25 Hydroxycholesterol Inhibits Adipogenesis and Expression of Adipogenic Transcripts in C3H10T1/2 Mouse Stem Cells Independent of Hedgehog Signalling Mechanism

by

Dorothy Moseti

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg

ABSTRACT

This study was conducted to assess the effects of specific oxysterols on the adipogenic differentiation and expression of adipogenic transcripts in C3H10T1/2 mouse stem cells. Four oxysterols namely; 20S, 22R, 22S and 25 hydroxycholesterol (25-HC) were tested to determine which one best inhibits adipogenesis in C3H10T1/2 mouse stem cells. Adipogenic differentiation was induced using an adipogenic media (DMITro) consisting of dexamethasone (DEX), 3-isobutyl-1-methyl-xanthine (IBMX), insulin and troglitazone (Tro). Inhibition of adipogenesis was assessed by treatment of cells with DMITro+20S, 22R, 22S or 25-HC for six days. Oil red O pictures and gene expression analysis showed that 25-HC was more effective in inhibiting the expression of adipogenic genes compared to the other oxysterols. Further investigation of the mechanisms of action of 25-HC showed that the inhibitory effects of 25-HC on adipogenesis in C3H10T1/2 cells are not mediated by hedgehog signalling.

ACKNOWLEDGEMENT

It is with great pleasure that I express my sincere gratitude to the people who have made completion of this thesis possible. First of all, my great appreciation goes to my advisor, Dr. Woo K. Kim for giving me this opportunity and the resources for my studies and for his valuable advice and guidance throughout my Master's programme. His patience, encouragement, understanding and desire to see this work completed is highly appreciated. I would like to extend my appreciation to my co-advisor Dr. Karmin O for her valuable guidance, advice and suggestions concerning my work. I would also like to thank Dr. Judith Anderson for accepting to be on my advisory committee and for all her suggestions concerning my work. I thank my lab members Dr. Alemu Regassa for his help and training in cell culture, real time PCR and microarray, and Yvette Shang and Bishwo Bandhu for all their input and help in labwork. I would also like to extend my heartfelt appreciation to fellow graduate students, technical and research staff at the Department of Animal Science for their friendship and support. I thank the administrative staff, Margaret Ann, Mei, Kathy, Catherine and Gwen for their assistance in administrative matters. My special thanks to Peris, Gustavo and Roseline for their friendship, encouragement and support.

This study was funded by Natural Science and Engineering Research Council-Discovery grant (NSERC-Discovery), Agri-food Research Development Initiative and University of Georgia Start-up Grant. Financial assistance received from IGSES and a Faculty of Graduate Studies conference travel award are highly appreciated.

I would also like to express my special thanks to my family who have supported and encouraged me throughout this programme. I thank my parents to whom this thesis is dedicated for their warm love and support. I thank my sisters and brother who have been a great source of inspiration and support. Finally, I thank God, through whom everything is possible.

DEDICATION

This thesis is dedicated to my parents; Wilfred Moseti and Florence Gekara for their constant love and support.

FOREWORD

This thesis is in a manuscript format and is composed of two manuscripts. Manuscript I will be submitted to Lipids Journal and manuscript II will be submitted to BMC Genomics. A literature review based on this thesis will be submitted to the International Journal of Molecular Science. Part of the work in manuscript I was presented at the Experimental Biology meeting in April 2014. The authors of manuscript I are Dorothy N. Moseti, Alemu Regassa, Karmin O and Woo K. Kim. The authors of manuscript II are Dorothy N. Moseti, Alemu Regassa and Woo K. Kim.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
BAT	Brown adipose tissue
BMP4	Bone morphogenic protein 4
C/EBPα	CCAAT/enhancer binding protein α
cAMP	cyclic adenosine monophosphate
DBD	DNA binding domain
DEX	Dexamethasone
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthetase
FBS	Fetal bovine serum
GLUT4	Glucose transporter IV
IBMX	3-isobutyl-1- methylxanthine
KLFs	Kruppel like factors
LPL	Lipoprotein lipase
LXR	Liver x receptor
MCE	Mitotic clonal expansion
MSCs	Mesenchymal stem cells
NF1	Nuclear factor 1
OCN	Osteocalcin
PBS	Phosphate buffered saline
PEPCK	Phosphoenol pyruvate carboxykinase
PPARγ	Peroxisome proliferator-activated receptor γ
RXR	Retinoid x receptor
SCD	Stearyl-CoA-desaturase
SREBF1	Sterol-regulatory element-binding factor 1
TNFα	Tumor necrosis factorα

T2DM	Type 2 diabetes mellitus
WAT	White adipose tissue
ZFP423	Zinc finger protein 423

CHAPTER ONE

1.0 INTRODUCTION

Obesity is a major contributor to the global burden of disease and has led to an increase in obesity-related disorders such as cardiovascular diseases, type 2 diabetes and several cancers (Haslam & James, 2005). The World Health Organization (WHO) defines obesity and overweight as excessive fat accumulation that may impair health, and estimates that in 2008, more than 1.4 billion adults globally were overweight. In 2013, WHO estimated that roughly 42 million children under the age of 5 were obese. Overweight and obesity were accepted by WHO as a global problem at the 1997 WHO expert consultation on obesity (WHO, 2013). The incidence of obesity continues to rise and is now being associated with conditions such as dyslipidaemia, osteoarthritis, pulmonary diseases and sleep apnea (WHO, 2000). The obesity epidemic is caused by various factors such as lack of physical exercise and changes in diet including excess food consumption, especially foods rich in sugars, fats and starch, leading to generation of additional fat cells or adipocytes (Curbing the obesity epidemic, 2006). Generally, obesity develops as a result of energy intake exceeding energy expenditure (Leibel, Rosenbaum, & Hirsch, 1995).

In the body, storage of fat in the adipose tissue represents the excess energy intake relative to energy expenditure. When energy intake is scarce, this stored fat is released into the blood stream as fatty acids and is in turn taken up by other body tissues and used as a source of energy (Siersbaek et al., 2010). As such, the adipose tissue serves as an important energy storage organ in healthy humans and is considered an essential regulator of energy balance and glucose homeostasis (Rosen & Spiegelman, 2006). However, excess increase in adipose mass, characterized by increase in number (hyperplasia) and size (hypertrophy) of adipocytes leads to obesity and associated conditions (Couillard et al., 2000). Thus, obesity and weight

gain have led to increased interest in adipose tissue and development of fat cells, a process known as adipogenesis (Lefterova & Lazar, 2009).

Adipocytes constitute majority of cells in the adipose tissue. Other cell types found in this tissue include adipogenic progenitor cells or preadipocytes and vascular stromal cells such as fibroblasts, smooth muscle cells, endothelial cells and pericytes (Katz, 2002). There are 2 types of adipose tissues in the human body; brown adipose tissue (BAT) and white adipose tissue (WAT). Of these two, WAT is the most abundant and is important in storage of energy in the form of triglycerides, while BAT is important in basal and inducible energy regulation through thermogenesis, which is important in heat generation in response to cold environments (Gesta et al., 2007). Studies show that obesity is mainly associated with the expansion of WAT commonly found under the skin and around visceral organs. Increase in size of white adipose cells in obesity leads to alteration of function such as disruption of hormones and release of cytokines and adipokines, which alter the normal energy homeostasis mechanisms leading to a wide array of disorders such as cardiovascular diseases (Farmer, 2008). Secreted adipokines directly interfere with insulin signalling by causing insulin resistance. This in turn leads to an increase in demand for insulin production, which leads to type 2 diabetes mellitus if production is not able to meet demand (Siersbaek et al., 2010).

It is thought that adipose tissue has a mesodermal origin just like bone, muscle or cartilage tissue. However, the specific mesodermal lineage is still not known (Gesta et al., 2007). Since obesity is considered a major public health problem, it is necessary to study the principal mechanisms associated with weight gain in order to treat and prevent obesity and associated disorders, which are becoming increasingly prevalent, especially in developed societies (James, 2008). An understanding of the process of adipose tissue formation and the

mechanisms that govern this process is therefore vital in providing valuable information that could be useful in the fight against the growing incidence of obesity (Farmer, 2006).

The use of *in-vitro* cell culture models of adipocyte differentiation such as mesenchymal stem cells has proven useful in studying adipogenesis and the mechanisms involved. Furthermore, molecular analytical techniques such as quantitative real-time PCR and Microarray have been used to study the expression profiles of transcription factors involved in adipocyte differentiation (Fu et al., 2005).

Oxysterols, which are products of cholesterol oxidation, have been identified as a possible means of regulating adipogenic differentiation of mesencymal stem cells (MSCs) and have been found to inhibit adipogenic differentiation while inducing osteogenic differentiation of these cells (Kha et al., 2004; Kim et al., 2007).

However, the effects of oxysterols on the adipogenic differentiation of C3H10T1/2 mouse stem cells are still poorly understood. Such information will be useful in assessing the potential of oxysterols as an intervention for treatment of excess fat accumulation and obesity.

We therefore hypothesized that;

1.) Specific oxysterols are able to inhibit adipogenesis and expression of adipogenic genes in C3H10T1/2 mouse stem cells
2.) Specific oxysterols inhibit adipogenic differentiation in C3H10T1/2 mouse stem cells through hedgehog signalling mechanism.

The main objective of this study was to assess the effect of four different oxysterols namely; 20S, 22R, 22S and 25 hydroxycholesterols on adipogenic differentiation and expression of adipogenic-gene transcripts in C3H10T1/2 cells. The specific objectives were to study;

1.) The effects of oxysterols on adipogenic differentiation of C3H10T1/2 cells at different time points.
2.) The mechanisms through which oxysterols inhibit adipogenic differentiation in C3H10T1/2 cells.
3.) The profile of genes/molecules associated with adipogenic differentiation and inhibition of adipogenesis in C3H10T1/2 cells.

Relevance of this study

Knowledge of the anti-adipogenic properties of oxysterols in stem cell differentiation may be clinically useful in inhibiting adipogenesis and therefore provide an intervention in excess fat accumulation associated with obesity.

This knowledge could also be applied in the poultry and animal production industries where excess accumulation of adipose tissue leads to reduced productivity and feed efficiency and causes diseases.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 An overview of adipogenesis

Adipogenesis involves the differentiation of preadipocytes into mature adipocytes that contain lipid droplets. This process also involves changes in cell morphology, induction of insulin sensitivity, expression of adipogenic differentiation markers and changes in secretory capacity of cells (Lefterova & Lazar, 2009). The increasing prevalence of obesity has led to increased research in the area of fat cell biology and mechanisms involved in adipogenic differentiation. Furthermore, the availability of reliable cell culture models of adipogenic differentiation has greatly enhanced studies on adipogenesis (Rosen et al., 2002). Differentiation of preadipocytes to adipocytes involves a transcriptional network consisting of markers responsible for expression of proteins that enhance mature adipocyte formation (Farmer, 2006).

The peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) are the main regulators of adipogenesis, and genome-wide studies have indicated an extensive overlap in their transcriptional targets (Lefterova & Lazar, 2009). PPARγ is induced during differentiation of preadipocytes to adipocytes and is necessary and sufficient for the process of adipogenesis (Rosen et al., 2000). Without it, precursor cells are unable to differentiate into mature adipocytes (Rosen et al., 2000). Furthermore, PPARγ is capable of promoting adipogenesis in C/EBPα-deficient cells. However, C/EBPα is not able to promote adipogenesis in PPARγ deficient cells demonstrating that PPARγ is the master regulator of adipogenesis (Rosen et al., 2002). Although cells deficient in C/EBPα are capable of differentiating into adipocytes, this differentiation is defective in that they accumulate fewer lipid droplets and do not induce expression of PPARγ, demonstrating that cross-regulation between C/EBPα and PPARγ is important for maintenance of a

differentiated state (Rosen et al., 2002; Z. Wu et al., 1999). Apart from PPARγ and C/EBPα, adipocyte differentiation involves the expression of several other transcription factors which interact in the different stages of adipogenesis to yield mature adipocytes (Kim et al., 1998). The expression pattern of genes and proteins involved in adipogenesis is in a coordinate fashion depending on the stage of adipogenesis. These proteins and genes regulate normal adipocyte differentiation and include glucose transporter IV (GLUT4), lipoprotein lipase (LPL), stearyl-CoA-desaturase (SCD) and fatty acid synthetase (FAS) (Student et al., 1980; Vu et al., 1996). The promoters of some of the genes that are differentially expressed during the differentiation of preadipocytes to adipocytes have been shown to have binding sites for PPARγ and C/EBPα (Christy et al., 1989; Tontonoz et al., 1994).

2.2 The Peroxisome proliferator-activated receptor γ

PPARγ is a member of the PPAR nuclear hormone receptor superfamily of ligand activated transcription factors, which bind to the promoter of target genes leading to increase or decrease in DNA transcription upon binding of ligands or small lipophilic molecules (Kersten, 2002). PPARs consist of a non-conserved N-terminal domain, a highly conserved DNA binding domain (DBD), a hinge region and a C-terminal ligand binding domain. It is at the DBD that PPARs anchor to their binding sites on DNA templates from which they regulate gene expression (Bain et al., 2007; Chandra et al., 2008). PPARs govern various biological processes such as energy metabolism, cell proliferation and inflammation (Kersten, 2002). The PPAR family consists of three members namely; α, β and γ. The name Peroxisome proliferator-activated receptor derives from the ability of PPARα to respond to compounds that induce peroxisome proliferation (Kliewer et al., 1994). PPARα mRNA is thus mostly expressed in tissues that undergo peroxisomal proliferation such as heart, kidney and liver, where increase in peroxisomes increases β- oxidation (Dreyer et al., 1992; Nemali et al., 1988). In the liver, PPARα regulates nutrient metabolism including gluconeogenesis

and amino acid metabolism. It also mediates the uptake, activation and oxidation of fatty acids, synthesis of ketone bodies and apolipoproteins (Kersten, 2002). In addition, PPARα is highly expressed in the skeletal muscle and vascular wall (Bishop-Bailey & Wray, 2003). Natural ligands for PPARα include polyunsaturated fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, linoleic acid and linolenic acid (Wahli, 2002). PPARβ, on the other hand, is present in many tissues, but its functions are not very clear. However, it has been proposed to mediate fatty acid-controlled differentiation of preadipocytes (Bastie et al., 2000).

