EBPα, Thermo Fisher Scientific, USA). The conditions of the PCR reaction were set as follows: it was begun by a denaturation cycle at 95°C for 30 min, followed by 95°C 10 min and 60°C for 25 min, respectively. The mRNA expression was normalized with beta-actin. Relative gene expression was expressed as fold change in mRNA expression level compared with the control.

2.2.13. Statistical analysis

All experiments were repeated at least three times. The standard error of the mean (S.E.M.) of triplicates was utilized and significance was analyzed by one-way ANOVA or Student's Test. All statistical data reflect averages of the three independent experiments. Results with *P<0.01 and **P<0.0001 were considered statistically significant.

2.3. Results

2.3.1. Dehydroleucodine mostly inhibits early stages of adipocyte differentiation through attenuation of mitotic clonal expansion

Previous studies have demonstrated that DhL and their epimers inhibited the differentiation of 3T3–L1 preadipocytes into adipocytes (Galvis et. al., 2011). Structure of DhL is depicted in **Figure 11A**. Adipocyte differentiation occurs through the preadipocyte stages, which can be clearly divided into proliferation and mitotic cloning expansion (early stage) and terminal differentiation (late stage) (Ali et al. 2013). Accordingly, we examined whether DhL causes stage-dependent inhibition of adipocyte differentiation. 3T3-L1 cells were treated with 9 μM DhL at several time points after

induction with IM as shown in **Figure 11B**. Treatment with DhL at early stages (days 0-2 and 2-4) significantly inhibited adipogenesis (**Figure 11B**). The treatment of DhL after day 4 (days 4-6 and 6-8) showed moderate inhibition of adipogenesis for days 4-6 (16±2 % of inhibition), whereas the treatment for days 6-8 showed no inhibition at all. As expected, the addition of DhL on day 0, strongly inhibited differentiation of 3T3–L1 preadipocytes (**Figure 11B**). Consistent with these observations, when cells were treated with DhL during early stage (day 0-2) or during the whole process (day 0-8), the intracellular triglyceride content significantly decreased in a concentration-dependent manner (**Figures 11C** and **E**).

The model system that was utilized, 3T3-L1 preadipocytes, undergoes mitotic clonal expansion through the upregulation of C/EBPβ and C/EBPδ during the early stage of adipocyte differentiation (Wu et al. 1996). This process is followed by the activation of the downstream signaling molecules PPARγ and C/EBPα, which are critical for terminal adipocyte differentiation (Farmer 2005). Therefore, the effect of DhL on the expression of CBEPβ was examined during the first 48 hours after stimulation of 3T3-L1 preadipocytes with IM (**Figure 11D**). Expression of CBEPβ was significantly decreased in a concentration-dependent manner when cells were treated with DhL (**Figure 11D)**. In addition, PPARγ and C/EBPα expression in adipocytes treated with DhL strongly decreased compared with the control cells (**Figure 11F).**

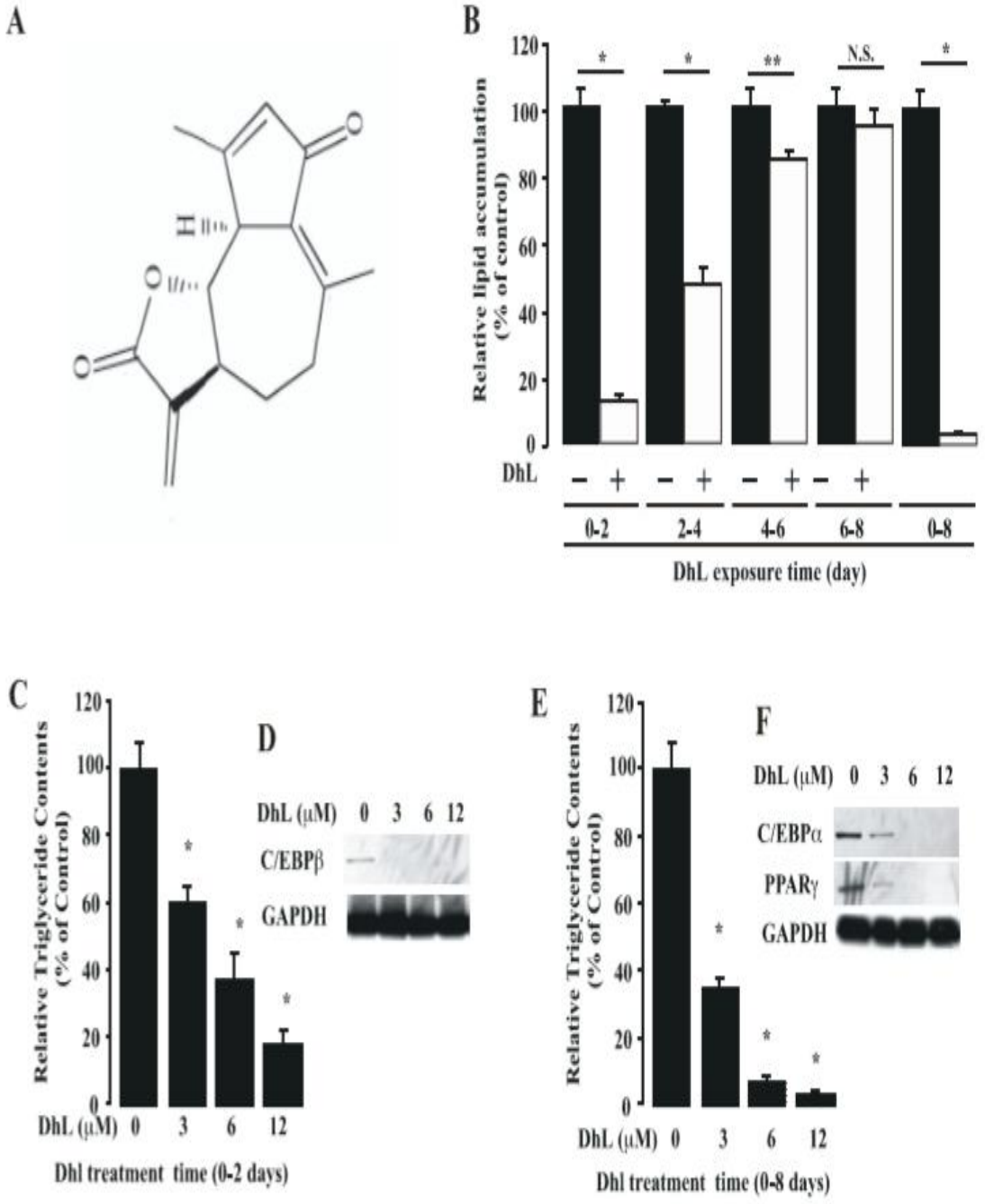

Figure 11. Inhibitory effect of dehydroleucodine on triglyceride content and adipogenic transcription factors during mitotic clonal expansion. Preadipocytes were cultured in differentiation medium containing 9 μM dehydroleucodine for day 0-2, 2-4,

4-6, 6-8 and 0-8, respectively. **(A)** Chemical structure of dehydroleucodine. **(B)** Preadipocytes were treated as described above. Cells were stained with Oil Red O and then visualized as described in Material and Methods. All values are presented as the mean ± SEM of three independent experiments performed in triplicate. **P*<0.01 vs. no dehydroleucodine treatment. Cellular triglyceride content of preadipocytes treated with 0, 3, 6, and 12 μM dehydroleucodine for day 0-2 (**C**) and 0-8 (**E**) was determined as described in Material and Methods using a Triglyceride Kit. Results are shown as relative percentage of control without dehydroleucodine. DMSO was used a vehicle control. All values are presented as the mean ± SEM of three independent experiments performed in triplicate. **P*<0.01 versus no dehydroleucodine treatment. Cells were incubated with dehydroleucodine, harvested and the lysates were subjected to western blot analysis for PPARγ, C/EBPα (**F**) and C/EBPβ (**D**). GAPDH was used as loading control. The Western blots were performed three times, and a representative image of the three independent experiments is shown (**D**, **F**).

2.3.2. Dehydroleucodine inhibits mitotic clonal expansion through down regulation of cyclins A and D and cyclin-dependent kinases 2 and 4

CCAT/Enhancer Binding Protein Beta, a specific transcriptional factor expressed in the early stage of adipogenesis, is also known to be required for MCE. Consequently, we determined whether DhL affects proliferation of 3T3-L1 preadipocytes after induction of differentiation with IM. As shown in **Figure 12A**, the treatment with DhL significantly decreased the cell numbers of IM-induced 3T3-L1 preadipocytes during day 1 as well as day 2 as compared to the cell numbers of control 3T3-L1 preadipocytes not treated with DhL. Thus, the effects of DhL that cause inhibition of differentiation of 3T3-L1 preadipocytes appear to occur during the early stage of adipogenesis.

I then investigated whether DhL modulates cell cycle progression. When 3T3–L1 preadipocyte differentiation was induced, addition of DhL arrested the cell cycle at the G_0/G_1 phase (**Figure 12B**). The percentage of preadipocytes in the G_0/G_1 phase was about 71 ± 1.9 % in the absence of IM, while 41 ± 2.5 % in the presence of IM. Interestingly, 72 ± 3.1 % of DhL-treated preadipocytes were in the G_0/G_1 phase. These

observations indicated that DhL inhibited clonal expansion of the cell by inducing G_0/G_1 phase arrest. Cyclin D1, cyclin A and CDK2 are the cell cycle regulatory proteins at G_0/G_1 phase. When the preadipocytes were treated with 9 μM DhL, the expression of cyclin D1, cyclin A, and CDK2 was strongly inhibited (**Figure 12C-E**). These observations suggest that DhL induces cell cycle arrest at the G_0/G_1 phase through the upregulation of p27 expression, respectively.

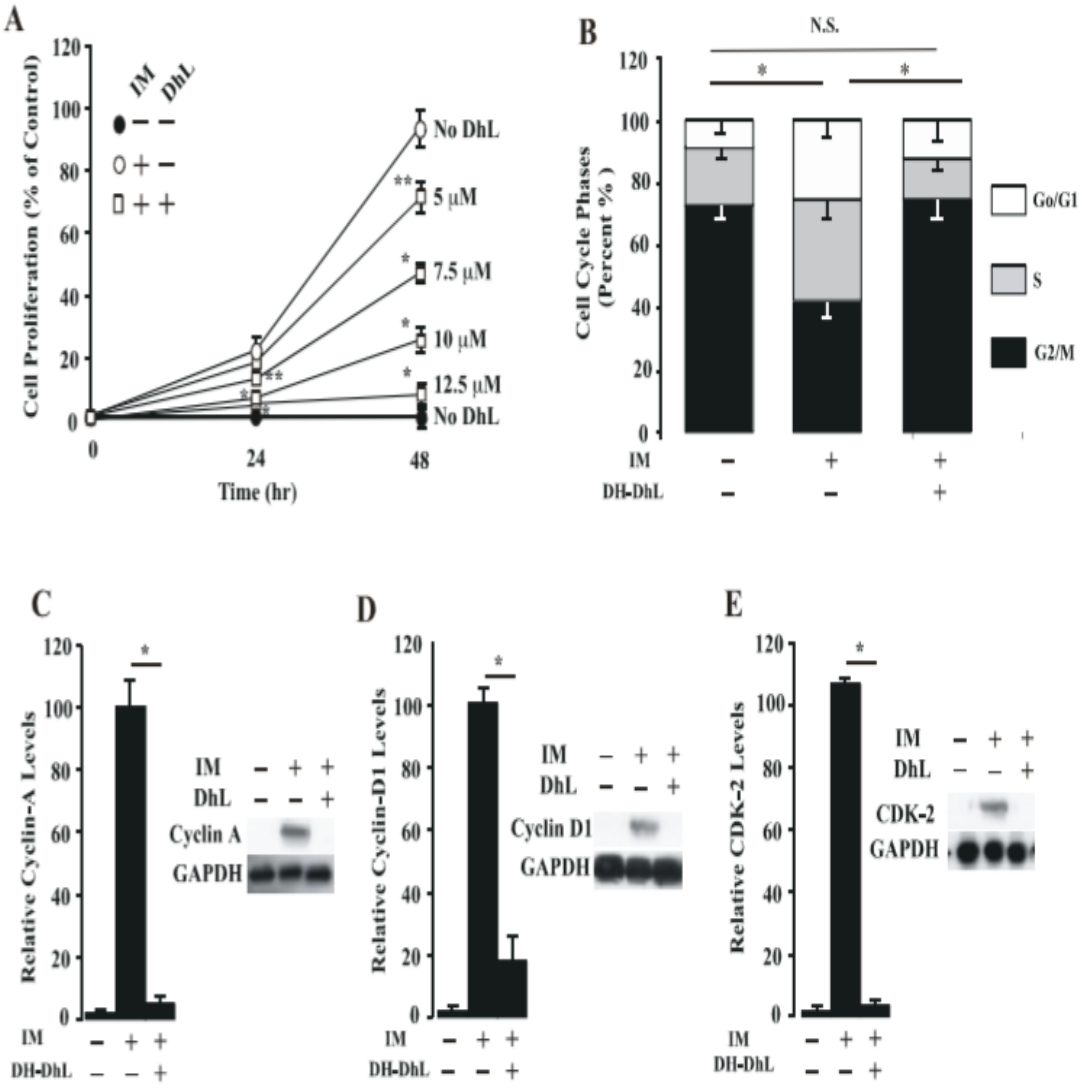

Figure 12. Dehydroleucodine inhibits proliferation of preadipocytes and cell cycle progression during mitotic clonal expansion. Post-confluent preadipocytes (day 0) were cultured in differentiation medium, and then treated once with different concentration of dehydroleucodine as indicated (**A**). The number of cells treated with dehydroleucodine for 24 and 48 h was determined using an automated cell counter machine. DMSO was used a vehicle control and preadipocytes incubated in the absence of differentiation medium was used a negative control. All values are presented as the mean ± SEM of three independent experiments performed in triplicate. *P<0.01, **P<0.001 vs. no dehydroleucodine treatment. Additionally, post-confluent preadipocytes (day 0) were cultured in the absence or presence of differentiation medium as indicated in the figure (**B**). After treatment of preadipocytes with 9 μM dehydroleucodine for 24 h, stained with a propidium iodine solution, and the cell population was analyzed by flow cytometry as described in Material and Methods. The percentage of cell population at each stage of the cell cycle was determined using Accuri C6 software (**B**) After treatment of preadipocytes with 9 μM dehydroleucodine for 24 h, cells were harvested and the lysates were subjected to Western blot analysis for Cyclin A (**C**), Cyclin D1 (**D**), CDK2 (**E**). Western blot analysis for GAPDH was used as loading control. The Western blots were performed three times, and a representative image of the three independent experiments was shown.

2.3.3. Dehydroleucodine blocks expression of C/EBPβ and histone demethylase JMJD2B, CDC25A and CDC451 genes

Several histone methyltransferases and demethylases have been reported to be involved in adipogenesis. These enzymes seem to regulate expression of C/EBPβ and PPARγ (Guo et al. 2012; Lee et al. 2008). To further assess the regulators involved in the DhL inhibition of MCE, I examined the expression of C/EBPβ in DhL-treated 3t3-L1 cells at the indicated time after IM induction as indicated in **Figure 13A**. Levels of expression of C/EBPβ mRNA was increased in the presence of IM treatment during the 48 hours. However, the addition of DhL clearly diminished the C/EBPβ mRNA level (**Figure 13A**), which expression has been correlated with the regulation of H3K9 demethylase JMJD2B and cell cycle genes (e.g., Cdc25c) genes. Thus, the expression of JMJD2B and the cell cycle genes in DhL-treated 3T3-L1 cells during the MCE process

was determined (**Figure 13B-D**). The JMJD2B mRNA level was also reduced by DhL treatment (**Figure 13D).** Interestingly, I also observed a dramatic inhibition of the expression of mRNA levels of cell cycle genes Cdc25A and Cdc451 during MCE (**Figure 13B-C**).

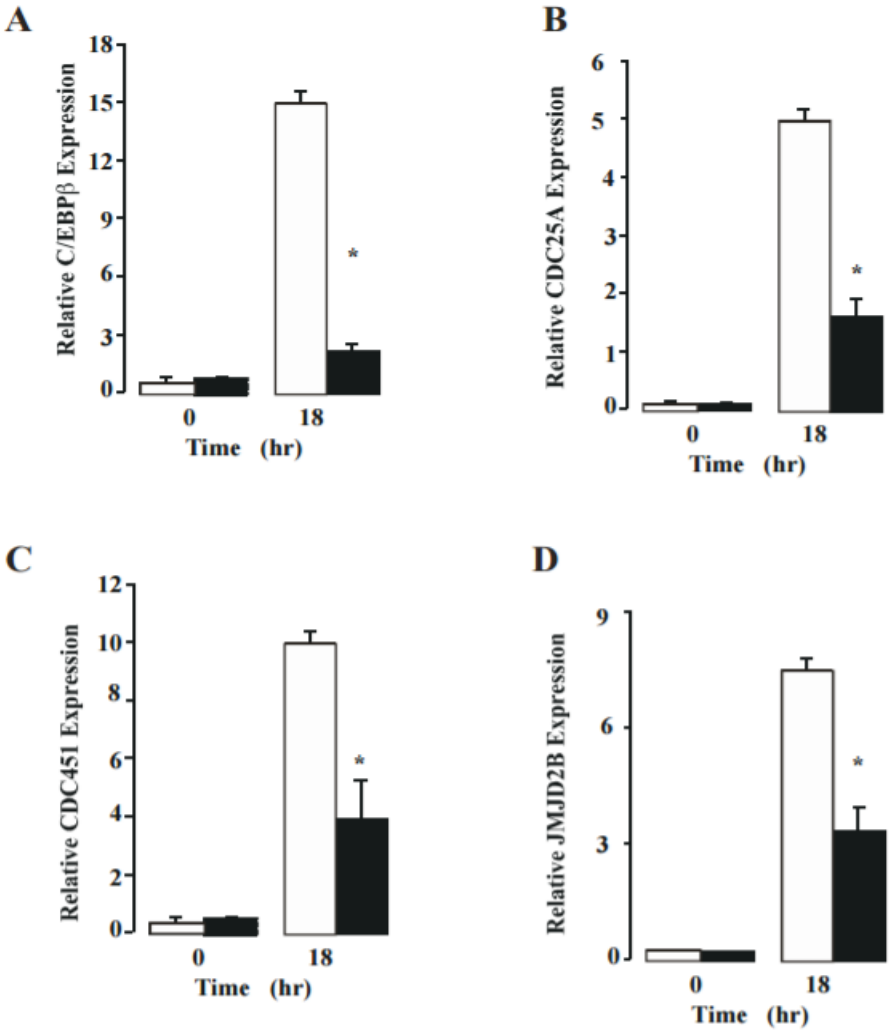

Figure 13. Dehydroleucodine downregulates the expression of C/EBPβ, histone demethylase JMJD2B, CDC451 and CDC25A genes. Post-confluent preadipocytes (day 0) were cultured in the presence of differentiation medium containing 9 μM dehydroleucodine for 24 h, then total RNA was extracted as indicated in Material and Methods. mRNA levels of C/EBPβ, histone demethylase JMJD2B, CDC451 and

CDC25A were determined by qPCR (**A**-**D**). The qPCR data are represented as the mean ± SEM of there independent experiments. **P*<0.01 vs. no dehydroleucodine treatment.

Next, the effect of DhL on the expression of PPARγ as well as methytransferases involved in its expression during adipogenesis, were examined. To this end, the levels of expression of MLL3, MLL4 and G9a in DhL–treated 3T3-L1 cells was determined at the indicated time after IM induction (**Figure 14A-D**). As shown in **Figure 14**, DhL clearly inhibited the expression of PPARγ and MLL4 (**Figure 14A and C**), without affecting the expression of MLL3 and G9a, respectively (**Figure 14B and D**). These results suggest that the DhL inhibition of MCE might be epigenetically regulated through downregulation of C/EBPβ and PPARγ.

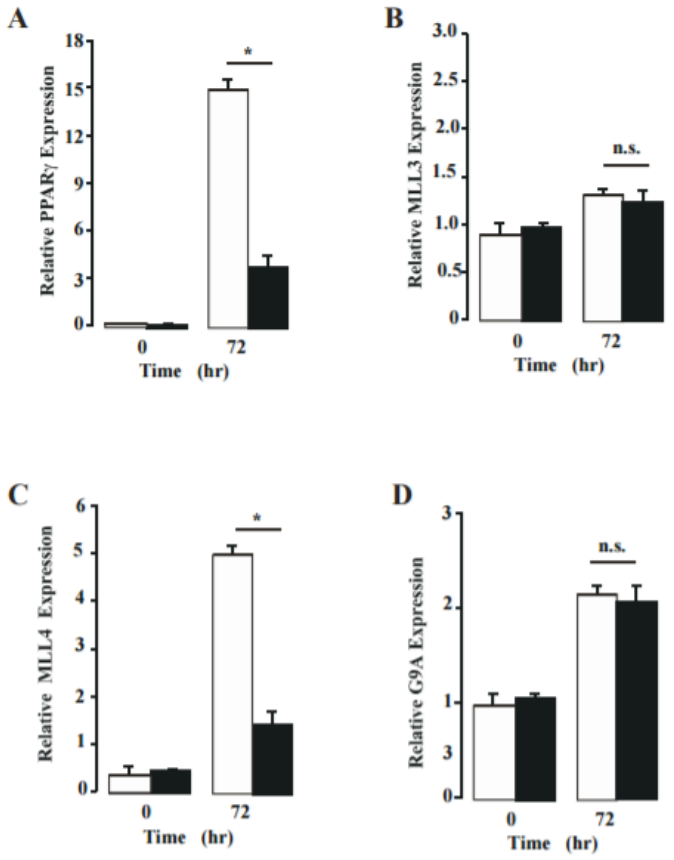

Figure 14. Dehydroleucodine represses the expression of PPARγ, MLL3, MLL4 and G9A genes. Post-confluent preadipocytes (day 0) were cultured in the presence of differentiation medium containing 9 μM dehydroleucodine for 24 h, then total RNA was extracted as indicated in Material and Methods. mRNA levels of PPARγ, MLL3, MLL4 and G9A were determined by qPCR (**A**-**D**). The qPCR data are represented as the mean ± SEM of three independent experiments. **P*<0.01 vs. no dehydroleucodine treatment, and N.S.: not statistically significant as compared with no dehydroleucodine treatment.

2.4. Discussion

Approximately 50% of modern pharmaceuticals derive from traditional plant compounds (Bilia et al. 2017). A deeper understanding of the anti-adipogenic mechanism of action of the sesquiterpene lactone DhL, derived from *Artemisia douglasiana*, may

thus have pharmacological import in the continuing search for therapeutic modalities to ameliorate the effects of the global obesity epidemic. The aim of this line of experiments was to further elucidate the potential mechanisms of action of DhL, that previous work revealed acts upon middle stage, and possibly late stage, differentiation of 3T3-L1 preadipocytes (Galvis et al. 2011).

It is evident from the results that DhL has a multiplicity of effects on adipogenesis, and therefore it is likely that DhL interacts with multiple molecular actors and/or acts upstream of significant processes responsible for the progression of signaling cascades necessary for MCE. I established that DhL has a marked effect on lipid accumulation when applied to 3T3-L1 cells between 0 and 2 days post induction of differentiation (**Figure 11C**). When applied from day 4 through day 8 post-induction, the anti-adipogenic affect was drastically reduced (**Figure 11B**), indicating that one single dose of DhL acts by inhibiting the early stage of adipogenesis, during which MCE occurs. Results from a triglyceride assay confirmed these findings, demonstrating that within this 0-2 day post-induction period, 12 μM of DhL worked most effectively in decreasing triglyceride content, an observable marker of successful progression through the adipogenic program (**Figures 11C** and **E**). Observable decreases in triglyceride content were also found with as little as 3 μM of DhL.

CCAT/Enhancer Binding Protein Beta is expressed within 2 to 4 hours post induction (Tang et al. 2003), and I observed that 3 μM of DhL led to a drastic downregulation of protein levels of C/EBPβ (**Figure 11D**). As the acquisition of DNA binding activity of C/EBPβ beginning at approximately 12 hours post induction is essentially required for the progression of MCE (Tang et al. 2003), this represents strong

evidence that DhL inhibits adipogenesis by obstructing the MCE process. As activated C/EBPβ, along with C/EBPδ, triggers the expression of C/EBPα, which leads to the expression of PPARγ (Farmer et al. 2006), the inhibitory effects of DhL on C/EBPβ would be expected to result in a downregulation of protein levels of PPARγ and C/EBPα. This is precisely what was observed when 9 μM of DhL was applied, which drastically downregulated PPARγ and C/EBPα in an inversely correlated manner as concentration of DhL was increased to 6 μM and beyond (**Figure 11F**).

Flow cytometry revealed that DhL arrested the cell cycle at the G_0/G_1 phase, decreased cyclins A, D1 and CDK2 (**Figure 12C-E**). Consistent with this arresting of cell cycle progression, DhL inhibited the proliferation of preadipoyctes during MCE in a concentration dependent manner with the greatest effect observed at 12.5 μM of DhL.

Dehydroleucodine also repressed the expression of CDC25A, CDC451, and JMJD2B (**Figure 13B-D**), which all act downstream of C/EBPβ to facilitate progression through the cell cycle (Guo et al. 2012; Aressy and Ducommun 2008; Turowski et al. 2003; Goloudina et al. 2003). Messenger RNA levels of the H3K4me1/2 methyltransferase MLL4 was downregulated upon application of DhL (**Figure 14C**), and PPAR*γ* mRNA was also downregulated, as would be expected from the literature reporting that MLL4 is part of the activating signal cointegrator-2 -containing complex (ASCOM), which co-activates PPARγ (Lee et al. 2008; Qi et al. 2003; Kuang et al. 2002; Hong et al. 2001). No significant effects were found on mRNA levels upon application of DhL for the expression of MLL3 (**Figure 14B**), also a part of the ASCOM complex (Lee et al. 2008), or the expression of G9A (**Figure 14D**), which represses PPARγ expression (Wang et al. 2013).