PPARγ mRNA is abundantly expressed in white and brown adipose tissue, colon, cecum and macrophages and its expression increases during adipocyte differentiation (Braissant et al., 1996). PPARγ plays a dominant role in adipogenic differentiation, glucose metabolism, inflammation and other physiological processes, and is also a receptor of an important class of antidiabetic drugs (Kliewer et al., 1994; Tontonoz & Spiegelman, 2008). These drugs, known as Thiazolidinediones (TZDs), include rosiglitasone and troglitazone and are generally considered to be agonists/ligands of PPARγ (Kersten, 2002). In patients with type 2 diabetes mellitus (T2DM), which is associated with lack of insulin responsiveness in peripheral tissues, activation of PPARγ by the synthetic TZD drugs enhances insulin sensitivity and leads to enhanced glucose uptake and thus a reduction in concentration of plasma glucose (Willson et al., 2001). PPARγ forms a heterodimer with retinoid X receptor (RXR), enabling it to bind to DR-1 sites on target sequences (Tontonoz et al., 1994). Activation of PPARγ has been shown to facilitate the process of adipogenesis, leading to increase in number of small and insulin sensitive adipocytes (Okuno et al., 1998). In addition, activation of PPARγ also up-regulates the adipose-derived hormone adiponectin which improves insulin sensitivity in the liver and muscle (Nawrocki et al., 2006).

A wide range of compounds including fatty acids, prostaglandins and oxidized phospholipids have been proposed to act as PPAR ligands in vitro (Kersten, 2002). Although most PPARγ agonists such as 9, 10-dihydroxyoctadecenoic acid, and 15-deoxy-Delta (12,14)-PGJ(2) act by promoting adipogenesis while inhibiting osteogenesis, not all agonists achieve a similar effect. For example, the thiazolidine acetamide partial agonist GW0072, inhibits osteogenesis but does not stimulate adipogenesis. In contrast, 9-hydroxyoctadecadienoic acid stimulates adipogenesis but has no effect on osteoblast differentiation, indicating that the adipogenic and anti-osteoblastogenic effects of PPARγ are mediated by distinct pathways that are modulated by the nature of ligand involved (Lecka-Czernik et al., 2002).

PPARγ is expressed in two main protein isoforms: PPARγ1 and PPARγ2, which occur as a result of alternate promoter usage and splicing (Zhu et al., 1995). Both isoforms are abundantly expressed in the adipose tissue. In addition, PPARγ1 is also broadly expressed in the colon, retina and hematopoietic cells and has also been detected in low levels in the liver, spleen and heart (Kersten et al., 1999; Shimoike et al., 1998; Vidal-Puig et al., 1996). PPARγ2 is identical to PPARγ1 except that its N-terminus contains an additional 30 amino acids. The functional differences between these two isoforms in adipocyte differentiation have been studied by blocking PPARγ2 expression in 3T3-L1 cells using artificial zinc finger repressor proteins (Ren et al., 2002). Cells with a 95% reduction in PPARγ2 expression failed to undergo adipogenic differentiation but exogenous delivery of PPARγ2 into the cells restored adipogenic differentiation (Ren et al., 2002). On the other hand, exogenous reactivation by PPARγ1 had no effect on adipogenic differentiation, suggesting that PPARγ2, not PPARγ1 plays a key role in adipogenesis (Ren et al., 2002). PPARγ2 has been described as an adipocyte-specific nuclear hormone receptor which is capable of activating the adipocyte-specific ap2 enhancer in heterologous cells, and can be transcriptionally activated by lipids, including naturally occurring polyunsaturated fatty acids (Tontonoz et al., 1994).

In vivo and *in vitro* loss-of-function studies have demonstrated that PPARγ is both necessary and sufficient for adipogenic differentiation and induction of an adipose phenotype, which is marked by accumulation of lipid and expression of adipocyte differentiation markers (Barak et al., 1999; Rosen et al., 1999; Rosen et al., 2000). PPARγ knockout mice are known to die during embryogenesis due to interference with terminal differentiation of the trophoblast and placental insufficiency (Barak et al., 1999). In an experiment comparing adipogenic differentiation between PPARγ wild type and PPARγ knockout mice, it was demonstrated that adipose tissue derives preferentially from wild type cells while PPARγ-null cells were unable to contribute to fat cell formation in the mice (Rosen et al., 1999). Furthermore, embryonic fibroblasts derived from PPARγ deficient fetuses were not able to differentiate into adipocytes in an in vitro model (Kubota et al., 1999).

Since PPARγ plays an important role in adipogenic differentiation and is a receptor for insulin-sensitizing drugs, regulation of its expression is of importance with respect to nutrition, obesity and diabetes. Tissue expression and potential for regulation of PPARγ have been studied both in vivo and in vitro (Desvergne & Wahli, 1999). In an *in vivo* study using mice, it was reported that PPARγ mRNA and protein levels are down-regulated by fasting and insulin-deficient diabetes whereas a diet rich in fatty acids increased adipose tissue expression of PPARγ in normal mice and induced PPARγ2 expression in the liver of obese mice (Vidal-Puig et al., 1996). Fasting for 48 hours was shown to reduce the expression of both PPARγ isoforms in subcutaneous and visceral adipose tissues of rats (Shimoike et al., 1998). In an *in vitro* study, treatment of isolated human adipocytes with insulin and corticosteroids was shown to induce the expression of PPARγ mRNA (Vidal-Puig et al., 1997). In contrast, treatment of 3T3-L1 cells with Tumor necrosis factor alpha (TNFα), a polypeptide hormone with pleiotropic effects on cellular differentiation, down-regulated the expression of PPARγ (Xing et al., 1997).

2.3 CCAAT/enhancer-binding proteins (C/EBPs)

The C/EBP transcription factors also play an important role in adipocyte differentiation. They belong to a family of highly conserved basic leucine zipper transcription factors consisting of six members, of which three family members (C/EBPα, -β, and –δ) have an established role in adipogenesis. In particular, C/EBPα is commonly expressed in the adipose tissue, liver, lungs, adrenal glands and placenta (Birkenmeier et al., 1989; Yeh et al., 1995). C/EBPα and PPARγ are involved in a single adipogenic differentiation program, in which PPARγ is the dominant factor. C/EBPα is important in terminal differentiation of adipocytes, as absence of this factor leads to insulin resistance in *in vitro* experiments and hinders formation of WAT *in vivo*. In contrast, development of BAT is independent of C/EBPα (El-Jack et al., 1999; Linhart et al., 2001). C/EBPβ and –δ have been postulated to be the first transcription factors induced during induction of adipogenesis, and therefore play an important role in directing the differentiation process (Darlington et al., 1998).

The importance of C/EBPβ and –δ has been demonstrated in loss-of-function and gain-of-function studies where embryonic fibroblasts from mice lacking these two markers are unable to differentiate in response to hormonal induction. Consequently, these cells fail to express other important adipogenic markers such as C/EBPα , PPARγ or fatty acid binding protein 4 (FABP4), suggesting that in vitro adipocyte differentiation proceeds according to the proposed transcriptional cascade in which C/EBPs and PPAR families of transcriptional factors are activated sequentially leading to formation of mature adipocytes (Tanaka et al., 1997). In contrast, *in vivo* studies show that induction of C/EBPα and PPARγ can take place without the expression of C/EBPβ and C/EBPδ. However, adipogenesis in double knock out C/EBPβ, δ-null mice is severely impaired. This suggests that though *in vivo* induction of C/EBPα and PPARγ can take place without the expression of C/EBPβ and C/EBPδ, co-

expression of C/EBPα and PPARγ is not sufficient for complete adipocyte differentiation in the absence of C/EBPβ and C/EBPδ (Tanaka et al., 1997).

2.4 The process of Adipocyte differentiation

Adipogenic differentiation is characterized by chronological changes in the expression of various genes that lead to the establishment of the adipocyte phenotype. These changes include the appearance of early, intermediate and late mRNA/protein markers and accumulation of triglycerides (Farmer, 2006). The process of adipogenesis, which occurs in four main stages namely; growth arrest, mitotic clonal expansion (MCE), early differentiation and terminal differentiation, involves the expression of the transcriptional markers such as PPARγ and C/EBPs family (Farmer, 2006). Cell/cell contact is important for adipocyte differentiation. Cultured preadipocytes undergo proliferation before entering the growth arrest stage, at which point they begin to express early markers of differentiation. It is possible that cell/cell contact activates mechanism(s) that induce early differentiation markers (Dani et al., 1990; Tong & Hotamisligil, 2001).

Appropriate inducers are required for the cells to proceed to the mitotic clonal expansion stage and subsequent differentiation (MacDougald & Lane, 1995). 3T3-L1 fibroblasts are able to differentiate into fat-laden adipocytes in a span of approximately one week upon induction using fetal bovine serum (FBS), Dexamethasone (DEX), 3-isobutyl-1-methyl-xanthin (IBMX) and Insulin (Farmer, 2006; Green & Kehinde, 1975). This cocktail activates the adipogenic program in these cells, which are then directed into the different stages of adipogenesis. In particular, DEX and MIX are identified as direct inducers of genes responsible for the expression of C/EBPδ and C/EBPβ respectively (Cao et al., 1991). Insulin acts by stimulating the cells to take up glucose, which is stored in the form of triacylglycerol (Summers et al., 1999). During the early stages of differentiation, there is a high expression of C/EBPδ and C/EBPβ in response to hormonal induction. The two markers play early

catalytic roles in the adipogenic differentiation pathway and diminish during late stages of differentiation and are replaced by PPARγ and C/EBPα (Cao et al., 1991; Yeh et al., 1995). Other studies have shown that ectopic expression of C/EBPβ in NIH 3T3 fibroblasts, alone or in combination with C/EBPδ, leads to expression of PPARγ2 and eventual conversion of fibroblasts to adipocytes. However, these cells do not express C/EBPα despite an accumulation of abundant lipid droplets in response to activation of PPARγ (Wu et al., 1995; Wuet al., 1996). MCE is a fundamental requirement for terminal differentiation. Blocking entry of 3T3-L1cells into S phase during MCE leads to inhibition of adipogenic differentiation because it is during the MCE stage that cells express various transcription factors and regulators that lead to expression of PPARγ and C/EBPα (Tang et al., 2003). Upon activation, PPARγ induces the expression of other target genes involved in adipogenesis. PPARγ also induces the expression of C/EBPα, which can bind on the promoter region of PPARγ thus providing for a stable, self-regulatory loop (Tontonoz & Spiegelman, 2008). C/EBPα induces the activation of a number of adipocyte-specific genes including phosphoenol pyruvate carboxykinase (PEPCK) FABP4 and GLUT4, which contain C/EBP-binding sites in their promoter region (Christy et al., 1989; Park et al., 1993; Yeh et al., 1995). On the other hand, target genes for PPARγ include those coding for ap2, lipoprotein lipase, acyl-CoA synthase, PEPCK, fatty acid transport protein and adipisin, the promoters of which contain regulatory elements for PPARγ (Rosen et al., 1999; Tontonoz et al., 1994; Wahli, 2002).

Cooperative gene expression between C/EBPα and PPARγ has been demonstrated where ectopic expression of either transcription factor alone leads to expression of the other, suggesting that at the final stage of adipogenesis, C/EBPα and PPARγ function in a cooperative manner to induce adipocyte-specific genes that establish the mature adipocyte phenotype (Tanaka et al., 1997). The terminal differentiation stage is thus characterised by

acquisition of machinery that is necessary for lipid transport and synthesis, production of lipid droplets, insulin action, secretion of adipocyte specific proteins and expression of various metabolic programs that define differentiated cells (Farmer, 2006). Maintenance of terminal differentiation is facilitated by the sustained expression of C/EBPα, which is able to transactivate various adipocyte genes (Christy et al., 1989). Furthermore, C/EBPα contains a C/EBP binding site within its proximal promoter that allows auto-activation of its own expression, which is important in enhancing continual expression of this marker (Christy et al., 1991; Lin et al., 1993). Quantitative expression profiling using both micro array and qRT-PCR analysis of mRNAs obtained from adipocyte differentiation cultures have demonstrated the presence of many transcriptional proteins and receptors involved in the process of adipogenesis. Some of these receptors include liver x receptor (LXR) and retinoid x receptor α (RXRα), which play key roles in differentiation and maintenance of mature fat cells (Fu et al., 2005).