It is plausible that this multitude of effects which arrest the necessary step of MCE, stem from the upstream inhibitory effect DhL has directly on C/EBPβ, or on factors necessary to express C/EBPβ or activate C/EBPβ's DNA binding activity. Such targets could possibly include Rb, which DhL decreased phosphorylation of in this study, and which binds to C/EBPβ and facilitates its activation (Chen et al. 1996). It is also possible that DhL acts redundantly and directly on numerous processes downstream of C/EBPβ activation, such as obstructing MLL4, which was downregulated in my experiments and is a component of the ASCOM complex, which is responsible for the activation of PPARγ and C/EBPα. In a similar fashion, DhL may act directly on the epigenetic modifiers as shown in these experiments, which include CDC25A, CDC45l, and JMJD2B (**Figure 13B-D**).

In summary, dehydroleucodine inhibits the accumulation of lipid droplets and decreased the elevations of triglycerides, and inhibition of adipogenesis occurred during the early stage of adipogenesis. Thus, not only did dehydroleucodine inhibit the expression of C/EBPα and PPARγ, it also strongly blocked the expression of C/EBPβ, an early stage biomarker of early adipogenesis, in a concentration-dependent manner. The proliferation of preadipocytes was dramatically suppressed when dehydroleucodine was added to the medium as early as 24 hours. These results indicate that dehydroleucodine may specifically affect mitotic clonal expansion to inhibit preadipocytes dedifferentiation. Dehydroleucodine arrested the cell cycle at the G_0/G_1 phase, increased p27 and decreased both cyclins A and D and their partners (e.g., CDK2 and CDK4). Furthermore, dehydroleucodine downregulated expression of histone demethylase JMJD2 as well as repressed the expression of histone methyltransferase MLL4, which in turn

diminished the expression of C/EBPβ and PPARγ respectively. Collectively, the results indicate that dehydroleucodine inhibits preadipocyte differentiation by blocking mitotic clonal expansion via cell cycle arrest, which may be mediated by regulation selective histone methylation/demethylation in transcription activation during the early stage of adipogenesis.

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III. 11,13-DIHYDRO [11R]DEHYDROLEUCODINE BLOCKS MITOTIC CLONAL EXPANSION DURING PREADIPOYCTE DIFFERENTIATION THROUGH CELL CYCLE ARREST

3.1. Introduction

As discussed in the previous chapter, it was previously found that the sesquiterpene lactone dehydroleucodine (DhL), extracted from the aerial parts of *Artemisia douglasiana*, decreased phenotypic (lipid content) and molecular markers (PPARγ and C/EBPα) of adipogenesis, indicating that this compound inhibited differentiation of preadipocytes (Galvis et al. 2011). Enantiomers of 11,13-dihydro-dehydroleucodine were found to inhibit 3T3-L1 preadipoycte differentiation (Galvis et al. 2011), but their effects on the various signaling molecules underlying the adipogenic differentiation program were not fully investigated.

A selective reduction of dehydroleucodine generates its 11,13-dihydro derivative of (11,13-dihydro-dehydroleucodine), which in turn shows very weak biological activity. Thus, it is likely that the functional groups in dehydroleucodine are required for the biological activities or that the cyclopentenone has more structural requirements for its optimal pharmacological activity (Galvis et al. 2011).

Similarly, it was found that the addition of 11,13-dihydro-dehydroleucodine during 3T3-L1 differentiation and HeLa cell proliferation, exhibited a weak effect compared with the inhibitory effect observed for dehydroleucodine. More importantly, one of the epimers of the reduced form of dehydroleucodine (i.e., 11*R*,13-dihydro-dehydroleucodine) inhibited these processes, indicating that the α-methylene-γ-lactone group was not required for such inhibition. However, the reduced form of dehydroleucodine showed no toxicity in these biological assays (Galvis et al. 2011).

In contrast, reduction of α,β-unsaturated carbonyl groups in other sesquiterpene lactones (i.e., helenalin, hymentin, mexicanin, custonolide and parthonolide) showed a differential biological effect: treatment with 11,13-dihydro-helenalin does not result in a reduced biological activity, while 11,13-dihydro-hymentin had reduced biological activity (Schmidt 1996).

Thus, by studying the effect of gene expression of 11,13-dihydro-dehydroleucodine in 3T3-L1 differentiation, and comparing these results to the gene expression of DhL, it is likely that the anti-adopogenic structural and conformational requirements for anti-adipogenic therapeutics could be greater elucidated. This new evidence could help aid researchers in synthesizing novel anti-adiopogenic compounds. Additionally, on its own merits, such evidence will help evaluate the potential benefits and deficits of 11,13-dihydro-dehydroleucodine as an adipogenic inhibitor, as well as further clarify its mechanism of action.

In this series of experiments, I examined the effects of 11,13-dihydro-[11R]-dehydroleucodine (11,13DH-[11R]-DhL), on the timing and expression of molecular markers underlying the adipogenic program, including the process of MCE, in 3T3-L1 preadipocytes, in order to further elucidate the action, underlying mechanism, and therapeutic import of dehydro derivatives of dehydroleucodine.

3.2. Materials and Methods

To examine whether DH-DhL modulates early steps of the differentiation of 3T3–L1 preadipocytes into adipocytes, post-confluent preadipocytes were treated with different concentrations of DH-DhL and the effect on lipid droplets were observed. Cells were stained with Oil Red O and morphological changes of 3T3–L1 preadipocytes were

monitored, and then photographed after 9 days from the onset of differentiation to quantify lipid droplets, followed by spectrophotometry. Cell viability was determined by the MTT assay.

Next, to further determine the effects on 3T3-L1 cells of DH-DhL, the effect of DH-DhL on the PPARγ and C/EBPα expression was assayed via western blot. Expression of additional adipogenic factors, FAS, and AMPK, were also assayed via western blot.

To determine the stage of adipogenesis upon which DH-DhL exerts its effects on 3T3-L1 cells, DH-DhL was applied at various time points after induction with IM, and the lipid content of the 3T3-L1 cells was then measured. Since, CBEPβ, a specific transcriptional factor expressed in the early stage of adipogenesis, is also known to be required for MCE, I examined the effect of DH-DhL on the expression of CBEPβ via western blotting, during the first 48 hour after stimulation of 3T3-L1 preadipocytes with IM. To more fully characterize the effects of DH-DhL on 3T3-L1 preadipocytes, the effect of DH-DhL on proliferation was also assayed.

To even further elucidate the effects of DH-DhL on 3T3-L1 preadipoyctes, I investigated whether DH-DhL modulates cell cycle progression, via treatment with DH-DhL followed by flow cytometry to determine cell cycle. As additional evidence of what phase of cell cycle arrest resulted from DH-DhL treatment, western blot was utilized to assay levels of cyclin D1, CDK4, cyclin A, and CDK2, cell cycle regulatory proteins at the G_0/G_1 phase, Rb, and p27, an upstream effector of the cell cycle regulatory proteins.

As will be elaborated upon more fully in the Results and Discussion sections of this chapter, as these experiments seemed to indicate that DH-DhL inhibited MCE, I then

sought to determine the signaling pathway through which DH-DhL inhibited MCE by investigating the expression as well as phosphorylation of Erk1/2 and Akt, proteins involved in cell cycle progression, via western blotting.

The reagents, cell culture and differentiation, treatments, cell viability assay, Oil Red O staining, microscopy, spectrophotometry, triglyceride assay, cell proliferation assay, western blotting, flow cytometry, high-performance liquid chromatography, compound extraction, purification, identification, and statistical analysis, were prepared and performed as in Chapter 2, above.

Previous studies have demonstrated that DhL and their epimers inhibited the differentiation of 3T3–L1 preadipocytes into adipocytes (Galvis et al. 2011). I further examined whether the 11,13DH-[11R]DhL epimer (**Figure 15A**) modulates early steps of the differentiation of 3T3–L1 preadipocytes into adipocytes.

3.3. Results

3.3.1. 11,13-dihydro-[11R]-dehydroleucodine inhibits 3T3–L1 adipocyte differentiation

Post-confluent preadipocytes were treated with 0, 10, 20, 40, 80, 120 and 160 μM 11,13DH-[11R]-DhL every other day for 9 days. 11,13DH-[11R]-DhL decreased the accumulation of lipid droplets in a concentration-dependent manner (**Figure 15B**). In particular, 160 μM 11,13DH-[11R]-DhL completely inhibited the formation of lipid droplets in the adipocytes with a half-maximal inhibitory concentration of 71.0 ± 4.1 μM. Interestingly, cell viability was not affected by 11,13DH-[11R]-DhL even at 200 μM (**Figure 15C**).

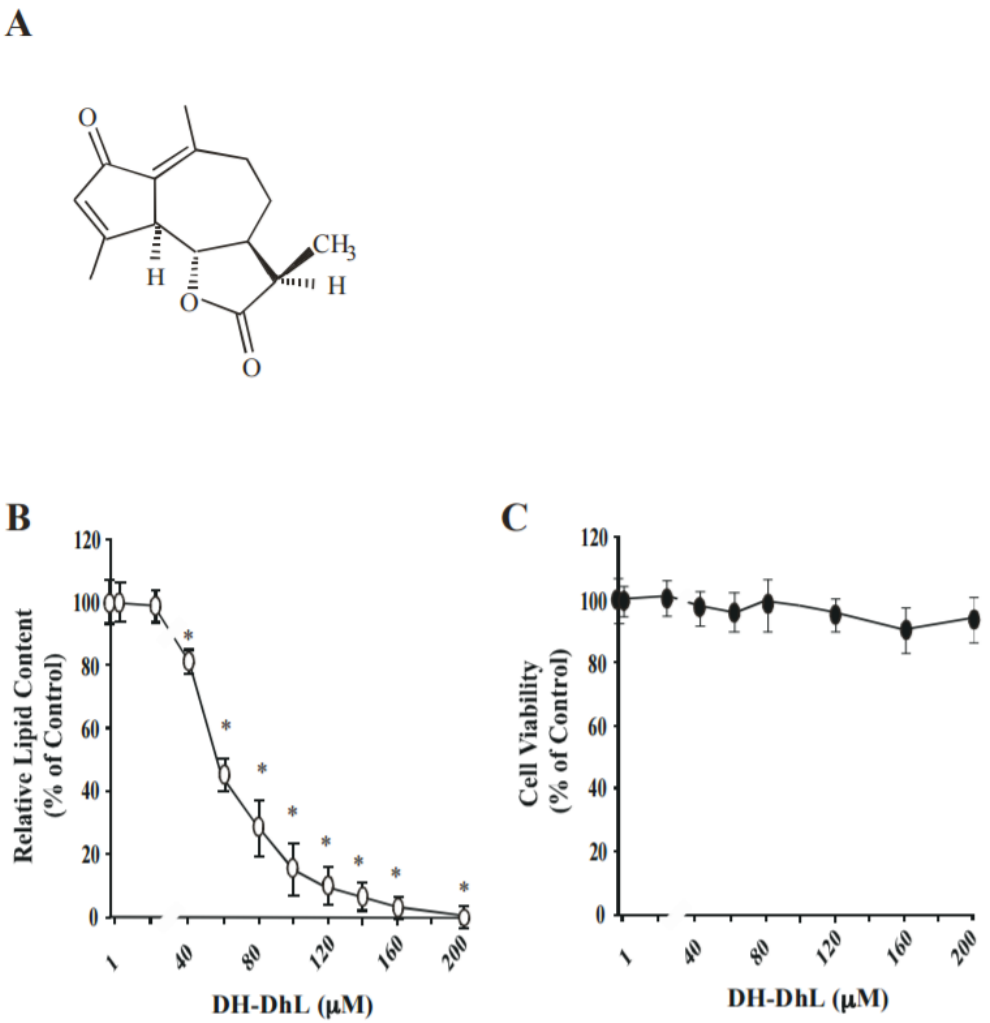


Figure 15. Inhibitory effect of 11,13-dihydro-[11R]-dehydroleucodine on adipocyte differentiation. (A) Chemical structure of 11,13DH-[11R]DhL. **(B)** Preadipocytes were cultured in induction medium containing different concentrations of 11,13DH-[11R]DhL as indicated in the figure for 9 days. Cells were stained with Oil Red O and morphological changes of 3T3–L1 preadipocytes were monitored, and then photographed (x100) after 9 days from the onset of differentiation. The percentages presented are relative to the 11,13DH-[11R]DhL-free control in the presence of induction media (100%). **(C)** Preadipocytes were cultured in induction medium containing different concentrations of 11,13DH-[11R]DhL for 3 days. Cell viability was determined by the MTT assay. All values are presented as the mean ± SEM of three experiments performed in triplicate. **P* < 0.01 vs no 11,13DH-[11R]DhL treatment.

Quantification of intracytoplasmic triglyceride during preadipocyte differentiation aids in evaluating the effect of 11,13DH-[11R]DhL. When the adipocytes were exposed to 30, 60 and 120 μM 11,13DH-[11R]DhL for 9 days, cellular triglyceride content

significantly decreased in a concentration-dependent manner (**Figure 16A**). The relative triglyceride contents of the adipocytes treated with different concentration of 11,13DH-[11R]-DhL were 31.1 ± 2.1%, 59 ± 2.8% and 85 ± 3.1%, respectively, with respect to the control. The half-maximal inhibitory concentration of 11,13DH-[11R]-DhL for the effect on triglyceride content was 69.3 ± 3.2 μM. Transcription factors such as PPARγ and C/EBPα are critical for terminal adipocyte differentiation. PPARγ and C/EBPα expression in adipocytes treated with 11,13DH-[11R]-DhL strongly decreased compared with the control cells as well as other adipogenic factors (e.g., FAS, and AMPK). Specifically, expression of FAS was decreased, while phosphorylation of AMPK was increased (**Figure 16B-E**).

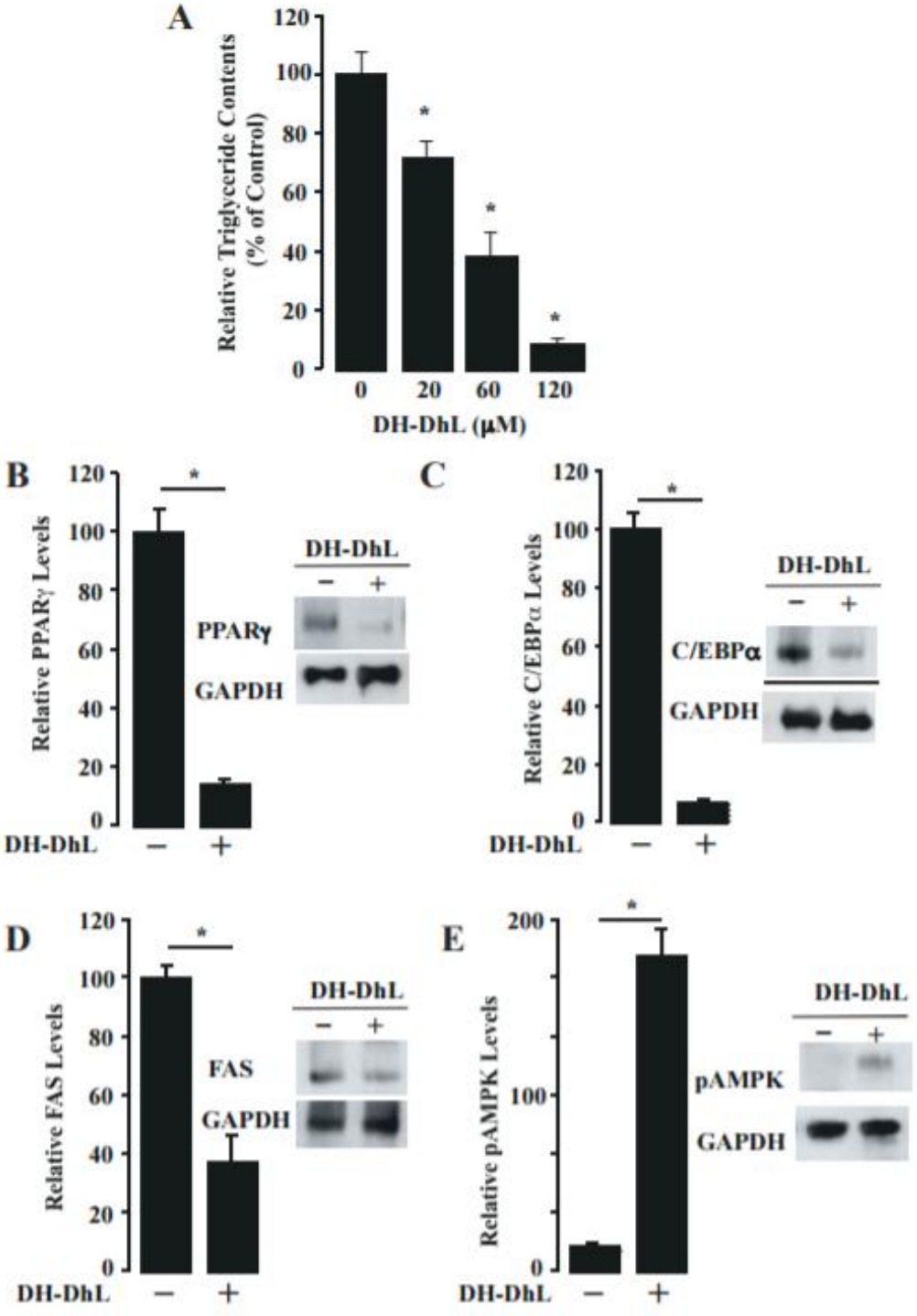


Figure 16. Inhibitory effect of 11,13-dihydro-[11R]-dehydroleucodine on triglyceride content and adipogenic markers. Preadipocytes were cultured in differentiation medium containing 100 μM 11,13DH-[11R]DhL for 9 days. (**A**) Cellular triglyceride content was

determined and the percentages presented for the adipocytes treated with 100 μM 11,13DH-[11R]DhL are relative to the control (100%). *P<0.01 vs no 11,13DH-[11R]DhL treatment. (**B-E**) Preadipocytes treated with 100 μM 11,13DH-[11R]DhL for 9 days were harvested and the lysates were subjected to western blot analysis for (B) C/EBPα, (C) PPARγ, (D) FAS and (E) phospho(p) AMPK. The percentages presented are relative to the 11,13DH-[11R]DhL-free control without inducers (100%). All values are presented as the mean ± SEM of three experiments performed in triplicate. *P<0.01 vs no 11,13DH-[11R]DhL treatment. GAPDH was used as loading control. The Western blots were performed three times, and a representative image of the three independent experiments was shown.

In addition, 11,13DH-[11R]-DhL treatment significantly inhibited adipogenic morphology (i.e., transition from a fibroblast-like shape to an increasingly rounded-up appearance with an accumulation of lipid droplets (**Figure 17** inset). Consistent with these observations, it was found that 11,13DH-[11R]-DhL decreased the number as well as the sizes of lipid droplets. The histogram was clearly asymmetric and showed a long tail in the positive axis reflecting the presence of lipid droplets with sizes much higher with respect to the mean of the population. Lipid droplets have a diameter in the range of 0.5 to 4.3 μm. Analysis of the distribution of the numbers of lipid droplet sizes showed a distinct shift of the lipid droplet size toward smaller lipid droplets following incubation with 11,13DH-[11R]-DhL (**Figure 17**). These results suggest that 11,13DH-[11R]-DhL may inhibit differentiation of 3T3-L1 preadipocytes by decreasing the expression of several transcriptional factors (e.g., PPARγ and C/EBPα), consistent with the robust effect of 11,13DH-[11R]-DhL on the number as well as the size of the lipid droplets.

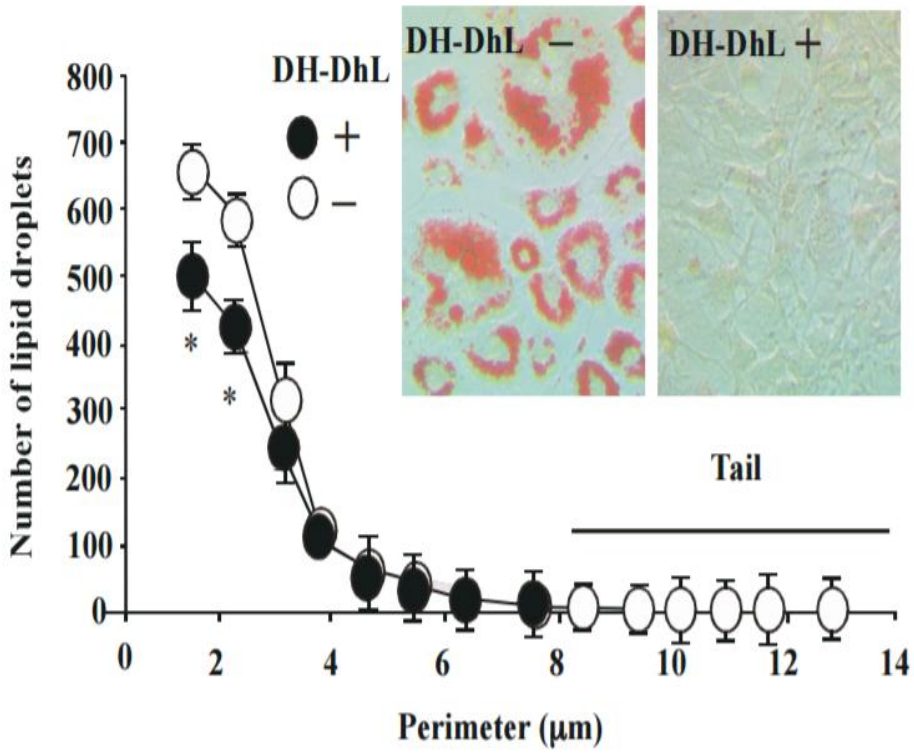


Figure 17. 11,13-dihydro-[11R]-dehydroleucodine decreased the number and the size of lipid droplets during adipogenesis. Preadipocytes were cultured in induction medium containing 100 μM of 11,13DH-[11R]DhL for 9 days. After differentiation, cells were stained with Oil Red O, and then photographed. The total number of lipid droplets was quantified from, at least, 100 cells. The perimeters of 1784 lipid droplets from 48 control cells and 1392 lipid droplets from 52 11,13DH-[11R]DhL-treated cells were obtained by using NIH Image software. The mean perimeter and the relative variance for the lipid droplets from control cells are 6.54 (2.08 diameter) and 0.51, while the corresponding values for lipids droplets the 11,13DH-[11R]DhL-treated cells are 4.74 (1.51 diameter) and 0.45, respectively. **P*<0.01 vs no 11,13 [R]-dihydro-dehydroleucodine treatment.

3.3.2. 11,13-dihydro-[11R]-dehydroleucodine suppresses clonal expansion during the early stage of adipogenesis

Adipocyte differentiation occurs through the preadipocyte stage and progresses through several stages, which can be clearly divided between proliferation and mitotic cloning expansion (early stage) and terminal differentiation (late stage). Thus, we

examined whether 11,13DH-[11R]-DhL causes stage-dependent inhibition of adipocyte differentiation. 3T3-L1 cells were treated with 120 μM 11,13DH-[11R]-DhL at several time points after induction with IM as shown in **Figure 18.**

Treatment with 11,13DH-[11R]-DhL at early stages (days 0-2 and 0-4) strongly inhibited adipogenesis by 75 ± 2.1% and 82 ± 3.1 % respectively (**Figure 18A**). The treatment of 11,13DH-[11R]-DhL after day 2 (days 2-8) showed moderate inhibition of adipogenesis by 48 ± 2.5 %, whereas the treatment after day 4 (i.e., days 4-8 and 6-8) showed only 20 ± 1.5 % inhibition or no statistically significant inhibition at all (**Figure 18A**). Consistent with these observations, when cells were treated with different concentrations of 11,13DH-[11R]-DhL during early stage, the intracellular triglyceride content significantly decreased in a concentration-dependent manner (**Figure 18B**). In the early adipogenic stage, the number of preadipocytes greatly increases as a result of MCE. CBEPβ, a specific transcriptional factor expressed in the early stage of adipogenesis, is also known to be required for MCE. Therefore, we examine the effect of 11,13DH-[11R]-DhL on the expression of CBEPβ, during the first 48 h after stimulation of 3T3-L1 preadipocytes with IM. Expression of CBEPβ was significantly decreased in a concentration-dependent manner when cells were treated with 11,13DH-[11R]-DhL (**Figure 18C).** In addition, I also determined whether 11,13DH-[11R]-DhL affects proliferation of 3T3-L1 preadipocytes after induction of differentiation with IM. As shown in **Figure 18D**, the treatment with 11,13DH-[11R]-DhL significantly decreased the cell numbers of IM-induced 3T3-L1 preadipocytes during day 1 as well as day 2 as compared to the cell numbers of control 3T3-L1 preadipocytes not treated with 11,13DH-[11R]-DhL. Thus, the effects of 11,13DH-[11R]-DhL that cause inhibition of

differentiation of 3T3-L1 preadipocytes appears to occur during the early stage of adipogenesis.