2.5 Positive regulators of PPARγ expression and adipogenesis

2.5.1 Kruppel like factor family (KLF4, KLF5, KLF9, KLF15)

In addition to PPARγ and C/EBPα which play central roles in adipogenic differentiation, other transcription factors have been identified and shown to be important in adipocyte differentiation. They include the Kruppel like factors (KLFs), zinc finger protein 423 (ZFP423), Nuclear factor 1 (NF1) and sterol-regulatory element-binding factor 1 (SREBF1) (Siersbaek et al., 2010). The KLF transcription factors that are induced during adipogenesis in 3T3-L1 cell line include KLF4, KLF5, KLF9 and KLF15. KLF4 has been characterized as an early marker of adipogenic differentiation. In 3T3-L1 cells, KLF4 is expressed within the first 30 minutes and peak at around 2h after exposure to an adipogenic cocktail consisting of insulin, glucocorticoids and IBMX (Birsoyet al., 2008). Further analysis shows that knockdown of KLF4 inhibits adipogenesis and down regulates the expression of C/EBPβ

(Birsoyet al., 2008). KLF5 is induced by C/EBPδ/β during the early stages of adipogenesis in 3T3-L1 preadipocytes and is followed by expression of PPARγ2, suggesting that KLF5 mediates both the early and late stages of adipogenic differentiation (Oishi et al., 2005). KLF5 has also been shown to bind directly to the PPARγ2 promoter and cooperate with C/EBPs to induce PPARγ2 expression (Oishi et al., 2005). This study also shows 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). The expression of KLF9 is up-regulated during the middle stage of adipogenic differentiation, and inhibition of this factor by RNA interference has been shown to inhibit adipogenesis (Pei et al., 2011). Just like KLF5, KLF9 binds directly to the PPARγ2 promoter and directly activates it by binding to C/EBPα (Pei et al., 2011).

2.5.2 SREBF1/ADD1

The adipocyte determination and differentiation-dependent factor 1 (ADD1), also termed as SREBF1, is a basic helix-loop-helix (bHLH) leucine transcription factor that is associated with adipocyte differentiation and cholesterol homeostasis (Yokoyama et al., 1993). SREBF1/ADD1 is expressed in different types of tissues but is predominantly expressed in brown adipose tissue in vivo (Tontonoz et al., 1993). As a member of bHLH transcription factor family, SREBF1/ADD1 has dual DNA binding specificity, in that it can bind to an E-box motif and a sterol regulatory element (SRE). Thus, when expressed in fibroblasts, SREBF1/ADD1activates transcription through both the E-box motif and SRE, thus providing a novel mechanism to coordinate different lipid metabolism pathways (Kim et al., 1995; Yokoyama et al., 1993). SREBF1/ADD1 is also described as a sequence-specific transcriptional activator in that it is able to stimulate the expression of chloramphenical acetyltransferase vector that displays multiple SREBF1/ADD1 binding sites, but is not able to stimulate expression of myosin light-chain enhancer, which contains multiple binding sites

for MyoD, another bHLH factor (Tontonoz et al., 1993). It has been shown that SREBF1/ADD1 plays an important role in adipocyte gene expression by enhancing a step in PPARγ-mediated transcription. This transcription factor also controls the expression of fatty acid synthase and lipoprotein lipase, important genes involved in fatty acid metabolism (Kim & Spiegelman, 1996). A different study suggests that expression of SREBF1/ADD1increases the activity of PPARγ but not that of PPARα or PPARδ. This activation is thought to occur through production of endogenous ligands, which are likely to be derivatives of fatty acids (Kim et al., 1998). Moreover, the lipid molecules produced due to expression of SREBF1/ADD1 bind to PPARγ, eventually leading to displacement of radioactive thiazolidinedione ligands (Kim et al., 1998). Expression of ADD1/SREBF1, coupled with use of hormonal inducers leads to stimulation of adipogenesis in cells (Kim & Spiegelman, 1996).

2.5.3 Cyclic AMP response element-binding protein (CREB)

CREB has been proposed to have a possible role in the control of adipogenesis. 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). Alternatively, transfection of 3T3-L1 preadipocytes with a dominant-negative form of CREB blocks adipogenic differentiation (Reusch et al., 2000). Further study shows that expression of CREB is stimulated by differentiation-inducing agents such as dexamethasone, insulin and dibutyryl cAMPs (Reusch et al., 2000).

2.5.4 Zinc Finger Protein 423 (ZFP423)

ZFP423 is a transcription factor that was recently identified as a regulator of preadipocyte cell determination and is abundant in preadipose compared to non-preadipose fibroblasts (Gupta et al., 2010). Ectopic expression of ZFP423 in non-adipogenic NIH3T3 fibroblasts

induces expression of PPARγ in undifferentiated cells and promotes adipogenesis once cells have been induced to differentiate. Conversely, inhibition of ZFP423 in 3T3-L1 cells inhibits PPARγ expression and adipogenic differentiation (Gupta et al., 2010). It has also been shown that adipocyte differentiation is greatly impaired in ZFP423-deficient mouse embryos (Gupta et al., 2010). Furthermore, ZFP423 stimulates adipogenic differentiation of bovine stromal vascular cells as shown by accumulation of lipids and expression of PPARγ and C/EBPα (Huang et al., 2012). The molecular mechanism by which ZFP423 regulates PPARγ expression is not clear, although it is proposed that it acts in part through amplification of the BMP signalling pathway (Gupta et al., 2010).

2.5.5 The Nuclear Factor 1 (NF1)

The nuclear factor I family of transcriptional factors have been identified as possible regulators of adipocyte differentiation. During adipogenesis in 3T3-L1 cells, NFIA and NFIB are significantly expressed, and knockdown of either of these factors has been shown to reduce adipogenic differentiation in these cells (Waki et al., 2011). However, it is necessary to do further investigation to elucidate the molecular mechanisms of NFI on adipogenesis.

2.6 Negative regulators of PPARγ expression and adipogenesis

2.6.1 The Kruppel like factor 2 (KLF2)

Several transcription factors that repress adipogenesis have also been identified. They include KLF2 and several member s of the GATA-binding family (Banerjee et al., 2003). KLF2 is a negative regulator of adipocyte differentiation. Cell line studies using 3T3-L1 adipocytes show that KLF2 is expressed in preadipocytes but not in mature adipocytes and that over expression of KLF2 inhibits the expression of PPARγ but not C/EBPβ and C/EBPδ (Banerjee et al., 2003). KLF2 binds directly to the CACCC region on the PPARγ2 proximal promoter thereby repressing promoter activity. Furthermore, mutation on the KLF2 binding site does

not block the KLF2-mediated repression of PPARγ promoter, indicating that other mechanisms of KLF2 activity are involved (Banerjee et al., 2003).

2.6.2 GATA2 and GATA3 zinc fingers

GATA2 and GATA3 are zinc-finger DNA binding proteins involved in development. These proteins are expressed in preadipocytes and down regulated during the terminal differentiation process (Tong et al., 2000). Expression of GATA2 has been shown to decrease adipocyte differentiation, while embryonic stem cells lacking GATA2 display enhanced adipogenic differentiation potential. Consequently, defective GATA2 and GATA3 expression is associated with obesity, while expression of GATA2 and GATA3 inhibits adipogenesis and traps cells at the preadipocyte stage, which could be as a result of direct suppression of PPARγ (Tong et al., 2000). Furthermore, GATA2 and GATA3 form protein complexes with C/EBPα and C/EBPβ leading to suppression of adipocyte differentiation (Tong et al., 2005).

2.7 In vitro models of adipocyte differentiation

Establishment of *in vitro* cellular models for adipogenic differentiation has greatly increased the understanding of the molecular basis of differentiation. Two types of *in vitro* experimental cell culture models have been used to study the mechanisms involved in adipocyte proliferation and differentiation: preadipocyte cell lines, which are already committed to the adipocyte cell line and multipotent stem cells, which have not undergone commitment to the adipocyte lineage, but have the capacity to do so. Multipotent cell lines are able to commit to different lineages such as adipocytes, osteoblasts, or myoblasts upon appropriate induction (Moreno-Navarrete and Fernández-Real, 2012). Preadipocyte cell lines include 3T3-F442A, 3T3-L1 and Ob1771 cell lines which have undergone determination and can either remain as preadipocytes or undergo differentiation to adipose tissue upon hormonal induction. These preadipocyte cell lines have been used to extensively study adipogenesis and the molecular mechanisms involved. 3T3-L1 and 3T3-F442A cell lines were isolated

from nonclonal Swiss 3T3 cells and were selected for their propensity to accumulate lipid droplets and are generally considered established pre-adipose cell lines. 3T3-L1 in particular, is one of the best characterized and widely used *in vitro* model of adipocyte differentiation (Green & Meuth, 1974; Green & Kehinde, 1975). These clonal cells are homogenous in their cellular population and display uniformity in every differentiation stage, thus making them an appropriate research tool that is complementary to animal models. Preadipose cells are similar in morphology to fibroblasts and once induced to differentiate they become spherical in shape and acquire lipid droplets in their cyctoplasm as they develop into adipocytes (Moreno-Navarrete and Fernández-Real, 2012).

Preadipocyte cell lines are generally considered a faithful model of preadipocyte differentiation. This is evidenced by *in vivo* transplantation studies where subcutaneous injection of 3T3-F442A preadipocytes into Balb-C Athymic mice led to the formation of normal fat pads at the site of injection within 5 weeks (Green & Kehinde, 1979). Further evidence shows that adipocytes derived from preadipocyte cell lines display metabolic patterns similar to those of adipocytes isolated from adipose tissue and that accumulation of lipid droplets in the preadipocytes closely correlates with *de novo* fatty acid biosynthesis. (MacDougald & Lane, 1995).

Mesenchymal stem cells (MSCs) are an example of multipotent stem cells. MSCs, also known as multipotent marrow stromal cells, are a heterogeneous population of plastic-adherent, fibroblastike-like cells which in culture are able to self-renew and differentiate into bone, cartilage or adipose tissue. MSCs are found in a variety of tissues during human development including skeletal muscle and adipose depots, and were first identified in postnatal human bone marrow (Bruder et al., 1997; Mackay et al., 1998). Bone marrow derived MSCs are scarce but have the ability to expand in culture and exhibit multilineage potential (Chamberlain et al., 2007), while adipose tissue represents a widely used source of

MSCs as it is readily available, practical and found in abundant supply. Just like marrow stromal cells, adipose-derived stromal cells (ASCs) are able to differentiate towards multiple lineages upon appropriate induction (Niemela et al., 2008). In addition, adipose derived stem cells display morphology and differentiation characteristics similar to those of MSCs isolated from other sources (Levi & Longaker, 2011). Evidence indicates that commitment of stem cells to specific lineages is triggered by factors that induce expression of genes that direct entry of lineage –specific differentiation (Davis et al., 1987) Commitment of MSCs to the adipose lineage produces preadipocytes which upon induction, undergo mitotic clonal expansion and differentiate into adipocytes (Tang et al., 2003).

MSCs are considered the common progenitor for both osteoblasts and adipocytes (Caplan, 1994; Prockop, 1997). As a result, a decrease in number of osteoblasts as observed in aging and osteoporosis, is as a result of increased differentiation of progenitor cells into the adipose lineage rather than the osteoblast lineage (Chan & Duque, 2002). There is a reciprocal relationship between adipogenic and osteogenic lineage commitment and differentiation, such that differentiation towards an adipocyte lineage occurs at the expense of an osteogenic lineage and *vice versa*. This relationship is regulated by a number of regulatory pathways that involve two main transcription factors; PPARγ, the main regulator of adipogenic differentiation and Runt-related transcription factor 2 (Runx2), the main regulator of osteogenic differentiation (James, 2013). In aging it has been observed that the adipocyte volume in the bone marrow increases with decrease in osteoblast volume, especially in osteoporosis. MSCs thus prove to be a useful tool for studying not only obesity and adipogenesis but also aging and osteoporosis by inhibition of marrow adipogenesis and a subsequent increase in osteoblastogenesis (Nuttall & Gimble, 2000).

The availability of MSCs and preadipose cell lines has also facilitated knowledge of the molecular mechanisms that control adipogenesis and allowed studies on the adipogenic

induction potential of hormones and growth inducers. Hormones and growth factors that induce adipocyte differentiation such as insulin and insulin-like growth factor, do so by transducing external growth signals to differentiating adipocytes (Niemela et al., 2008). 3T3-L1 preadipocytes can be induced to differentiate into adipocytes using a defined adipogenic cocktail consisting of Insulin, IBMX and DEX during the first 48h. Insulin and IBMX, in the presence of fetal bovine serum, are important in elevating intracellular cyclic adenosine monophosphate (cAMP) levels, while DEX is important in stimulating the guanylyl cyclase (GC) receptor (Caprio et al., 2007). After the first 48h, IBMX and DEX are omitted as insulin alone is sufficient to continue the differentiation program from that point. Although DEX is an adipogenic inducer, it displays anti-adipogenic properties when added during later stages of adipogenic differentiation (Caprio et al., 2007).