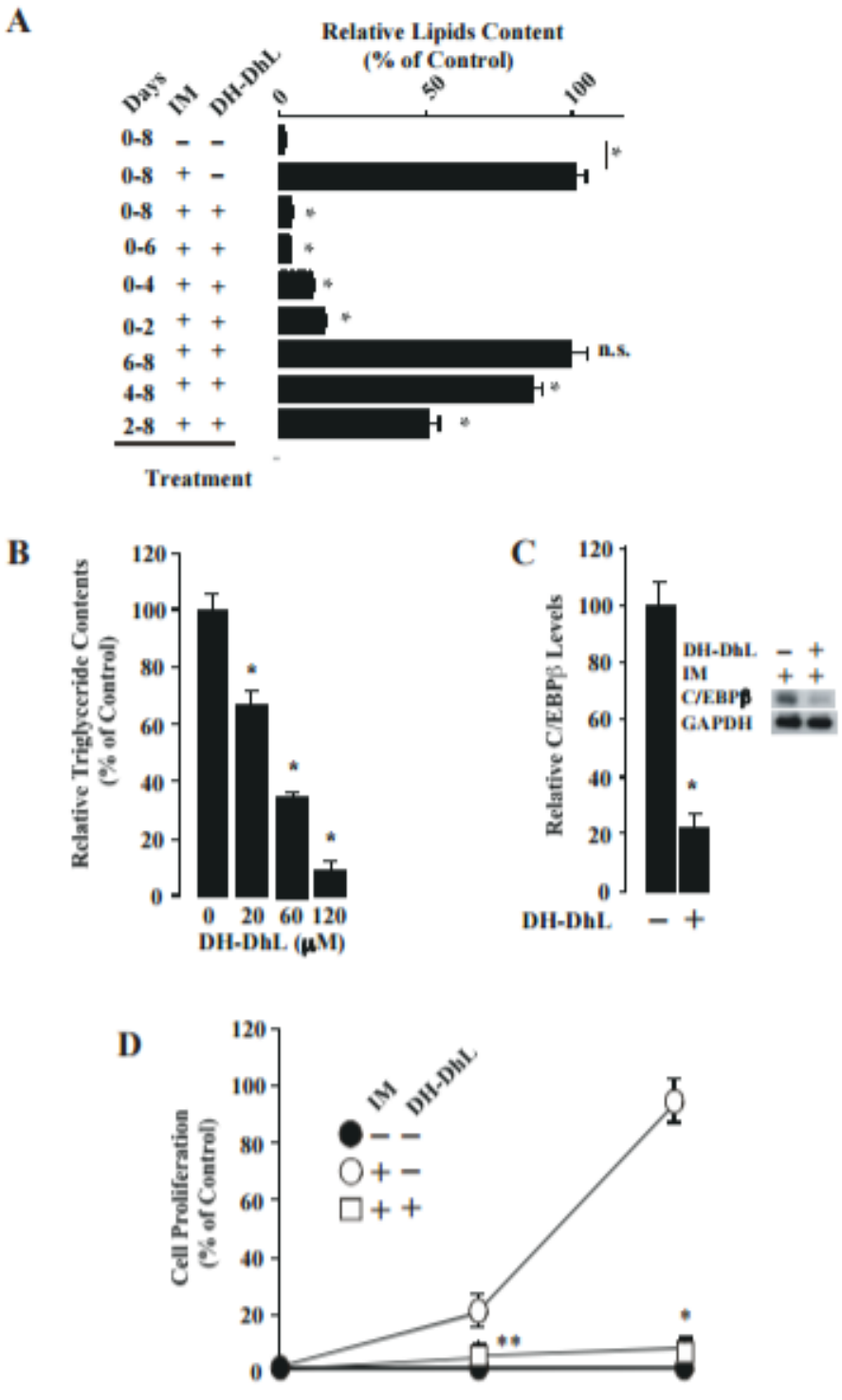


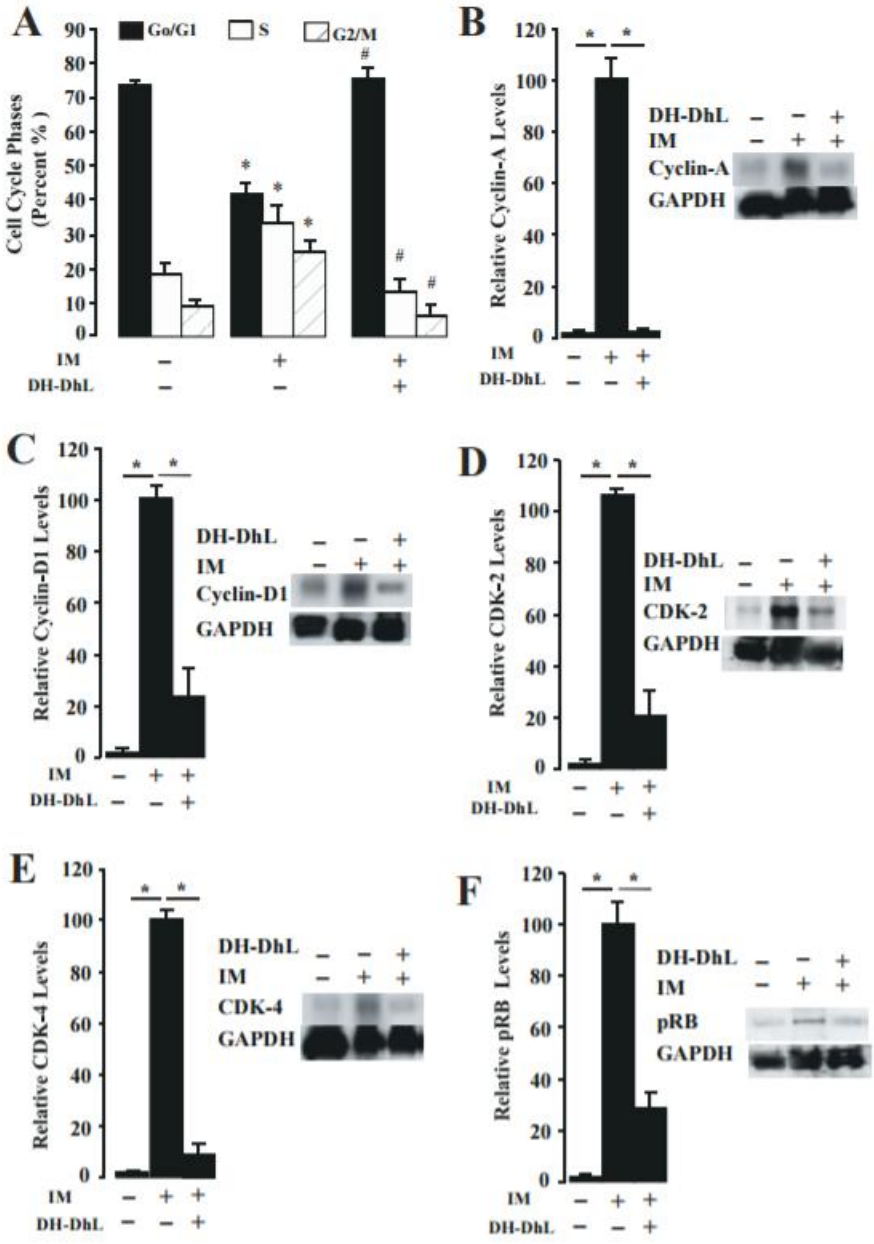
Figure 18. Inhibitory effect of 11,13-dihydro-[11R]-dehydroleucodine on mitotic clonal expansion during the early stage of adipogenesis. (A) Preadipocytes were cultured in differentiation medium containing 100 μM 11,13DH-[11R]DhL for day 0-2, 0-4, 0-6, 0-8, 2-8, 4-8 and 6-8, respectively. Cells were stained with Oil Red O and then visualized as described in the Material and Methods. All values are presented as the mean ± SEM of three independent. *P < 0.01 vs no 11,13DH-[11R]DhL treatment. N.S.: not statistically significant versus no 11,13DH-[11R]DhL treatment. **(B)** Cellular triglyceride content of preadipocytes treated with 100 μM 11,13DH-[11R]DhL for day 0-2 was determined as described in the Material and Methods using a Triglyceride Kit. Results are shown as relative percentage of control without 11,13DH-[11R]DhL. DMSO was used a vehicle control. All values are presented as the mean ± SEM of three independent experiments performed in triplicate. *P<0.01 vs no 11,13DH-[11R]DhL treatment. (C) Preadipocytes treated with 100 μM 11,13DH-[11R]DhL for day 0-2 were harvested and the lysates were subjected to western blot analysis for C/EBPβ. The percentages presented are relative to the 11,13DH-[11R]DhL -free control without inducers (100%). All values are presented as the mean ± SEM of three experiments performed in triplicate. *P<0.01 versus no 11,13DH-[11R]DhL treatment. GAPDH was used as loading control. The western blots were performed three times, and a representative image of the three independent experiments was shown. (D) Post-confluent preadipocytes (day 0) were cultured in differentiation medium, and then treated with 100 μM 11,13DH-[11R]DhL for 24 and 48 h. The number of cells treated with 11,13DH-[11R]DhL as well as control cells was determined using an automated cell counter machine. DMSO was used a vehicle control and preadipocytes incubated in the absence of differentiation medium was used a negative control. All values are presented as the mean ± SEM of three independent experiments performed in triplicate. *P<0.01, **P<0.001 versus no 11,13DH-[11R]DhL treatment.

3.3.3 11,13-dihydro-[11R]-dehydroleucodine induces G_0/G_1 phase arrest through p27 upregulation

I then investigated whether 11,13DH-[11R]DhL modulates cell cycle progression. When 3T3–L1 preadipocyte differentiation was induced, addition of 11,13DH-[11R]DhL arrested the cell cycle at the G_0/G_1 phase (**Figure 19A**). As expected, G_0/G_1 phase decreased and both S and G_2/M phases were statistically increased in the presence of IM. The addition of 11,13DH-[11R]DhL reversed the effect of IM on the cell cycle. Specifically, the percentage of preadipocytes in the G_0/G_1 phase was about 73 ± 1.5 %, while 47 ± 2.1 % of the untreated adipocytes, and 78 ± 3.1 % of the 11,13DH-[11R]DhL-treated preadipocytes were in the G_0/G_1 phase. These observations indicated that 11,13DH-[11R]DhL inhibited clonal expansion of the cell by inducing G_0/G_1 phase

arrest. Cyclin D1, CDK4, cyclin A, and CDK2 are the cell cycle regulatory proteins at the G_0/G_1 phase. Additionally, phosphorylation of the Rb protein is upregulated during cell cycle progression. When the preadipocytes were treated with 100 μM 11,13DH-[11R]DhL, the expression of cyclin D1, CDK4, cyclin A, CDK2, and p-Rb was strongly inhibited (**Figure 19B-F**). The expression of p27, an upstream effector of the cell cycle regulatory proteins, also decreased during adipogenesis but 11,13DH-[11R]DhL restored its expression (**Figure 19G**). These observations suggested that 11,13DH-[11R]DhL induces cell cycle arrest at the G_0/G_1 phase through the downregulation of pRB and upregulation of p27 expression, respectively.

To determine the signaling pathway through which 11,13DH-[11R]DhL inhibited the clonal expansion during the early stage of adipogenesis, the expression as well as phosphorylation of Erk1/2 and Akt, which are involved in cell cycle progression were examined. Adipogenic inducers increased the phosphorylation of Akt and Erk1/2. When the adipocytes were treated with 100 μM 11,13DH-[11R]DhL for 10 minutes or 3 hours, both Akt and Erk1/2 phosphorylation was significantly decreased, but not to the level demonstrated for cyclin protein abundance (**Figure 19H-J**).


A
Go/G1
S
G2/M
Cell Cycle Phases (Percent %)
IM
DH-DhL
B
Relative Cyclin-A Levels
DH-DhL
IM
Cyclin-A
GAPDH
C
Relative Cyclin-D1 Levels
Cyclin-D1
D
Relative CDK-2 Levels
CDK-2
E
Relative CDK-4 Levels
CDK-4
F
Relative pRB Levels
pRB