The mouse pluripotent cell line C3H10T1/2, which was established from 14- to 17-day-old C3H mouse embryos displays characteristics of MSCs. These cells display a fibroblastic morphology in culture when sub confluent and when fully confluent, show a change in cell shape into a flat epithelial-like pattern (Pinney & Emerson, 1989). C3H10T1/2 cell line is an immortalized fibroblast cell line which, like other immortalized fibroblast cell lines of murine origin, can be induced to differentiate into adipocytes using a mix of Insulin, DEX and IBMX, which mediates activation of multiple genes and gene families important in adipogenesis (Gregoire et al., 1998; Pantoja et al., 2008). In addition to the insulin, DEX and IBMX mix, several other compounds with a potential to induce adipogenic differentiation in C3H10T1/2 cells have been studied. Previous studies have shown that treatment of C3H10T1/2 cells with 5-azacytidine leads to hypo-methylation of genomic DNA, which activates regulatory genes that cause differentiation of these cells into cells that display features of bone, skeletal and adipose tissue (Konieczny & Emerson, 1984; Reznikoff et al., 1973). Furthermore, treatment of pluripotent C3H10T1/2 cells with bone morphogenic

protein 4 (BMP4) is capable of triggering commitment of these cells to the adipoyte lineage. BMP4 causes the cells to undergo lineage commitment into preadipocytes which then undergo terminal differentiation to form adipocytes (Tang et al., 2004). In addition, alkylphenols, a class of non-ionic surfactants of which 4-tert-octylphenol (OP) is a member (Kwack et al., 2002), have been shown to inhibit differentiation of C3H10T1/2 cells into osteoblasts (Miyawaki et al., 2008). Treatment of confluent C3H10T1/2 cells with OP induces the expression of PPARγ, a major adipogenic differentiation marker. OP acts as a PPARγ ligand, resulting in inhibition of osteoblastic differentiation (Miyawaki et al., 2008). Alkylphenols have been found to mimic the roles of estrogens (Kwack et al., 2002) , which are important in maintaining bone volume in the body (Suga et al., 2001). Induction of differentiation in C3H10T1/2 cells also leads to differentiation to non-adipogenic lineages. For example, a study involving interleukin 11 (IL-11), a cytokine that supports hematopoietic cell growth and is involved in bone resorption, demonstrated that IL-11 increases alkaline phosphatise activity and upregulates expression of osteocalcin in C3H10T1/2 cells. Furthermore, IL-11 did not have any effect on the expression of PPARγ and ap2, suggesting that IL-11 is capable of inducing osteoblastic differentiation in C3H10T1/2 cells (Suga et al., 2001). Moreover, differentiation of C3H10T1/2 cells to the myogenic lineage leads to activation of genes responsible for muscle-specific transcription factors such as myoD, myogenin, myf5 and mrf4 (Weintraub, 1993).

2.8 Regulation of MSCs differentiation by use of oxysterols

A potential strategy to regulate differentiation of mesenchymal stem cells involves the use of oxysterols (Kha et al., 2004). Oxysterols are 27-carbon oxygenated derivatives of cholesterol that contain a hydroxyl group on the side chain, and are formed either through auto-oxidation, as by products of lipid peroxidation or through enzymatic processes that involve mono-oxygenases (Russell, 2000). Oxysterols are considered as either intermediates or end products

of cholesterol breakdown. They are able to pass through cell membranes like the blood brain barrier with ease, and are thus important transportable forms of cholesterol (Bjorkhem & Diczfalusy, 2002). Oxysterols can also be obtained from food sources through auto-oxidation. Such examples include 7-hydroxycholesterols which are absorbed, though in small quantities and transported to the cells and tissues (van Reyk et al., 2006). Oxysterols play important roles in a number of biological processes including cholesterol turn over, lipid metabolism, apoptosis, inflammation, bile acid synthesis, sterol transport from the periphery to the liver and immune-suppression where they act as substrates or signal molecules. They also act as physiological mediators in many disorders that are associated with cholesterol metabolism (Bjorkhem & Diczfalusy, 2002).

Several reports have noted potential roles of oxysterols in cellular differentiation (Hanley et al., 2000; Hayden et al., 2002). The oxysterols 22R and 25 hydroxycholesterol stimulate keratinocyte differentiation *in vitro* and inhibit proliferation, demonstrating their therapeutic potential in treatment of cutaneous disorders that arise as a result of decreased differentiation and increased proliferation (Hanley et al., 2000). Furthermore, 7-ketocholesterol has been shown to induce monocyte differentiation in vitro (Hayden et al., 2002). Further studies have shown that specific oxysterols are able to regulate the differentiation of MSCs (Kha et al., 2004; Kim et al., 2007). In a study conducted using M2-10B4 pluripotent marrow stromal cell line to assess the effects of oxysterols, it was reported that the oxysterols 22R, 20S, and 22S induce osteogenic differentiation in marrow stromal cells. This was evidenced by induction of ALP activity, an early marker of osteogenesis and increased DNA binding activity of RunX2, a transcriptional regulator of osteoblast gene expression and increased expression of osteocalcin (OCN), an osteoblast specific-gene (Kha et al., 2004). Moreover, 20S inhibits adipogenic differentiation in M2-10B4 bone marrow stromal cells by inhibiting the expression of important adipogenic differentiation markers such as PPARγ (Kim et al.,

2007). These findings are an indication that oxysterols play an important role in the differentiation of mesenchymal stem cells and could have important implications in the field of stem cell biology (Kha et al., 2004) by providing interventions in stem cell-related diseases and disorders such as obesity and osteoporosis by shifting lineage development.

2.9 Signal transduction pathways that regulate adipocyte differentiation

The commitment and differentiation of MSCs towards an adipogenic or osteogenic lineage involves various transcription factors and signalling pathways which follow the inverse relationship between adipogenesis and osteogenesis to produce a pro-osteogenic or anti-adipogenic stimuli. They include: the β-catenin dependent Wnt signalling, Hedgehog signalling and bone morphogenic protein (BMP) signalling (James et al., 2013). These signalling cascades are influenced by the key regulators of adipogenis and osteogenesis; PPARγ and Runx2 respectively, which are responsible for mediating the effects of cytokines that lead to osteogenic or adipogenic MSC differentiation, where over expression of one factor inhibits the expression of the other (Valenti et al., 2011; Zhang et al., 2006). In addition to PPARγ and Runx2, MSC differentiation is governed by sequential activation of a number of other transcription factors that function downstream of signalling pathways leading to lineage establishment (Rosen & MacDougald, 2006).

2.9.1 Wnt signalling pathway

The Wnt pathway is a highly conserved signal transduction pathway that plays an important role in biological processes such as the regulation of cell proliferation and differentiation during embryonic development and tissue regeneration in adults. Signal transduction takes place through either β-catenin dependent (canonical) or β-catenin independent pathways (Kim et al., 2013). The name "Wnt signalling" is derived from *Wingless*, the *Drosophila melanogaster* segment-polarity gene, and *Integrase-1*, the vertebrate homologue. Wnts are secreted glycoproteins that bind to frizzled transmembrane receptors which may be coupled

to G proteins, and binding of Wnt proteins to the receptors initiates signalling (Huelsken & Behrens, 2002). These glycoproteins act through paracrine and autocrine mechanisms to influence cell differentiation and development. In the β-catenin dependent Wnt signalling, β-catenin acts as the main transcriptional co-activator enhancing extracellular signal transduction for the activation of target genes (Kim et al., 2013). Studies have shown that Wnt signalling inhibits adipocyte differentiation *in vitro*. Induction of wnt signalling inhibits adipogenic differentiation of 3T3-L1 preadipoctyes by blocking gene expression that is responsible for mitotic clonal expansion, thus leading to dysregulation of the cell cycle (Ross et al., 2002), and blocking the expression of PPARγ and C/EBPα. Furthermore, the expression of Wnt10b, an activator of Wnt signalling, is elevated in preadipocytes and down regulated upon induction of differentiation (Ross et al., 2000).

In C3H10T1/2 cells, Wnt proteins capable of stabilizing β-catenin have been shown to induce the expression of the osteoblast differentiation marker alkaline phosphatise (ALP) while Wnt3a inhibits that expression of FABP4 and PPARγ in the same cells (Rawadi et al., 2003). In contrast, disruption of Wnt signalling leads to adipogenic differentiation of pre-adipocytes and mesenchymal precursors of adipocytes. This is achieved through treatment of preadipocytes with an adipogenic medium consisting of DEX, IBMX, insulin and FBS which induce adipogenic differentiation while inhibiting Wnt signalling (Rosen & MacDougald, 2006). Dysregulation of Wnt/β-catenin signalling has been linked to a number of human diseases such as cancer, alzheimers and osteoporosis (Kim et al., 2013).

2.9.2 BMP and TGF-β signalling

Bone morphogenic protein (BMP) signalling has been identified as a downstream process of MSC differentiation that controls adipogenesis and osteogenesis (James, 2013). BMPs, which are members of transforming growth factor-β (TGF-β) super family are extracellular cytokines that induce ectopic chondrogenesis and osteogenesis (Wozney et al., 1988). BMPs

are involved in a number of regulatory processes such as cellular differentiation, embryonic development and patterning of bone and cartilage tissues (Chen et al., 2004). TGFβ and BMPs regulate the differentiation of various cell types, including adipocytes (Massague et al., 2005). BMPs display varied effects on differentiation of MSCs, depending on the concentration and type of BMP, type of precursor cells, and the presence or absence of differentiation regulators in the medium *in vitro*. For example, BMP4 commits pluripotent C3H10T1/2 cells to an adipose lineage, allowing these cells to express adipocyte markers and display adipocyte characteristics (Tang et al., 2004). BMP2 alone has little effect on adipogenesis but is able to interact with other differentiation factors such as TGFβ and insulin to stimulate adipogenesis in embryonic stem cells (Zur et al., 2005). Furthermore, BMP2 causes a dose-dependent differentiation of C3H10T1/2 cells where low concentrations favour adipocyte formation while high concentrations favour formation of chondrocytes and osteoblasts (Wang et al., 1993).

BMPs induce osteogenesis by binding to threonine-kinase receptors, enabling signal transduction to the nucleus through Smad proteins. Moreover, nuclear cofactors cooperate with the Smad proteins to regulate expression of target genes (von Bubnoff & Cho, 2001).The TGFβ signalling cascade is expressed in cultured adipocytes and adipose tissue. However, *in vitro* studies show that TGFβ inhibits pre-adipocyte differentiation. In a study to identify the adipogenic transcription factors that are targeted by TGFβ, the adipogenic factors PPARγ, C/EBPβ and C/EBPδ were over-expressed in NIH3T3 cells followed by blocking of adipogenesis using TGFβ. It was reported that TGFβ inhibits adipocyte differentiation by interacting with C/EBP and repressing its transcriptional activity (Choy & Derynck, 2003; Rahimi et al., 1998).

2.9.3 Hedgehog signalling pathway

Hedgehog (Hh) signalling has emerged as an important modulator of stem cell differentiation processes, including adipogenic differentiation and has been shown to play crucial roles in the developmental process in both vertebrates and invertebrates (McMahon et al., 2003). Hh was originally identified in *Drosophila melanogaster* in a genetic screen for mutations in segment number and polarity genes, which were found to alter the segmental pattern of developing larva. Loss of secreted Hh protein caused the embryos to develop as spiny balls resembling hedgehogs (Nusslein-Volhard & Wieschaus, 1980). Secreted signalling molecules are encoded by Hh genes and these molecules are important during embryonic and adult development in controlling pattern formation and cellular development (Nieuwenhuis & Hui, 2005). Dysregulation of the Hh pathway has been associated with severe physiological consequences including abnormal tissue regeneration, polydactyly, holoprosencephaly, craniofacial defects and skeletal malformations and cancers (Ingham & McMahon, 2001; McMahon et al., 2003). In verterbrates, Hh pathway activation is regulated by three main mammalian ligands; Sonic Hedgehong (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh) (Pathi et al., 2001). The Shh and Ihh ligands are more closely related to each other than to the Dhh ligand, which is closely related to *Drosophila* Hh (Varjosalo & Taipale, 2008). All mammalian Hh ligands have similar physiological effects, and differences in their developmental roles result from their diverse patterns of expression (McMahon et al., 2003). Dhh is mainly expressed in the gonads, including the Sertoli cells of testis where it plays an essential role in the regulation of mammalian spermatogenesis (Bitgood et al., 1996), while Ihh is expressed in the primitive endoderm where it induces formation of hematopoietic and endothelial cells (Dyer et al., 2001).

Shh is the most broadly expressed mammalian Hh protein, mainly in the embryo of vertebrates, where it plays a crucial role in patterning of embryonic tissues including the

brain and spinal cord. Deletion of Shh leads to defects in the ventral neural tube, distal limb malformation, cyclopia and absence of spinal column (Chiang et al., 1996). Hh signalling is initiated by binding of Hh ligand to patched (Ptc), a 12-pass transmembrane protein receptor, which frees smoothened (Smo), an adjacent 7-pass transmembrane protein, for downstream signalling. Active Smo in the phosphorylated form regulates the bi-functional transcription factor Cubitus interuptus (Ci), preventing its cleavage and enabling it to enter the nucleus to induce the transcription factors Gli2 and Gli3 (Cohen, 2003; Ruel et al., 2003). Gli1, a Hh signalling gene has been described as a reliable marker of Hh activity, as it is induced by Hh signals and creates a positive regulatory loop that enhances Hh responses (Hooper & Scott, 2005). In the absence of a ligand, Smo is inhibited by Ptc, preventing the activation of hedgehog signalling via cleavage of Ci. This cleavage of Ci results in a repressor form of Ci which enters the nucleus and blocks signal transduction (Cohen, 2003).

Purmorphamine, a 2,6,9-tri-substituted purine, is a Hh signalling agonist that acts by targeting the Smo transmembrane protein (Sinha & Chen, 2006). Activation of Hh signalling pathway by purmorphamine results in up- and downregulation of downstream target genes of the Hh pathway (Wu et al., 2004). In human bone marrow MSCs, activation of the Hh pathway by purmophamine up-regulates the expression of Smo, Ptc1, Gli 1 and Gli 2 (Oliveira et al., 2012). Another pharmacological modulator of Hh signalling, cyclopamine, has been described as a potent and specific Hh inhibitor. Cyclopamine is a steroidal alkaloid that displays antitumor activities due to its ability to block cellular responses to Hh signalling by directly binding to smo (Chen et al., 2002).