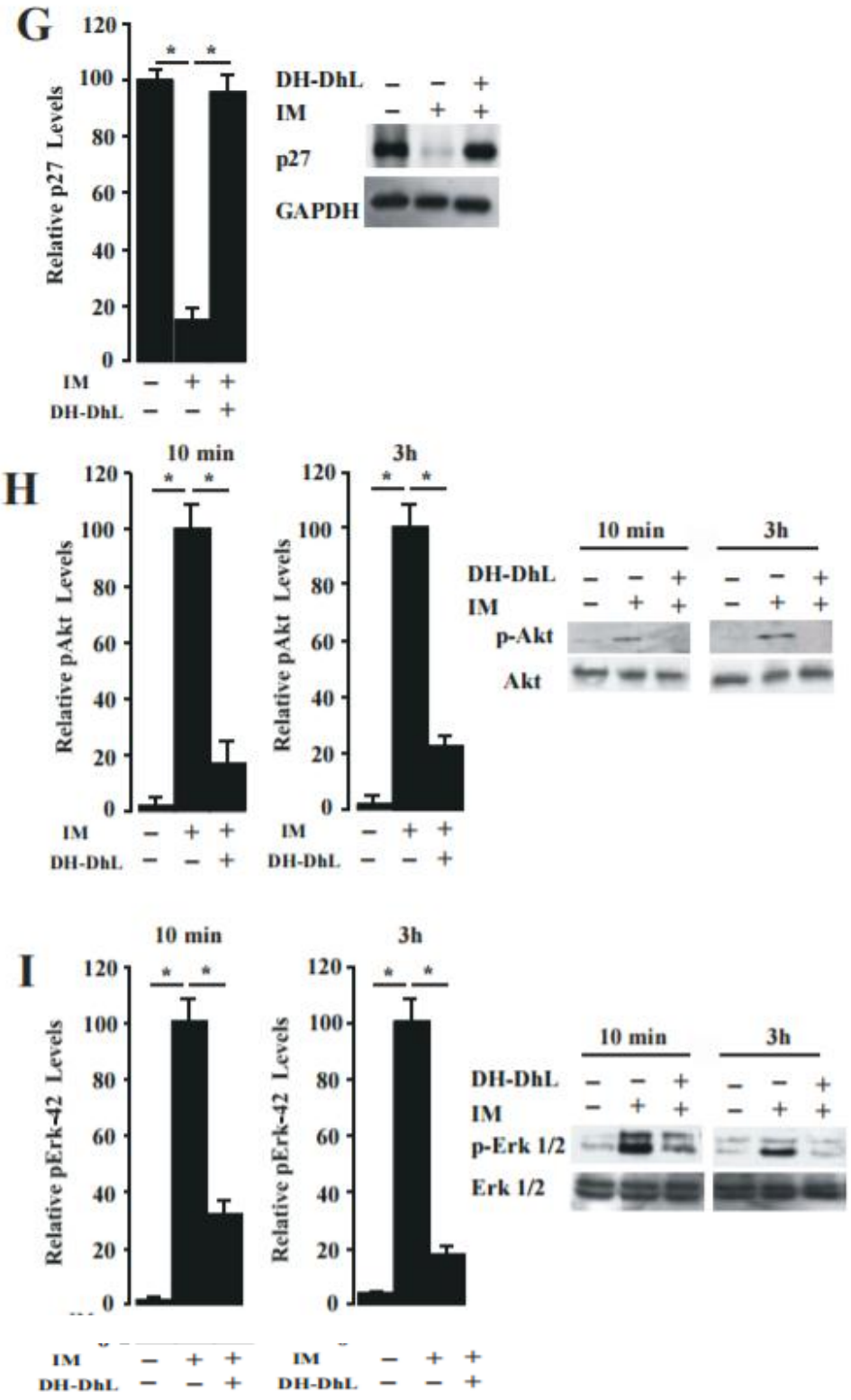


Figure 19. 11,13-dihydro-[11R]-dehydroleucodine regulates cell cycle progression. (**A**) 3T3–L1 preadipocytes were cultured in induction medium containing 100 μM 11,13DH-[11R]DhL for 24 h, stained with a propidium iodide solution, and analyzed by flow cytometry. The percentage

of cell population at each stage of the cell cycle was determined using the Modfit LT program. All values are presented as the mean ± SEM of three experiments performed in triplicate. *P < 0.01 vs control in the absence of IM; $^{\#}P$ < 0.01 vs control in presence of IM. (**B-D**) The cells were harvested and the lysates were subjected to western blot analysis for (**B**) cyclin A, **(C)** cyclin D1, **(D)** CDK2, **(E)** CDK4, **(F)** p-Rb, and (**G**) p27 by using specific antibodies as described in the Material and Methods. (**H**) Adipocytes treated with 100 μM 11,13DH-[11R]DhL for 10 min or 2 h were harvested and the lysates were subjected to western blot analysis for total(t)-Erk1/2, phospho(p)-Erk1/2, t-Akt, and p-Akt. The percentages presented are relative to the 11,13DH-[11R]DhL-free control with induction media (100%). All values are presented as the mean ± SEM of three experiments performed in triplicate. *P < 0.01 versus no 11,13DH-[11R]DhL treatment.

3.4. Discussion

In this series of experiments, the effect of 11,13DH[11R]-DhL on adipocyte differentiation in 3T3–L1 preadipocytes and the elucidation of the potential underlying mechanism, was investigated. Preadipocytes can differentiate into adipocytes, which possess a spherical shape and accumulate lipid droplets (Ruiz-Ojeda et al. 2016; Green and Kehinde 1975; Raajendiran et al. 2016; Otto and Lane 2005; Ntambi and Young-Cheul 2000; Tong and Hotamisligil 2001). 11,13DH[11R]-DhL inhibited lipid accumulation during adipocyte differentiation with a half-maximal inhibitory concentration value of 71 μM, as shown by the decreased numbers and sizes of lipid droplets as well as reduced triglyceride content.

Adipocyte differentiation is regulated by a complex network of transcription factors such as PPARγ and members of C/EBP family (C/EBPα, β, and δ) (Farmer 2006).

DhL, a sesquiterpene lactone compound abundant in the areal parts of *Artemesia douglasiana* inhibits adipogenesis by downregulating PPARγ signaling in 3T3-L1 cells (Galvis et al., 2011). 11,13DH[11R]-DhL strongly inhibited PPARγ expression, suggesting that this compound may inhibit adipocyte differentiation by downregulating the PPARγ pathway.

Adipogenesis can be divided into early and late stages. 3T3-L1 preadipocytes undergo mitotic clonal expansion through the upregulation of C/EBPβ and C/EBPδ during the early stage of adipocyte differentiation. This is followed by the activation of the downstream signaling molecules PPARγ and C/EBPα (Guo et al. 2015; Tang et al. 2003).

11,13DH[11R]-DhL inhibited the formation of lipid droplets and triglyceride accumulation, and suppressed C/EBPβ expression during the early differentiation stage (day 0–2). Clonal expansion occurs during the early stage of 3T3–L1 cell differentiation at which time the cell population is increased by, at least, one fold (Tang et al. 2003). In this study, 11,13DH[11R]-DhL inhibited adipocyte differentiation through the suppression of cell proliferation. These results indicate that the primary target of 11,13DH[11R]-DhL action for preventing adipocyte differentiation in 3T3–L1 cells may be clonal expansion during the early stage.

Cell proliferation during 3T3–L1 adipogenesis occurs through the G_1/S checkpoint as shown by the activation of CDK2-cyclin E/A and cyclin D1, turnover of p27, and hyperphosphorylation of the Rb protein (Chen 1996; Patel and Lane 2000; Sherr et al. 1999).

11,13DH[11R]-DhL arrested the cell cycle at the G_0/G_1 phase by decreased expression of cyclin D1, CDK2, cyclin A, CDK4, and phosphorylated Rb protein. 11,13DH[11R]-DhL also inhibits the growth of human colon carcinoma HT-29 cells through the upregulation of $p21^{cip1}$, and the downregulation of cyclin D1, cyclin A, followed by cell cycle arrest at the G_1 phase. Similarly, helenalin, a sesquiterpene isolated from *Arnica montana*, inhibits adipogenesis through G_1 arrest by increased p21

expression without change in mRNA transcripts, and increases its association with cdk2 in 3T3–L1 cells (Fernandes et al. 2008). The expression of the CDK inhibitor p27, an upstream effector of cell cycle regulatory proteins, was downregulated during adipogenesis, but sulforaphane restored the expression. These results suggest that 11,13DH[11R]-DhL may induce cell cycle arrest at the G_0/G_1 phase through the upregulation of p27 in 3T3–L1 cells.

The MAPK pathway is involved in cell cycle progression and these MAPKs act as a mitogenic signaling molecule in 3T3–L1 preadipocytes (Tang et al. 2003; Bouraoui et al. 2010; Roberts et al. 2002). Adipogenic inducers stimulate the MAPK pathway, followed by enhanced activity of C/EBPβ and induction of adipocyte differentiation (Prusty et al. 2002; Guo et al. 2015; Tong and Hotamisligil 2001).

Epigallocatechin gallate and matrine inhibit adipocyte differentiation by downregulating the ERK pathway (Xing et al. 2010; Hung et al. 2005).

The Akt pathway affects cell cycle progression, through regulation of cyclin D and p27 expression (Ouyang et al. 2006; Collado et al. 2000). Activation of the Akt pathway in 3T3–L1 preadipocytes can also induce adipocyte differentiation (Xu et al. 2004; Kohn et al. 1996; Cristancho et al. 2011).

In my experiments, 11,13DH[11R]-DhL decreased the phosphorylation of Erk1/2 and Akt, which were stimulated by adipogenic inducers in 3T3–L1 preadipocytes, but not to the level demonstrated for cyclin protein abundance. 11,13DH[11R]-DhL inhibits adipocyte differentiation by blocking clonal expansion via cell cycle arrest at the G_0/G_1 phase, which may be mediated by upregulation of p27 expression.

In conclusion, 11,13 dihydro-[11R]-dehydroleucodine blocked the accumulation of lipid droplets stained with Oil Red O and inhibited the increase of triglycerides in the adipocytes (IC_{50}: 71.0 ± 4.1 μM). Expression of PPARγ, C/EBPβ, and C/EBPα were significantly inhibited by 11,13 dihydro-[11R]-dehydroleucodine. in a time and concentration dependent manner. This is consistent with the inhibitory effect of 11,13 dihydro-[11R]-dehydroleucodine on the early stage of adipogenesis. The proliferation of preadipocytes were also suppressed by addition of 11,13 dihydro-[11R]-dehydroleucodine during the first 48 hours after induction. These results indicate that 11,13 dihydro-[11R]-dehydroleucodine may selectively affect mitotic clonal expansion to block preadipocyte differentiation. 11,13 dihydro-[11R]-dehydroleucodine arrested the cell cycle at the G_0/G_1 phase by diminishing the increase of phosphorylation of Rb, Erk1/2 and Akt while increasing the expression of p27. Collectively, the results indicate that the inhibition of early stage preadipocyte differentiation by 11,13 dihydro-[11R]-dehydroleucodine may be associated with cell cycle arrest at the G_0/G_1 phase through upregulation of p27 expression.

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IV. DEHYDROPARASHIN-B INHIBITS ADIPOYCTE DIFFERENTIATION

4.1. Introduction

Preliminary results from experiments on *Artemisia douglasiana* utilizing Nuclear Magnetic Resonance (NMR) and Mass Spectrometry indicated that Fraction 5 inhibited adipogenesis. This was evidenced by qualitative and quantitative analysis via ORO staining of lipid droplets in 3T3-L1 cells, observation of their morphology, and spectrophotometry. Preliminary results identified this compound as a sesquiterpene lactone acid. Despite the large number of sesquiterpenes documented for several families of plants,

sesquiterpene acids are only occasionally reported (Fraga et al. 2010). The presence of related unlactonized sesquiterpene acids in the genus *Artemisia* seems to be restricted to parishin-B isolated from *A. tridentata* ssp. *tridentata f. parishii* Beetle (Fraga et al. 2010; Kelsey and Shafizadeh 1979).

Based, on all this, the objective for this series of experiments was to identify the additional compound(s) accountable for inhibition of *in vitro* 3T3-L1 preadipocyte differentiation. As preliminary results showed that structures closely related to dehydroleucodine may be responsible, compound(s) were also tested for anti-adipogenic effects.

It has been reported (Priestap et al. 2012) that dehydroparishin-B (DhP), a sesquiterpene lactone acid of the guaianolide group, inhibits migration of B16 melanoma cells in a dose dependent manner (IC_{50} = 72.2 mM). In a similar fashion to experiments (Preistrap et al. 2012) which compared the effects of various sesquiterpene lactones, including DhL, DH-DhL, and DhP, on cancer cell migration, I tested the effects of DhP

on adipogenesis in 3T3-L1 preadipoyctes to elucidate whether structural differences in these sesquiterpene lactones could account for various differentials in their anti-adipogenic effects.

4.2. Materials and Methods

3T3-L1 preadipoyctes were incubated in the presence of various concentrations of DhP, and assayed lipid droplet concentration via an ORO assay. This experiment gaged whether DhP inhibited adipogenesis in the first place and its effect on the number and size of lipid droplets. The effect of DhP on the viability of 3T3-L1 preadipocytes by incubating cells with IM in the presence of 9 μ M DhP for 24 hrs (Day 1) or throughout the entire 9 days of differentiation (Day 9) was then examined. Cell viability was then measured using the incorporation of the trypan blue assay as described in the Material and Methods below. Next, western blotting was utilized to investigate DhP's effect on key transcriptional factors including C-EBPβ, PPARγ, FAS, and C-EBP α . These experiments were thus designed to garner both molecular and morphological evidence as to DhP's effects and mode of action.The reagents, cell culture and differentiation, treatments, cell viability assay, Oil Red O staining, microscopy, spectrophotometry, triglyceride assay, western blotting, flow cytometry, high-performance liquid chromatography, compound extraction, purification, identification, and statistical analysis, were prepared and performed as in Chapter 2, above.

4.3. Results

4.3.1. DhP blocks differentiation of 3T3-L1 preadipocytes

A fraction of *Artemesia douglasiana,* containing DhP and lacking DhL exhibited significant anti-adipogenic properties when applied to 3T3-L1 preadipocytes (see **Figure**

20).