Several studies have demonstrated the role of hedgehog signalling in MSC differentiation (Fontaine et al., 2008; Plaisant et al., 2009), although roles in cellular differentiation are still controversial as different results have been observed depending on cell lines used (Oliveira et al., 2012). In human MSCs, activation of Hh signalling inhibits osteoblast differentiation as

seen by the decrease in both mineralization and expression of osteoblastic differentiation genes such as Runx2, a key transcription factor that regulates early osteoblast differentiation (Plaisant et al., 2009). During human adipocyte differentiation, Hh signalling is down-regulated and activation of the pathway impairs adipogenesis and lipid accumulation by reducing the expression of C/EBPα. However, inhibition of this pathway is not sufficient to trigger adipogenesis (Fontaine et al., 2008). In 3T3-L1 preadipocytes, it has been shown that Shh protein inhibits adipogenesis and expression of adipogenic differentiation markers. Inhibiting the Hh signals using cyclopamine leads to an increase in adipogenic differentiation (Suh et al., 2006). Similarly, it has been reported that Hh signalling decreases during adipocyte differentiation of 3T3-L1 preadipocytes (Cousin et al., 2006). However, this down-regulation is not sufficient to trigger adipogenic differentiation. In a study using M2-10B4 pluripotent bone MSCs, Hh signalling was reported to be the molecular mechanism by which 20S inhibitsPPARγexpression and adipogenic differentiation (Kim et al., 2007).

In C3H10T1/2 pluripotent MSCs, Hh induces osteogenesis and expression of osteogenic differentiation factors. Hh also increases makers of terminal differentiation in these cells (Suh et al., 2006). Similar results have been reported by (Spinella-Jaegle et al., 2001) where sonic hedgehog was found to abolish adipogenic differentiation of C3H10T1/2 cells by reducing the expression of adipogenic transcription factors CEBPα and PPARγ, and increasing commitment of these cells to an osteoblastic lineage.

Summary

In light of the existing information as captured in this review, it is evident that stem cell differentiation is a vital process in cell biology and that oxysterols play an important role in the differentiation of these cells. The effects of specific oxysterols on the adipogenic differentiation of C3H10T1/2 mouse embryonic cells and the molecular mechanism by which the specified oxysterol affects adipogenic differentiation were therefore investigated. Using

microarray technology, a gene expression profile of genes that are up-regulated during adipogenic differentiation and inhibition of adipogenesis was generated in studies on C3H10T1/2 cells.

CHAPTER THREE

3.0 MANUSCRIPT 1

25 Hydroxycholesterol (25-HC) inhibits adipogenic differentiation of C3H10T1/2 mouse embryonic stem cells independent of hedgehog signalling mechanism.

3.1 ABSTRACT

In this study, we investigated the effects of specific oxysterols on adipogenic differentiation and expression of adipogenic transcripts in C3H10T1/2 cells. To assess induction of adipogenesis, cells were treated for six days with an adipogenic cocktail (DMITro) consisting of dexamethasone (DEX), 3-isobutyl-1-methly-xanthine (IBMX), insulin and troglitazone (Tro). To assess anti-adipogenic effects of different oxysterols, cells were treated with DMITro+20S hydroxycholesterol (20S), 25 hydroxycholesterol (25-HC), 22R hydroxycholesterol (22R) or 22S hydroxycholesterol (22S). Treatment of C3H10T1/2 cells with DMITro significantly induced mRNA expression of two key adipogenic factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα). This induction was most significantly inhibited by 25-HC among the tested oxysterols. To determine the mechanism by which 25-HC inhibits adipogenesis, we studied the effects of hedgehog (Hh) signalling, using a specific Hh pathway inhibitor, cyclopamine. Treatment of C3H10T1/2 cells with cyclopamine for 96h did not induce adipocyte differentiation, i.e. cyclopamine did not reverse the inhibitory effects of 25-HC on key adipogenic gene expression, suggesting that hedgehog signalling may not play a role in the anti-adipogenic effects of 25-HC. Our observations showed that 25-HC was the most potent oxysterol in inhibiting adipogenesis and the expression of key adipogenic transcripts in C3H10T1/2 cells, suggesting its potential for application to provide an intervention to reduce excess fat accumulation associated with obesity. We also report that the inhibitory effects of

25-HC on adipogenic differentiation in C3H10T1/2 cells are not mediated by hedgehog signalling but may be mediated in part by the SREBF1/ADD1 pathway.

Key words: Oxysterols, C3H10T1/2 stem cells, differentiation, PPARγ

3.2 INTRODUCTION

Obesity is associated with increase in adipose tissue and development of fat cells or adipocytes (Farmer, 2006). It is necessary to study the mechanisms of adipose tissue development and the transcription markers that influence maturation of adipocytes, not only to understand the pathogenesis of obesity but also to identify pathways and proteins that can be targeted for pharmacological interventions in order to combat the growing incidence of obesity (Lehrke & Lazar, 2005; White & Stephens, 2010).

Adipogenesis refers to the formation of fat cells from undifferentiated precussor cells, a process involving transcriptional networks with transcriptional factors that coordinate the expression of a number of proteins involved in mature fat cell formation (Rosen et al., 2002). The main transcriptional factors that directly influence fat cell formation are PPARγ and C/EBPα (Rosen et al., 2000). PPARγ has been described as a member of the nuclear hormone receptor super family of ligand-activated transcription factors which plays a central role in the regulation of gene expression of various physiological processes and is the dominant or "master" regulator of adipocyte biology (Lefterova et al., 2014; Rosen et al., 2000; Tontonoz & Spiegelman, 2008). Induction of expression of CEBPα and PPARγ mediates the entire adipocyte differentiation process involving formation of lipid droplets and expression of various metabolic programs associated with mature fat cells (Farmer, 2006). Mesenchymal stem cells (MSCs) are a reliable tool for studying differentiation of cells into adipocytes (Pinney & Emerson, 1989). These cells can be isolated from animal and human tissues, grown in culture and induced to differentiate into bone, cartilage, muscle or fat cells (Caplan

& Bruder, 2001). The mouse pluripotent cell line C3H10T1/2, established from 14- to 17-day-old C3H mouse embryos displays characteristics of MSCs (Konieczny & Emerson, 1984; Reznikoff et al., 1973). These cells display a fibroblastic morphology in culture when sub confluent and when fully confluent they change in into flat epithelial like-structures (Pinney & Emerson, 1989).

A potential strategy to regulate the differentiation of MSCs involves the use of oxysterols (Kha et al., 2004). Oxysterols are products of cholesterol oxidation, obtained through enzymatic and non-enzymatic processes, and are found in various human tissues and fluids (Bjorkhem & Diczfalusy, 2002; Brown & Jessup, 1999; van Reyk et al., 2006). Oxysterols are believed to be involved in regulation of gene expression associated with lipid metabolism and play important roles in differentiation, and developmental and inflammatory responses (Olkkonen et al., 2012). Oxysterols have been shown to inhibit adipogenic differentiation of MSCs while inducing their osteogenic differentiation. However, the effects of oxysterols on the adipogenic differentiation of C3H10T1/2 mouse embryonic stem cells are poorly understood. In this study, we evaluated the inhibitory effects of 25-HC on the adipogenic differentiation of C3H10T1/2 cells. We also report the role of hedgehog signalling and SREBF1/ADD1 pathways in the anti-adipogenic effects of 25-HC.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture

C3H10T1/2 mouse embryonic stem cells were purchased from ATCC (Manassas,VA), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and L-glutamate (Mediatech, Inc., Manassas, VA) and incubated at 37˚C with 5% CO2. Two days post-confluence (day 0), the medium was changed and cells were induced to differentiate into adipocytes in the presence of an adipogenic cocktail (DMITro) consisting of 500nM

dexamethasone (DEX), 0.5mM 3-isobutyl-1-methly-xanthine (IBMX), 20µg/ml Insulin + 10µM Troglitazone (Tro). Inhibition of adipogenesis was induced by use of oxysterols as follows; DMITro + 10µM 20S, 25, 22R or 22S hydroxycholesterol with 3 replications per treatment (n=3). The control treatment consisted of 10% FBS in DMEM. Two days after induction of differentiation, cells were re-treated with insulin and Tro. Three days later, cells were re-treated and allowed to differentiate for one more day to give a total of six days of adipogenic differentiation.

3.3.2 Oil red O staining

To examine lipid accumulation and formation of fat droplets, cell monolayers were rinsed with phosphate buffered saline (PBS) and fixed with 60% isopropanol for 2 minutes. The isopropanol was then removed from the cells and a working solution of oil red O stain added, followed by incubation for 20 minutes. The stain was rinsed off and the plates allowed to air dry before mounting and taking photographs (Parhami et al., 1999). Microscopic JPG images were taken using an EVOS xl core imaging system.the plates were also scanned using a CanoScan Tool box 4.6

3.3.3 RNA extraction and Quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Burlington, ON) according to the manufacturer's instructions. This was followed by single strand cDNA synthesis by reverse transcription quantitative polymerase chain reaction (RT-PCR) analysis using high capacity cDNA synthesis kit following the supplier's protocol (Applied Biosystems, Burlington, ON). Quantitative real-time PCR (qRT-PCR) was performed on a CFX Connect ^{TM} Real-Time PCR Detection instrument (Biorad). All qRT-PCR samples were prepared in duplicates and gene expression data were generated using the ΔΔCt method where expression of target genes

was normalized to the expression of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.3.4 Statistical analysis

The generated data were analyzed using the General Linear Model (GLM) procedure of the Statistics Analysis System (SAS) Institute version 9.2. Differences between groups were compared by one-way ANOVA and subsequent Tukey's studentized range test. A probability value of $p < 0.05$ was considered significant.

Table 3.1 Primer sequences for PCR reactions

Gene	Primer sequences	Size of PCR product
GAPDH	5'-ATGGACTGTGGTCATGAGCC-3' (sense) 5'-ATTGTCAGCAATGCATCCTG-3' (antisense)	190
PPARϒ	5'-TGAAACTCTGGGAGATTCTCCTG-3' (sense) 5'-CCATGGTAATTTCTTGTGAAGTGC-3' (antisense)	88
C/EBPα	5'-GGACAAGAACAGCAACGAGTACC-3' (sense) 5'-GGCGGTCATTGTCACTGGTC-3' (antisense)	146
FABP4	5'-AACACCGAGATTTCCTT-3' (sense) 5'-ACACATTCCACCACCAG-3' (antisense)	178
LPL	5'-AGGACCCCTGAAGACAC-3' (sense) 5'-GGCACCCAACTCTCATA-3' (antisense)	148
KLF2	5'-CTTACCCGCCACTACCGAAA-3' (sense) 5'-TTGTCCGGCTCTGTCCTAAG-3' (antisense)	224
ADD1/SREBF1	5'-CCTCCACTCACCAGGGTCT-3' (sense) 5'-CTCAGCAGCCCCTAGAACAA-3' (antisense)	206
ABCA1	5'-CTGTGTTGTGTGGGCTCCTC-3' (sense) 5'-GTCAGCGTGTCACTTTCATGG-3' (antisense)	205

3.4 RESULTS

To evaluate the effects of specific oxysterols on the adipogenic differentiation of C3H10T1/2 cells, we examined adipocyte formation in the different treatments. Treatment of C3H10T1/2 cells with the adipogenic cocktail (DMITro) for six days resulted in a significant accumulation of lipid droplets compared to the control **(Figure 3.1)**. The lipid droplets were

observed from day 2 post-treatment and increased with increasing duration of treatment. Treatment of cells with DMI or Troglitazone alone did not induce formation of fat droplets in C3H10T1/2 cells (data not shown). Treatment of cells with DMITro+10μM 25-HC significantly inhibited the adipocyte formation induced by DMITro as demonstrated by oil red O staining **(Figure 3.2)**. 20S, 22R and 22S did not inhibit formation of lipid droplets as shown by the oil red O pictures **(Figure 3.2)**.

We further evaluated the effects of specific oxysterols on the expression of key adipogenic transcripts. Treatment of cells with the DMITro for six days resulted in a significant increase in the mRNA expression of a key adipogenic gene, PPARγ. 25-HC significantly inhibited DMITro-induced PPARγ expression whereas 20S, 22R and 22S did not significantly inhibit the expression of PPARγ after six days of treatment **(Figure 3.3)**. The expression of C/EBPα was significantly increased by DMITro and this increase was significantly inhibited by 25-HC and 20S, whereas the expression of FABP4 was significantly inhibited by 25-HC, 20S and 22R hydroxycholesterols **(Figure 3.3)**. The expression of LPL was significantly increased by DMITro and this increase was inhibited by 25-HC, 20S and 22R **(Figure 3.3)** after six days of treatment. Of all the oxysterols tested, 25-HC was the most potent in inhibiting the expression of key adipogenic transcripts and adipogenesis in C3H10T1/2 mouse stem cells as shown in the oil red O staining and gene expression analyses, suggesting its potential application in reducing adipogenesis and obesity.

The Kruppel like factor 2 is an anti-adipogenic gene whose over expression inhibits PPARγ expression (Parhami et al., 1999). In this study, the expression of KLF2 was significantly inhibited by DMITro compared to the control. However, this inhibition was not significantly reversed by 25-HC **(Figure 3.3)**.