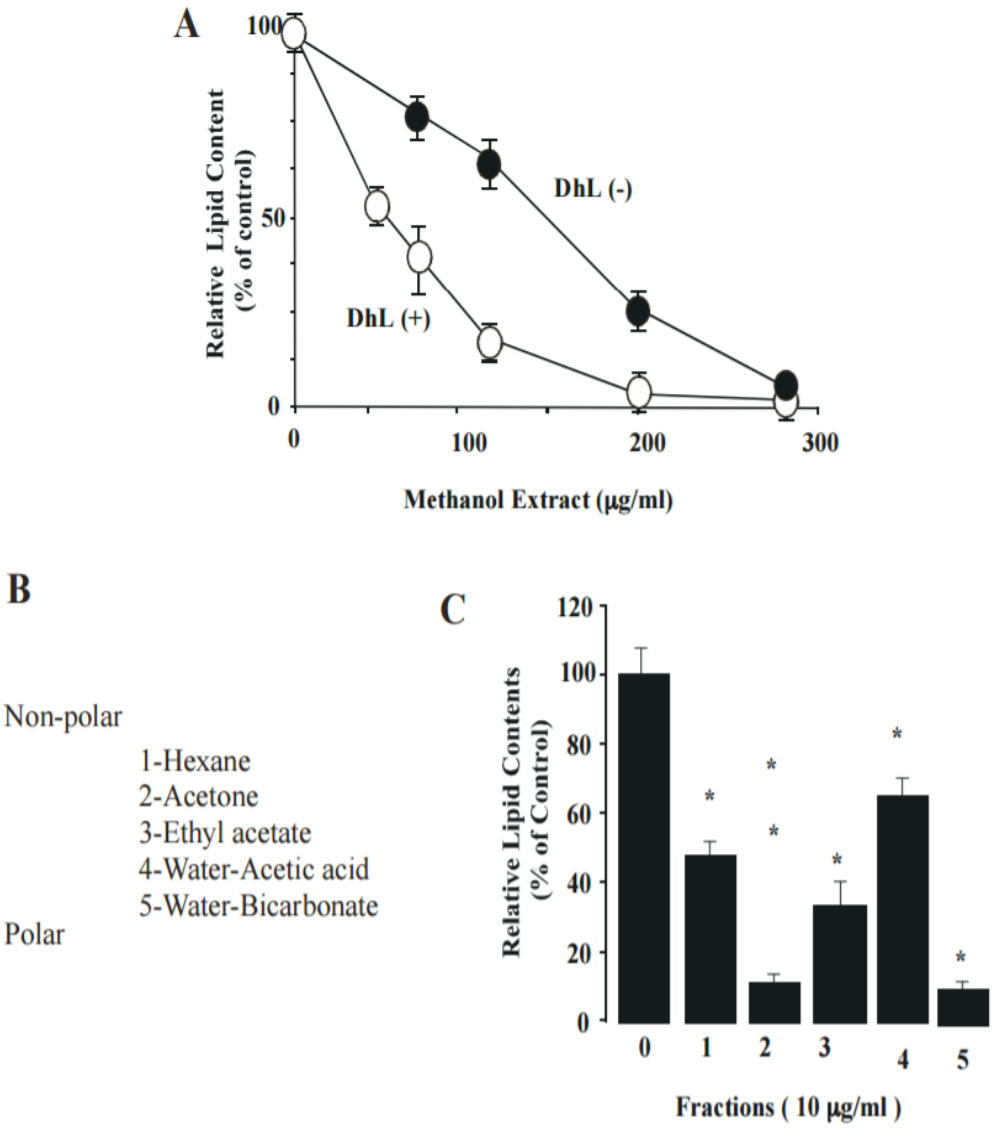

Figure 20. The Effect of *Artemisia douglasiana* methanol extract lacking DhL, and containing DhP inhibits differentiation of 3T3-L1 preadipocytes. The effect of *Artemisia douglasiana* extract lacking DhL and containing DhP on lipid content in 3T3-L1 preadipoyctes **(Figure 20A)**. Ethyl acetate and aqueous 5% NaCO3H which contains DhP strongly inhibits differentiation of 3T3-L1 preadipocytes **(Figure 20B** and **C)**.

To assess the possible role of DhP on the differentiation of 3T3-L1 preadipocytes, cells were incubated in the presence of various concentration of DhP. **Figure 21** (below), shows that the presence of DhP inhibited the lipid content in a dose dependent manner

with an IC50 of 2.5±06 μM, and more importantly, that the addition of 0.2% DMSO does not affect 3T3-L1 preadipocytes differentiation.

The effect of DhP was then examined on the viability of 3T3-L1 preadipocytes. Cells were incubated with induction media in the presence of 9 μM DhP for 24 hour (Day 1) or throughout the entire 9 days of differentiation (Day 9). Cell viability was measured by utilizing the trypan blue assay as described in Material and Methods. DhP did not significantly affect 3T3-L1 preadipocytes cell viability (compare 0.2 % DMSO control-cells 94.5±3.6 cell viability with 92.5±2.8 DhP-treated cells) These results suggest that, up to a maximum concentration of 9 μM, DhP has a strong inhibitory activity of 3T3-L1 preadipocytes differentiation without significant effect on cell viability.

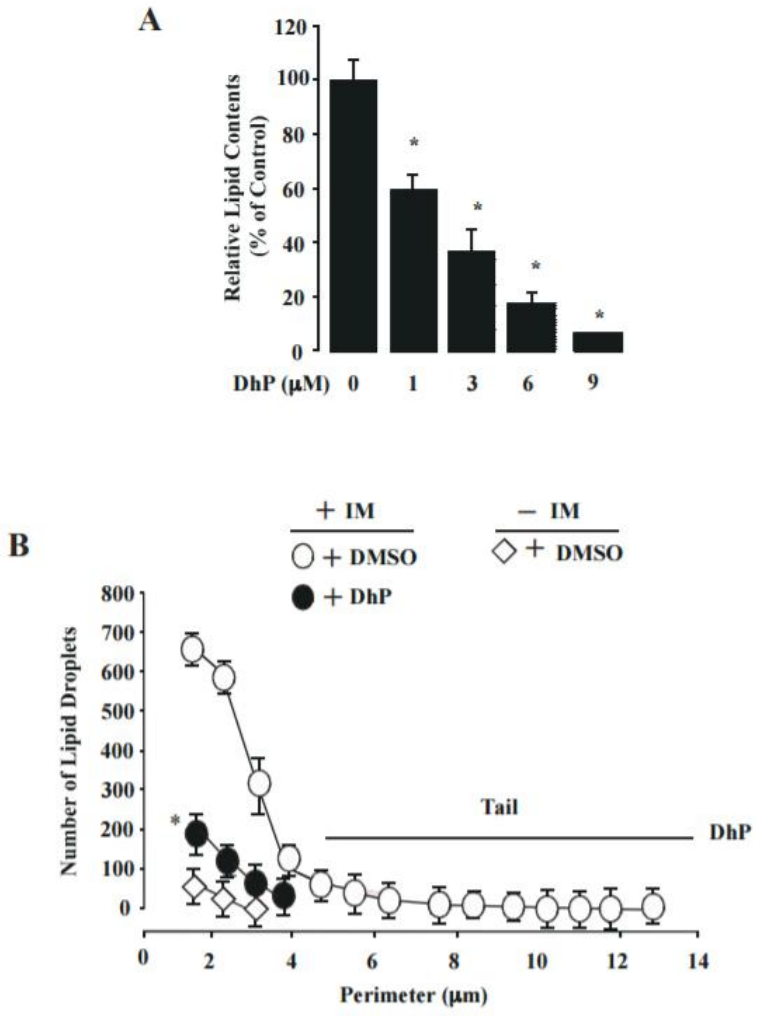

Figure 21. DhP inhibits lipid content in a dose dependent manner

4.3.2. DhP diminishes the number as well as the size lipid droplets

Adipocyte differentiation can be also monitored by formation of intracellular lipid droplets (Rosen and Spiegelman, 2000). As described above, 3T3-L1 preadipocytes were differentiated into adipocytes with induction media in the absence or presence of 9 μM DhP. At day 9, Oil Red O staining in untreated cells showed an abundant number of lipid droplets suggesting a significant lipid accumulation in untreated differentiated cells. However, lipid droplets were difficult to observe in untreated non-differentiated cells

(**Figure 21B** above). These observations were further supported with the quantitative measurement of lipid content by determining the absorbance at 540 nm (**Figure 21A** above).

4.3.3. DhP inhibits accumulation of triglyceride.

Given that DhP inhibited differentiation to 3T3-L1 preadipocytes and also affected the formation of lipid droplets, we next considered whether DhP would inhibit triglyceride accumulation. Cells were treated as described in the Material and Methods in the presence of several concentration of DhP. In **Figure 22A**, a statistically significant decrease in the intracellular triglyceride treated with DhP as compared with control cells, is displayed. As expected, DMSO-treated cells did not affect the formation of triglyceride accumulation as compared with cells incubated with induction media alone (**Figure 22A**).

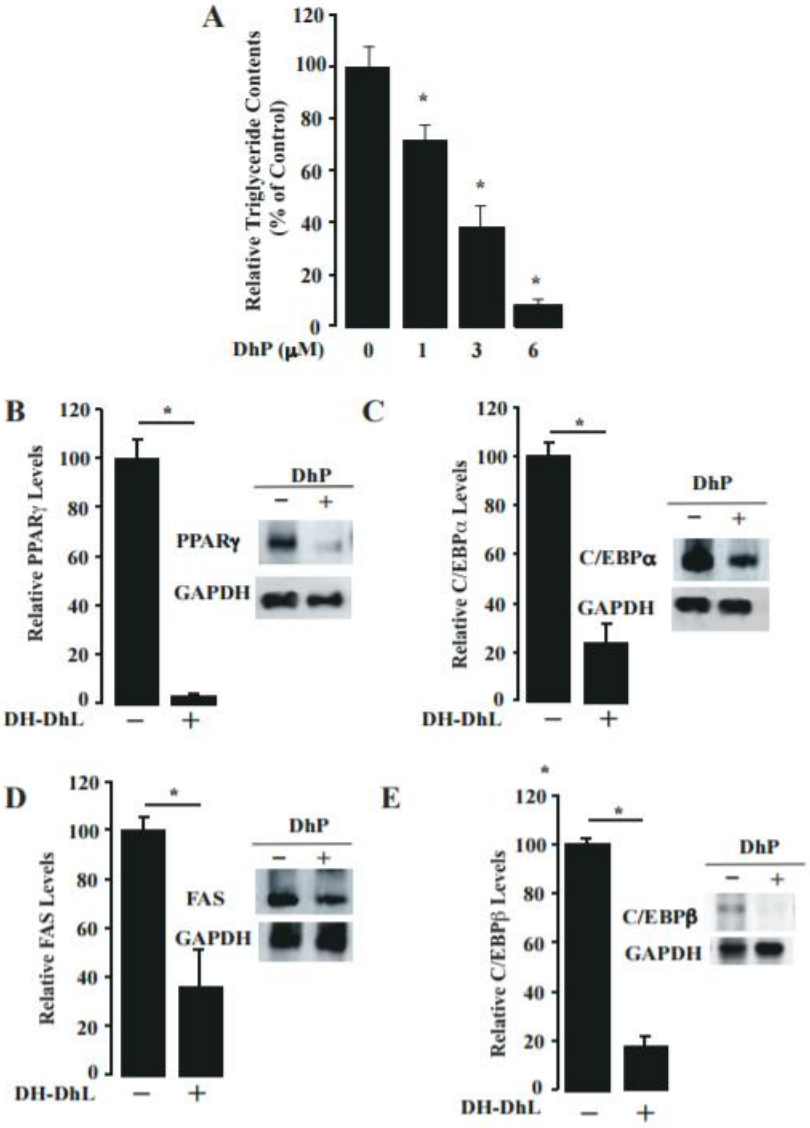

Figure 22. DhP inhibits key adipogenic transcription factors

4.3.4. DhP inhibits key adipogenic transcriptional factors

Adipogenesis is a highly regulated process requiring coordinated expression and activation of key transcriptional factors and signaling molecules (Rosen and Spiegelman, 2000). To investigate whether DhP affects the expression of C-EBPβ, PPARγ and C-EBPα, 3T3-L1 preadipocytes were incubated with induction media in the absence or

presence of 9 μM DhP as, and then harvested at day 9 for Western blot analysis using specific antibodies. In **Figure 22**, it can be seen that the addition of DhP clearly attenuated the expression of C-EBPβ, PPARγ, FAS, and C-EBPα. Taken together, this data suggests that DhL selectively blocks expression of several transcriptional factors during the differentiation of 3T3- L1 preadipocytes.

4.4. Discussion

The addition of DhP significantly decreased the accumulation of lipid content by a dramatic inhibition of the differentiation 3T3-L1 preadipocytes in a concentration and time dependent manner. The presence of DhP downregulates the expression of adipogenic-specific transcriptional factors C/EBPβ, PPARγ and C/EBPα as well as FAS.

Interestingly, I also found that the addition of DhP inhibited the number as well as the size of the lipid droplets during the differentiation of 3T3-L1 preadipocytes. But at what time point, and through which molecular targets is DhP exerting its anti-adipogenic effects?

Since the acquisition of C/EBPβ DNA binding activity corresponds with the initiation of the early adipogenic process of mitotic clonal expansion (MCE) (Tanaka et al. 1997, Tang et al. 2012), and since the experiments revealed a downreulation of C/EBPβ, it is likely that DhP inhibits adipogenesis at this early stage.

The downregulation of PPARγ and C/EBPα, pro-adipogenic markers in the early stage of adipogenesis, comprises further evidence suggesting that the anti-adipogenic effects of DhP observed in the experiments are temporally acting on early adipogenesis and the process of MCE.

Taken together, this data suggests that DhP has an important inhibitory effect on cellular pathways regulating adipocyte differentiation at the early stage of adipogenesis during MCE.

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V. CONCLUSIONS AND FUTURE DIRECTIONS

5.1. Conclusions and Future Directions for DhL, DH-DhL, and DhP

5.1.1. Hypothesized Model of Binding Action of DhL, DH-DhL, and DhP

As depicted in **Figure 21** below, when comparing all three sesquiterpene lactones investigated, DhL, DH-DhL, and DhP, all three displayed inhibitory effects on adipogenesis. This was evidenced in multiple experiments including the Oil Red O assays where both lipid droplet number and size were quantified after treatment with the respective sesquiterpene lactone. However, although both number of lipid droplets and size were reduced under all treatment conditions, DhP produced the most dramatic effect (**Figure 23** below).

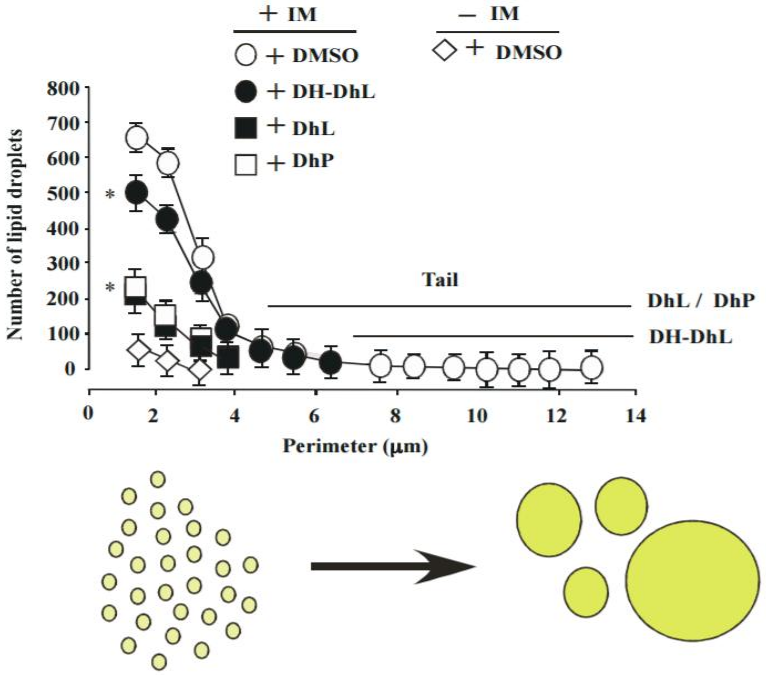

Figure 23. Comparative effects of DhL, DH-DhL, and DhP on lipid droplet quantity and size

As it is evident that differential structural moieties determine binding differences which then lead to functional demarcations, comparisons of points of structural differences between all three sesquiterpene lactones may garner a hypothesized explanatory model for these effects.

As depicted in **Figure 9,** DhL belongs to the guaianolide group of sesquiterpene lactones (Chaturvedi 2011), and contains an α-methylene-γ-lactone ring. It has been previously reported that the α**,** β–unsaturated carbonyl group of sesquiterpene lactones like DhL can undergo a Michael-type addition of a suitable nucelophile, such as nucleophilic attack of cysteine sulfhydryl groups (Heinrich et al. 1998). However, it has also been reported that the exocyclic double bound of sesquiterpene lactones like DhL can undergo nucleophilic attack by cysteine sulfhydryl groups (Blanco et al. 2001). Thus the presence of the exocyclic double bond of DhL, and its corresponding absence in DH-DhL and DhP, could be a key structural difference responsible for different binding modes of action and functional effects.

DhL may affect a multitude of proteins in the adipogenic cascade through this exocyclic double bond binding, to arrest adipogenesis. It is plausible that this multitude of effects which arrest the necessary step of MCE, stem from the upstream inhibitory effect DhL has directly on C/EBPβ, or on factors necessary to express C/EBPβ or activate C/EBPβ's DNA binding activity. Such targets could possibly include Rb, which DhL decreased phosphorylation of in this study, and which binds to C/EBPβ and facilitates its activation (Chen et al. 1996). It is also possible that DhL acts redundantly and directly on numerous processes downstream of C/EBPβ activation, such as obstructing MLL3, which was downregulated in this study and is a component of the ASCOM complex, which is

responsible for the activation of PPARγ and C/EBPα. In a similar fashion, DhL may act directly on the epigenetic modifiers as shown in this study, which include CDC25A, CDC45l, and JMJD2B (Figure 5). Further studies can better clarify these mechanistic issues.

It is also plausible that DhL induces different effects on 3T3-L1 cells in a concentration dependent manner. In D384 astrocytoma cells, lower concentrations of DhL induced a cytostatic effect, similar to that reported for other lactones such as parthenolide, deoxyelephantopin, isodeoxyelephantopin and costunolide (Bailon-Moscoso et al. 2015). Higher concentrations of DhL induced an apoptotic effect on D384 cells (Bailon-Moscoso et al. 2015).

The plausibility of this biphasic effect is enhanced by DhL's similar structure to parthenolide, which has been hypothesized to result in similar functions for the two sesquiterpene lactones (Ordonez et al. 2016). The functional moiety responsible for this activity is likely the exocyclic methylene group (Ordonez et al. 2016). Both parthenolide and DhL possess this moiety, which is lacking (and the only structural difference between it and DhL) in leucodine. Parthenolide and DhL both exhibit cytotoxic activity against acute myeloid leukemia (AML) cell lines by inducing apoptosis, whereas leucodine does not (Ordonez et al. 2016). Both compounds downregulate the p65 transcription factor, which is essential for NF-κB activation (Ordonez et al. 2016). Furthermore, surface signature analysis of both molecules using their X-ray crystal structures reveals that the areas of greatest functional similarity include the excoyclic methylene group, and consist of the convex lipophilic parts of each molecule (Ordonez et al. 2016). This may indicate

that the binding areas of DhL to molecular actors in the adipogenic program may consist of lipophilic regions.

Additionally, previous studies revealed that DhL induced apoptosis in D384 astrocytoma cells by affecting specific agents in the apoptotic cascade. c-ABL phosphorylates TAp73α on tyrosine Y99, which is required for the pro-apoptotic activity of TP73 and its ability to transcriptionally activate BAX. Activated BAX then translocates to the mitochondria to induce cytochrome c to release from the mitochondria, a necessary step in the apoptotic program (Melino et al. 2004; Wang et al. 2000; Yuan et al. 1999). DhL induced apoptosis in the astrocytoma D384 cell line, where increasing expression of the transcription factor TP73, the apoptotic activator BAX and the cell cycle inhibitor CDKN1A (p21), increasing phosphorylation of TP53 and TP73 at the S46 and Y99 amino acid residue, and phosphorylation of the DNA damage marker, γ-H2AX (p-S139-H2AX) were observed (Bailon-Moscoso et al. 2015). Further research is needed to elucidate whether the lipophilic moiety, which includes the exocyclic methylene group of DhL, acts upon similar agents directly or indirectly in 3T3-L1 cells to induce apoptosis.

In addition to oxidizing these possible protein targets in the adipogenic cascade, DhL may also react in a similar covalent binding fashion with the cysteine sulfhydryl groups of glutathione (GSH), thus rendering it inactive. By inhibiting GSH's function in preventing damage to cellular components involved in the adipogenic program by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals (Pompella et al. 2003), DhL could interfere with the normal progression of the adipogenic process.

In contrast to DhL, both DH-DhL and DhP lack DhL's exocyclic methylene moiety. In one study of a similar dihydro derivative of a sesquiterpene lactone to DH-DhL, chemical reduction of the double bond did not affect the capacity of the compound to inhibit aromatase activity (Blanco et al. 2001). Likewise, my results have shown that the lack of DH-DhL's and DhP's exoyclic methlyene moiety does not negate their anti-adipogenic effects. Unlike with DhL, it is likely that DH-DhL and DhP similarly bind non-covalently to binding pockets with conformational and electrostatic structures that favor such interactions.

Future elucidations of the potential binding sites and target molecules of these sesquiterpene lactones in the adipogenic cascade would be a fertile area for future study.

5.1.2. Hypothesized Mechanisms of Inhibitory Action on Adipogenesis of DhL, DH-DhL, and DhP

This binding activity of DhL, DH-DhL and DhP to signaling molecules in the adipogenic cascade, likely impairs factors responsible for facilitating the gene expression that results in the formation and expansion of lipid droplets.

Lipid droplet size reflects a balance of lipogenesis (triglyceride synthesis) and lipolysis (Miyoshi et al. 2008). Since my investigations revealed a decrease in lipid droplet size for all three sesquiterpene lactones, the next step would be to investigate the etiology of these transformations. At least four possibilities are evident: an arresting of lipid droplet formation, an arresting of lipid droplet fusion, a facilitation of lipophagy, or a facilitation of lipolysis (see **Figure 24** below).

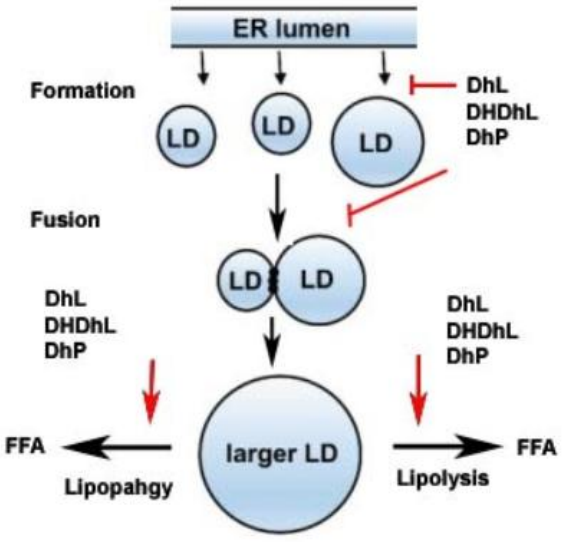

Figure 24. Factors influencing lipid droplet genesis and morphological transformation

Thus, mRNA and/or protein level investigations of gene expression underlying these processes would be another fertile area of research that would further elucidate the mechanistic picture painted by my investigations.

This line of research could include *in vitro* treatment of 3T3-L1 cells in the manner described in this thesis, and then measurement of perilipin A levels, the most abundant phosphoprotein on the surface of adipocyte lipid droplets which has a crucial role in lipid storage and lipolysis (Miyoshi et al. 2008). Additionally, adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) the major rate-determining enzymes for lipolysis in adipocytes (Miyoshi et al. 2008), could also be assayed, as could perilipins which may protect lipid droplets from lipolysis by shielding the triglyceride core of lipid droplets from lipases (Thiam et al. 2013). In addition, levels of enzymes which mediate triglyceride synthesis at the surface of lipid droplets such as GPAT4,

AGPAT3 and DGAT2 (Miyoshi 2008), could be assayed. Other proteins which affects lypolysis include lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), and cell death-inducing DFF45-like effector A (Cidea) (Manickam et al. 2010), which would all be promising to measure. Phosphodiesterase-3B (PDE3B), GTP binding protein ($G_i\alpha_1$), hormone-sensitive lipase (HSL), and tumor necrosis factor α (TNF-α) also affect lypolysis (Davis et al. 2012) and could be measured to gain insight into which process the sesquiterpene lactones are inhibiting.

Additionally, measuring levels of proteins such as Fsp27 and EPA, which are required to form unilocular lipid droplets from smaller lipid droplets during adipocyte differentiation (Thiam et al. 2013), could be undertaken to investigate the possibility of DhL, DH-DhL, and/or DhP interfering with the process of fusion. Lipid droplet protein targeting signals such as amphipathic α helices (such as hepatisis virus core protein) and hydrophobic hairpins (Thiam et al. 2013), could also be studied. Upregulation or downregulation upon treatment of the sesquiterpene lactones of proteins that facilitate formation of lipid droplets such as BSCL2/seipin and FIT proteins (Manickam et al. 2010), could also be assayed.

These and other experimental investigations can carry forward the inquiry I have undertaken in this thesis, just as I have attempted to carry forward the foundational inquiries that have preceded my work, and to which I owe a debt of appreciation.

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International Conference for Tropical Botany Presentation 2017

Florida International University Plant Symposium 2017

Florida International University History and Religious Studies Deparatment 2017

Biomedical Properties and Origins of Sesquiterpene Lactones, with a Focus on Dehydroleucodine (*Natural Product Communications*, in press)

The Birth of the Adipocyte: Early Adipogenesis, the MAPK Pathway, and Mitotic Clonal Expansion (*Current Topics in Biochemical Research*, in press)

Other potential original paper publications based on Chapter 2, 3, and 4 of this thesis are submitted for review