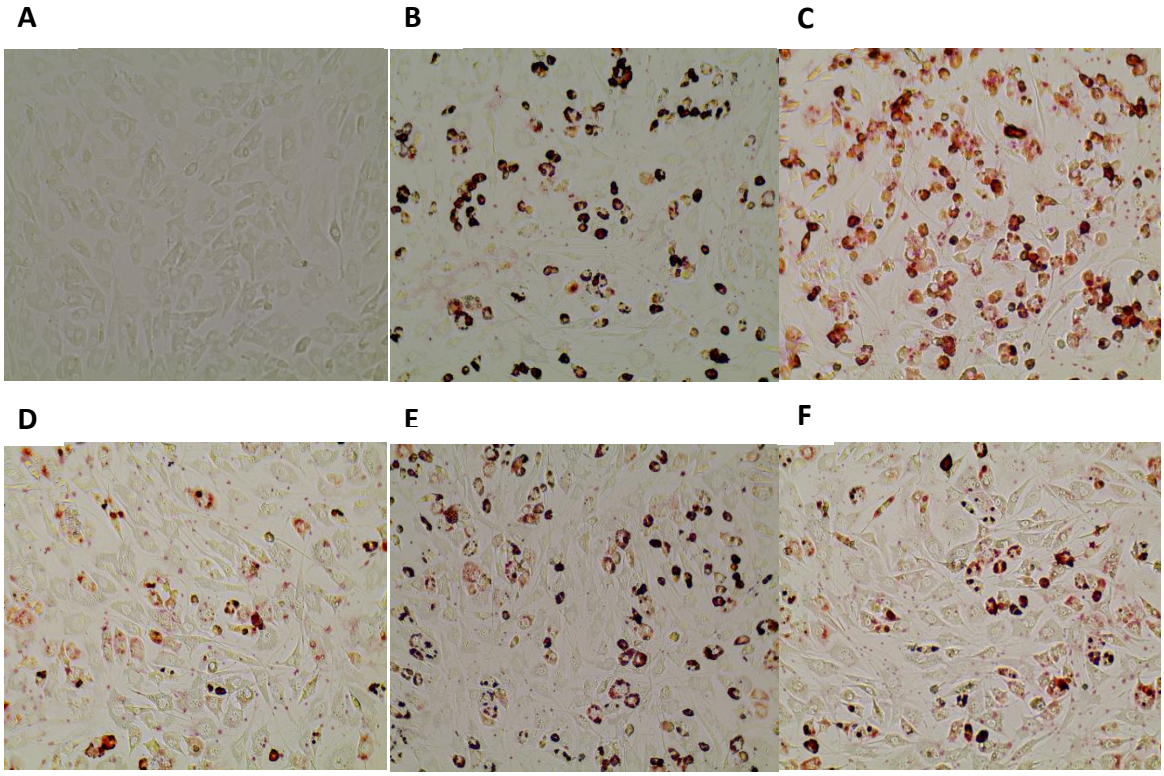

Figure 3.1. Representative images of C3H10T1/2 mouse cells treated with control (A), DMITro (B), DMITro+20S (C), DMITro+25-HC (D), DMITro+22S (E) and DMITro+22R (F). Cells were treated for six days and adipocyte formation examined after oil red O staining.

Figure 3.2. Representative image of C3H10T1/2 mouse cells. Cells were treated with a control vehicle, DMITro, DMITro+25-HC, 20S, 22S or 22R hydroxycholesterols for six days and lipid accumulation examined by oil red O staining. The plates were scanned using CanoScan Toolbox 4.6.

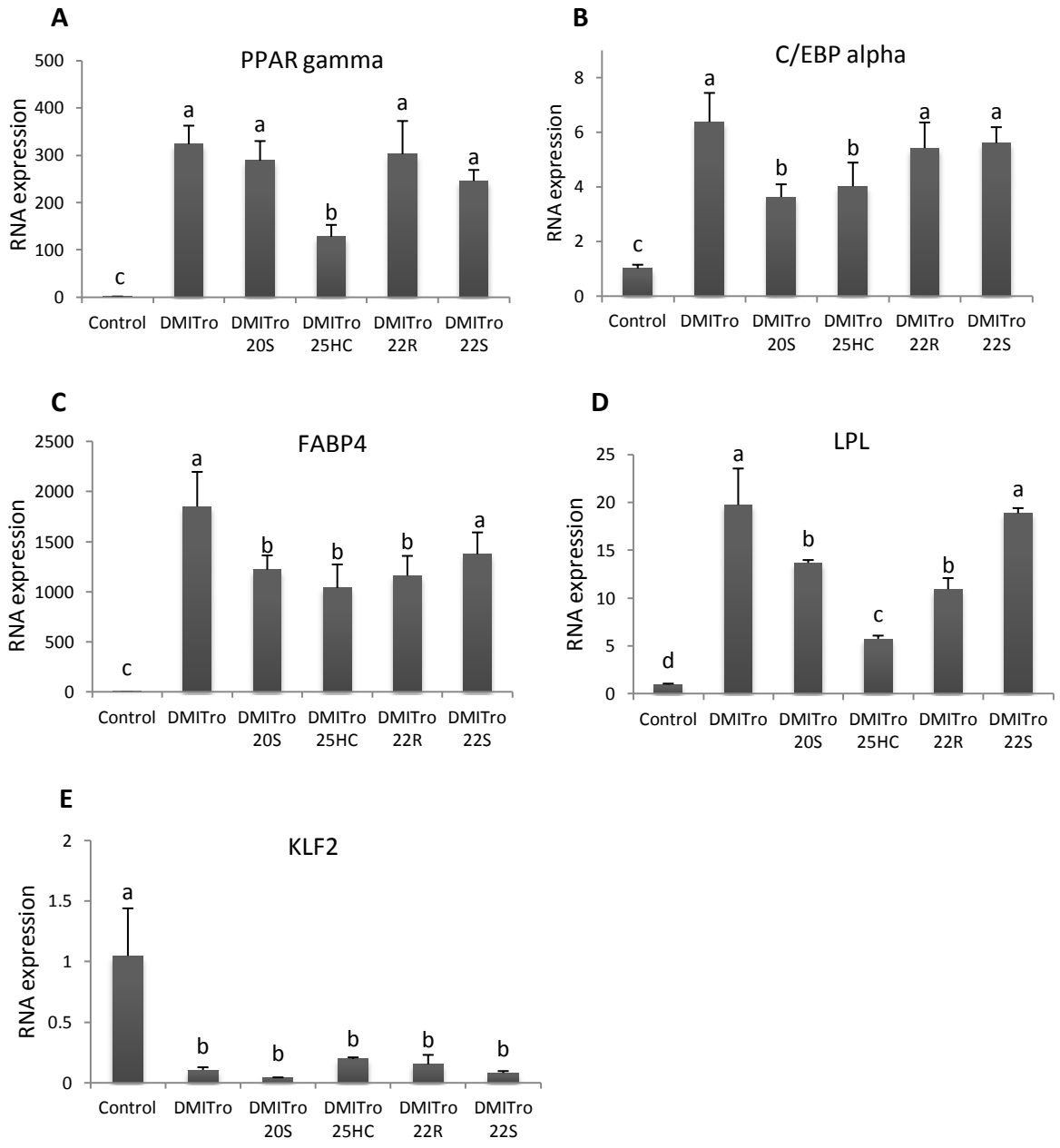

Figure 3.3. The effects of 20S, 25-HC, 22R and 22S hydroxycholesterols on (A) PPARγ, (B) C/EBPα, (C) FABP4, (D) LPL and (E) KLF2 mRNA expression induced by DMITro. Cells were treated with a control vehicle, an adipogenic cocktail DMITro or DMITro + 20S, 25, 22R or 22S hydroxycholesterol for six days. Gene expression was measured by qRT-PCR and the measured RNA levels were normalized to the expression of GAPDH. Fold changes

were determined by the ΔΔCT method. The results show the average values of three replicates (n=3) and the SD of the means.

3.4.1 Effects of 25-HC on the differentiation of C3H10T1/2 cells at different time points

To study the effects of 25-HC on the expression of key adipogenic genes at different time points, C3H10T1/2 cells were treated with DMITro in the presence or absence of 25-HC followed by collection of mRNA at 24h, 48h and 96h post treatment. Expression of mRNA was determined by RT-qPCR. Treatment of cells with DMITro caused a significant increase in the expression of PPARγ at 24-96 hours. This increase in PPARγ expression was not significantly inhibited by 25-HC at 24h, but was significantly inhibited at 48-96h **(Figure3.4)**. Treatment of cells with DMITro caused a significant increase in expression of C/EBPα at 24-96h. This increase in expression was not inhibited by 25-HC at 24h but was significantly inhibited at 48-96h **(Figure 3.4)**. The expression of FABP4 at 24h was not significantly inhibited by 25-HC but was significantly inhibited at 48-96h **(Figure 3.5)**. Similarly, LPL expression was not significantly inhibited by 25-HC at 24h but was sifnificantly inhibited at 48-96h **(Figure 3.5)**.

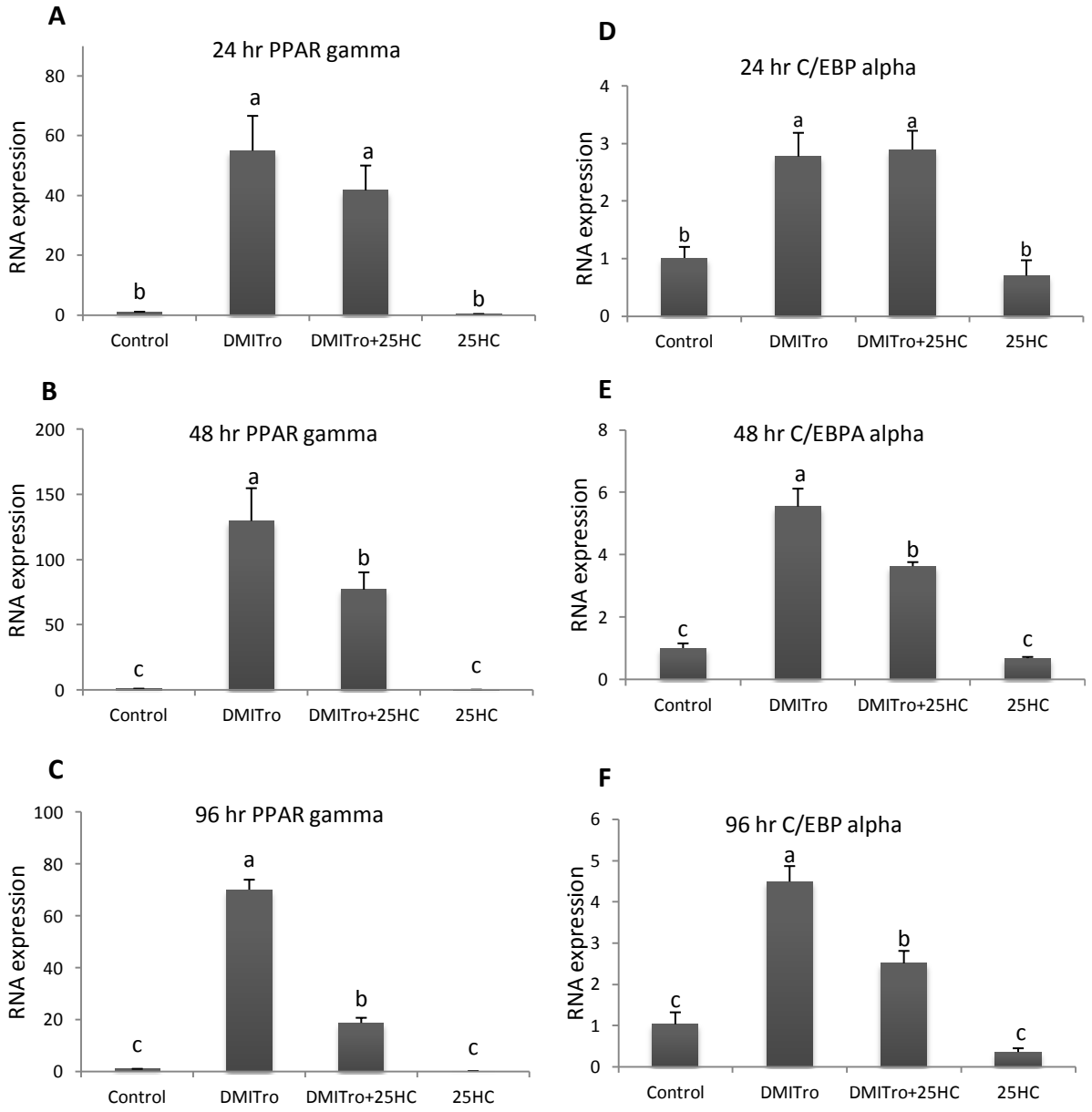

Figure 3.4. The effects of 25-HC on the expression of PPARγ (A,B and C) and C/EBPα (D, E and F) mRNAs. C3H10T1/2 cells at confluence were treated with control vehicle, an adipogenic media DMITro, DMITro+25-HC or 25-HC alone for 24, 48 and 96h. Gene expression was measured by RT-qPCR and RNA levels were normalized to the expression of GAPDH. Fold changes were determined by the ΔΔCT method. The results show the average values of three replicates (n=3) and the SD of the means.

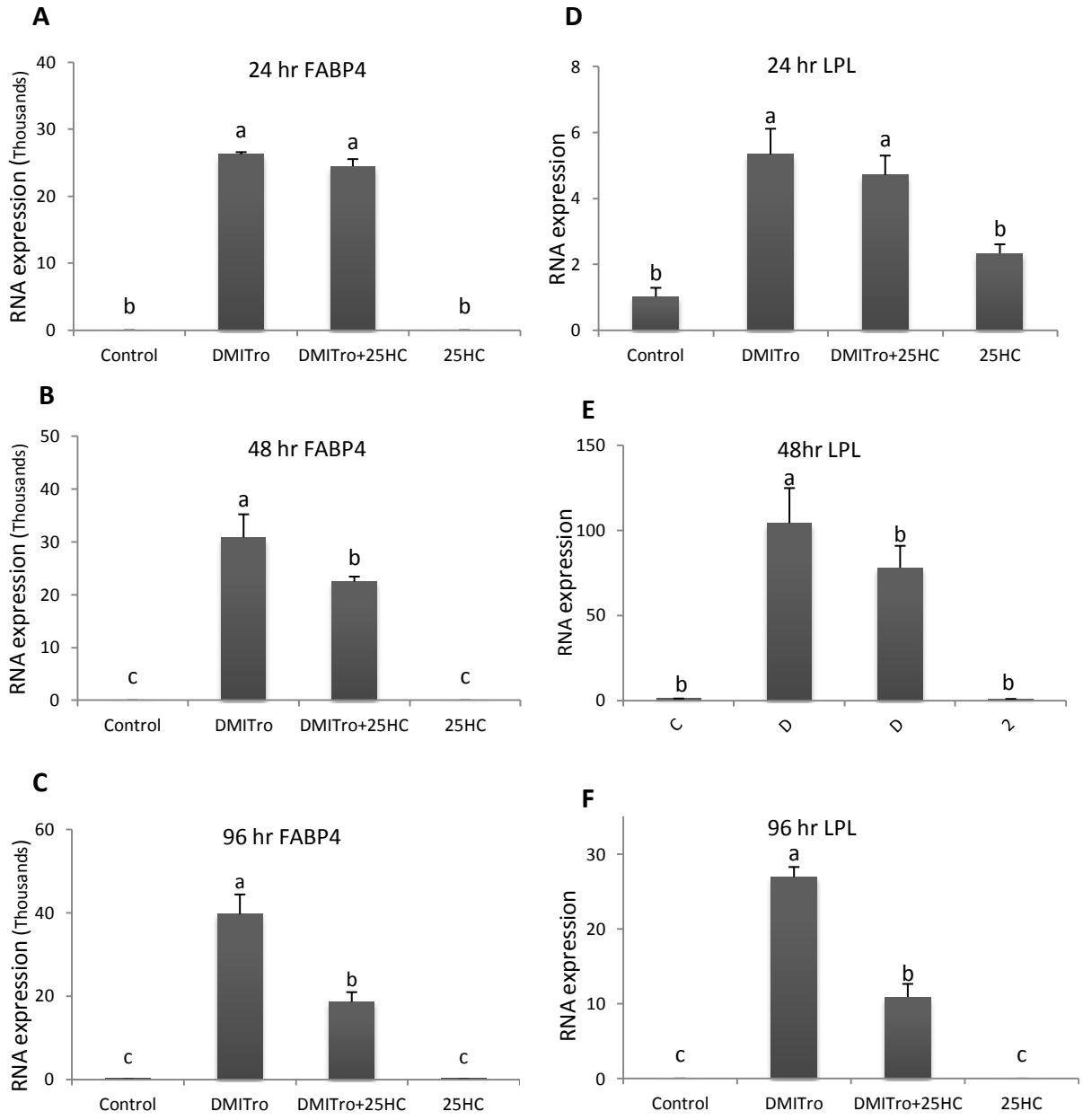

Figure 3.5. The effects of 25-HC on the expression of FABP4 (A,B and C) and LPL (D, E and F) mRNAs. C3H10T1/2 cells at confluence were treated with control vehicle, an adipogenic media DMITro, DMITro+25-HC or 25-HC for 24, 48 and 96h. Gene expression was measured by RT-qPCR and RNA levels were normalized to the expression of GAPDH. Fold changes were determined by the ΔΔCT method. The results show the average values of three replicates (n=3) and the SD of the means.

3.4.2 Role of hedgehog signalling on the anti-adipogenic effects of 25-HC in C3H10T1/2 cells

To study the mechanism by which 25-HC inhibits adipogenic differentiation in C3H10T1/2 cells, we studied the effects of the hedgehog pathway inhibitor cyclopamine, on cells treated with 25-HC. C3H10T1/2 cells were treated with the adipogenic cocktail DMITro or DMITro+25, with or without cyclopamine for 96h. The effects of cyclopamine on the expression of adipogenic genes were analysed by RT-qPCR. Treatment of cells with the adipogenic media DMITro greatly increased PPARγ mRNA expression after 96h of treatment. Addition of cyclopamine inhibited the expression of PPARγ **(Figure 3.6)**. Treatment of cells with DMITro+25-HC significantly inhibited the expression of PPARγ compared to the DMITro treatment. Addition of cyclopamine further inhibited the expression of PPARγ **(Figure 3.6)**. Similarly, addition of cyclopamine inhibited the expression of C/EBPα in cells treated with 25-HC. The same results were observed with the other adipogenic genes FABP4 and LPL **(Figure 3.6)**.

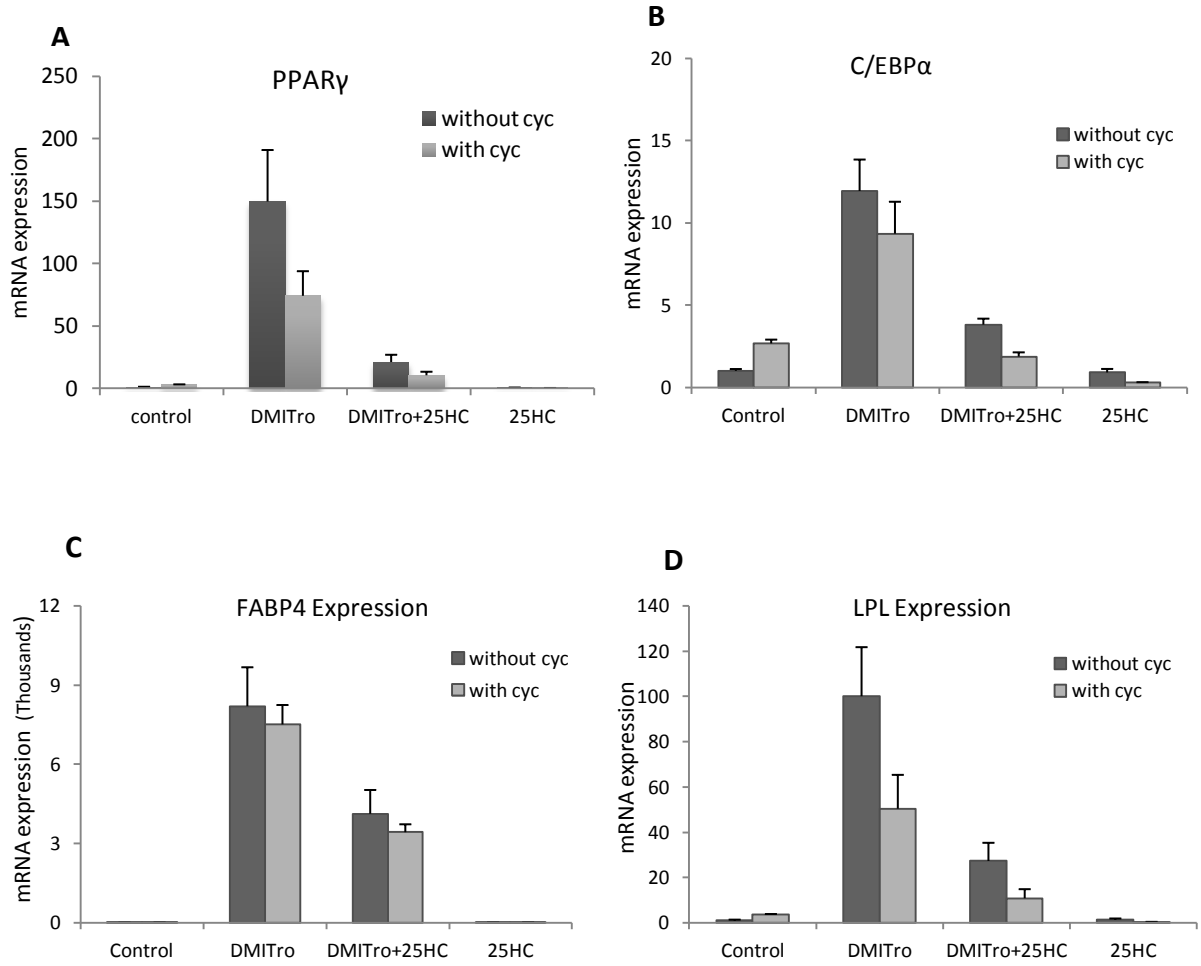

Figure 3.6. The effect of the Hedgehog pathway inhibitor, cyclopamine on the anti-adipogenic effect of 25-HC and mRNAexpression of PPARγ (A), C/EBPα (B), FABP4 (C) and LPL (D). C3H10T1/2 cells at confluence were treated with control vehicle, DMITro (500nM DEX, 0.5mM IBMX, 20µg/ml Insulin and 10µM Tro), DMITro+ 10µM 25-HC or 25-HC, with or without 4µM cyclopamine (cyc). After 96h of treatment, mRNA was collected and expression of adipogenic genes carried out by quantitative real-time PCR. Fold changes were determined by ΔΔCT method. The results show the average value of three replicates (n=3) and the SD of the means.

3.4.3 Role of Liver X Receptor on the anti-adipogenic effects of 25-HC in C3H10T1/2 cells

Liver X receptors (LXRs) are nuclear hormone receptors that play important roles in the regulation of cholesterol and fatty acid metabolism, and are activated by oxysterols including 22R and 20S (Edwards et al., 2002; Peet et al., 1998). To assess the possible role of LXRs in mediating the anti-adipogenic effects of 25-HC, we examined whether activation of LXRs by GW3965 had effects similar to those of 25-HC in C3H10T1/2 cells. The effects of purmorphamine, a Hh agonist, on the adipogenic differentiation of C3H10T1/2 cells were also examined. C3H10T1/2 cells at confluence were treated with GW3965, 25-HC or purmorphamine, alone or in combination with DMITro. The effects of the treatments on gene expression were analysed using qRT-PCR. Initial oil red O images showed an increase in lipid accumulation in cells treated with DMITro compared to non-treated cells **(Figure 3.7)**. Treatment of cells with DMITro+GW3965 showed a further increase in lipid accumulation compared to cells treated with DMITro whereas treatment with DMITro+purmorphamine showed a decrease in lipid accumulation compared to cells treated with DMITro **(Figure 3.7)**.

To assess activation of LXRs in C3H10T1/2 cells, we analysed the expression of ABCA1 gene, a target gene of LXRs activation (Edwards et al., 2002). GW3965 induced the expression of ABCA1 in non-treated (control) cells, demonstrating that LXRs are present in C3H10T1/2 cells **(Figure 3.8)**. ABCA1 was also induced in cells treated with 25-HC alone, but not in cells treated with DMITro+25-HC **(Figure 3.8)**.

Consistent with earlier results, treatment of cells with DMITro caused a significant induction in the expression of PPARγ and addition of 25-HC significantly inhibited this induction. However, in contrast to the effects of DMITro+25-HC, treatment of cells with DMITro+ GW3965 further increased the expression of PPARγ **(Figure 3.8)**. A similar effect was seen in the expression of the other adipogenic genes i.e. C/EBPα, AP2 and LPL **(Figure 3.8).**

Thus, the anti-adipogenic effects of 25HC are not mediated by LXRs since activation of LXRs by GW3965 did not inhibit adipogenesis; instead it enhanced the expression of adipogenic genes in C3H10T1/2 cells.

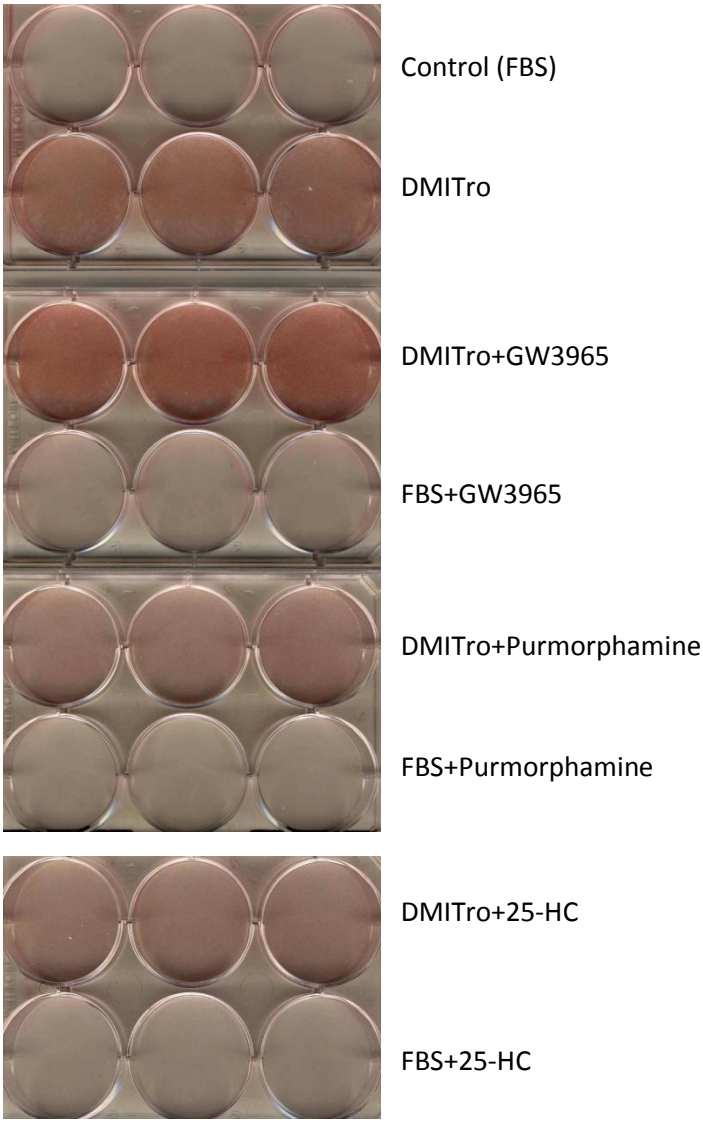

Figure 3.7. Liver X receptor agonist, GW3965, enhances lipid accumulation in cells treated with the adipogenic media, DMITro. C3H10T1/2 cells at confluence were treated for 4 days with a control, 2µM GW3965, 10µM 25-HC or 2µM purmorphamine, with or without DMITro. Oil red staining was then carried out to assess the extent of lipid accumulation in the treatments.

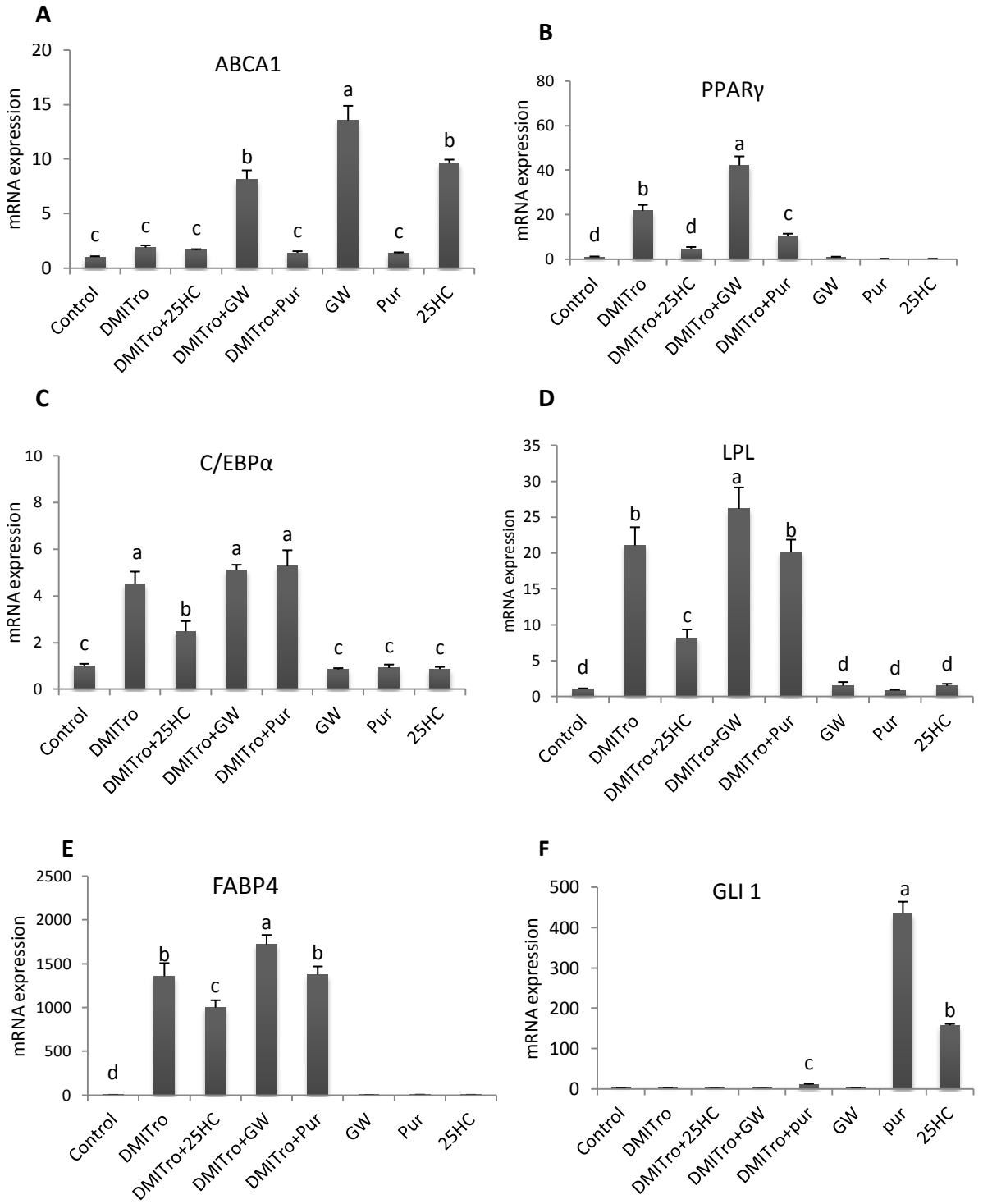

Figure 3.8. Effect of liver X receptor agonist, GW3965, on the expression of ABCA1 (A), PPARγ (B), C/EBPα (C), LPL (D), FABP4 (E) and GLI1 (F). C3H10T1/2 cells at confluence were treated control, 2μM GW3965, 10μM 25-HC or 2μM purmorphamine, with or without DMITro, for 4 days. Gene expression analysis was carried out by quantitative real-time PCR.

Fold changes in gene expression were calculated using ΔΔct method and reported as the mean of triplicate determination.

3.4.4 Role of SREBF1/ADD1 in the anti-adipogenic affects of 25-HC in C3H10T1/2 cells

The Sterol regulatory element binding factor 1/adipocyte differentiation and determination factor 1 (SREBF1/ADD1) is a member of the basic helix-loop-helix-leucine zipper (bhlh-lz) family of transcription factors that is associated with adipocyte development and cholesterol homeostasis (Kim & Spiegelman, 1996b). The SREBF family of transcription factors has been implicated in controlling the expression of PPARγ during lipid metabolism (Fajas et al., 1999). We therefore investigated whether the anti-adipogenic effects of 25-HC in C3H10T1/2 cells are mediated by SREBF1. C3H10T1/2 cells at confluence were treated with 25-HC alone or in combination with DMITro, or GW3965 alone or in combination with DMItro for 4 days, followed by gene expression analysis using qRT-PCR. Treatment of C3H10T1/2 cells with DMITro induced the expression of (SREBF1/ADD1) gene. This induction in expression was significantly inhibited by 25-HC **(Figure 3.9)**.

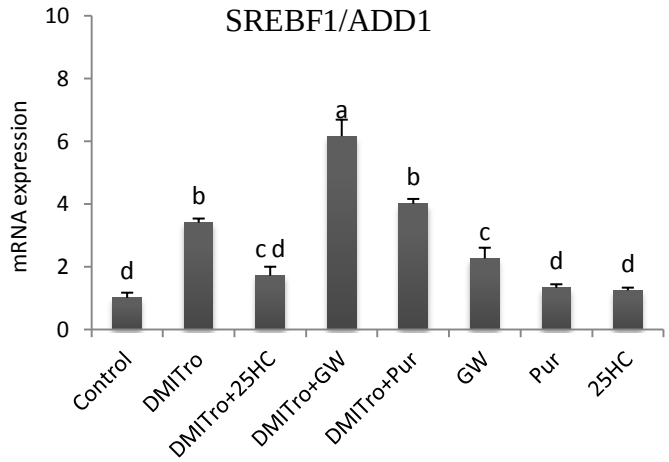

Figure 3.9. mRNA expression of SREBF1/ADD1. C3H10T1/2 cells at confluence were treated with C3H10T1/2 cells at confluence were treated with control, 2µM GW3965, 10µM

25-HC or 2μM purmorphamine, with or without DMITro for four days. Gene expression analysis was done by quantitative real time PCR. Fold changes in gene expression were calculated using ΔΔct method and reported as the mean of triplicate determination.

3.5 DISCUSSION

Previous studies have shown that mesenchymal stem cells are able to commit to either adipose, bone, cartilage or muscle lineages upon appropriate induction (Pinney & Emerson, 1989). Stem cells undergo mitotic clonal expansion (MCE) leading to differentiation of preadipocytes to adipocytes (Tang et al., 2003). Commitment of stem cells to either bone, cartilage or muscle lineages is as a result of expression of proteins that promote this lineage specific development (Davis et al., 1987)

Our findings show that the adipogenic cocktail, DMITro induces the differentiation of C3H10T1/2 mouse embryonic fibroblasts into adipocytes as shown by the accumulation of lipid droplets within the cells and the expression of adipogenic genes. We further demonstrate that 25-HC inhibits adipogenic differentiation in C3H10T1/2 cells by inhibiting the expression of PPARγ and downstream adipogenic genes. Of the four oxysterols tested in this study, 25-HC proved to be the most potent in inhibiting accumulation of cyctoplasmic lipid droplets and expression of adipocyte protein markers in the cells. 25-HC was able to inhibit the expression of PPARγ, which is the main regulator of adipogenesis (Rosen et al., 2000). In contrast, 20S, 22R and 22S hydroxycholesterols did not inhibit the expression of PPARγ after 6 days of treatment.

25-HC also inhibited the expression of C/EBPα, another key adipogenic gene which interacts with PPARγ2 to stimulate the cell differentiation process and is able to activate the promoter region of other genes involved in adipogenesis (Christy et al., 1989; Tontonoz et al., 1994). Fatty binding proteins (FABPs), including FABP4 are important in transport of fatty acids

during the early stages of adipocyte differentiation (Samulin, Berget, Lien, & Sundvold, 2008). In this study, treatment of cells with 25-HC inhibited the expression of FABP4 after 2 days of treatment. The expression of the adipogenic gene, LPL was also inhibited by 25-HC after 2 days of treatment. 20S has previously been shown to inhibit PPARγ expression in bone marrow stromal cells (Kim et al., 2007). In the current study, 20S did not inhibit PPARγ expression in C3H10T1/2 cells. However, 20S was able to inhibit the expression of C/EBPα, FABP4 and LPL in C3H10T1/2 cells whereas 22S did not inhibit any of the adipogenic genes that were analysed here. These results suggest that the inhibitory potential and mechanisms of different oxysterols may be different depending on cell types and cell source.

Our findings also demonstrate that the anti-adipogenic effects of 25-HC on C3H10T1/2 cells are not mediated by Hedgehog (Hh) signalling. Hh controls a number of biological processes including adipogenic differentiation of mouse cells (Fontaine et al., 2008). Studies on Hh signalling and adipocyte development are still controversial as different results have been observed depending on cell lines used. Cyclopamine, a specific inhibitor of Hh signalling, is useful in studying the role of Hh in normal development. Hh signalling has been shown to inhibit adipogenesis in mammalian cells while inhibition of this pathway increases adipogenesis in the same cells (Suh et al., 2006). However, in the present study, inhibition of the Hh pathway by cyclopamine did not reverse the anti-adipogenic effects of 25-HC on C3H10T1/2 cells, indicating that the anti-adipogenic effects of 25-HC are not mediated through Hh signalling activation. Down-regulation of the Hh pathway has been observed during differentiation of 3T3-L1 cells into adipocytes (Cousin et al., 2006). A study using 3T3-L1 cells demonstrated that blocking Hh signalling using cyclopamine increases adipogenesis and expression of the adipogenic genes PPARγ and FABP4 (Suh et al., 2006). In contrast, our findings show that blocking the Hh signalling with cyclopamine does not increase adipogenesis or expression of adipogenic genes in C3H10T1/2 cells. These findings

are consistent with a study carried out using murine 3T3-L1 cells where inhibition of Hh signalling using cyclopamine did not induce adipogenesis or expression of adipogenic differentiation markers (Cousin et al., 2006). In our findings, treatment of C3H10T1/2 cells with DMITro increased the expression of the core adipogenic marker, PPARγ and addition of cyclopamine decreased the expression of this marker, suggesting that inhibition of Hh signalling does not trigger adipogenesis in these cells. Treatment of C3H10T1/2 cells with DMITro+25-HC decreased the expression of PPARγ and addition of cyclopamine further inhibited PPARγ expression, suggesting that the anti-adipogenic effects of 25-HC are not mediated through hedgehog signalling **(Figure 3.6)**. These findings are also similar to experiments performed using human mesenchymal stem cells where inhibition of the Hh pathway by cyclopamine was not sufficient to trigger adipocyte differentiation (Fontaine et al., 2008).

We also demonstrate that the anti-adipogenic effects of 25-HC are not mediated by LXRs. LXRs are important in the regulation of cholesterol, where they regulate a set of genes associated with cholesterol catabolism, absorption and transport (Edwards et al., 2002; Peet et al., 1998). In addition, LXRs also regulate several genes involved in fatty acid metabolism by either regulating the expression of SREBF1/ADD1 or by directly binding the promoters of specific lipogenic genes (Amemiya-Kudo et al., 2000; DeBose-Boyd et al., 2001; Joseph et al., 2002). Naturally produced oxysterols such as 22R and 24S hydroxycholesterol have been shown to activate LXRs (Janowski et al., 1996; Lehmann et al., 1997). In the present study, both 25-HC and the LXR agonist GW3965 activated the LXRs in undifferentiated C3H10T1/2 cells as shown by the induction of expression of the ABCA1 gene **(Figure 3.8)**, a target gene for LXR activation. However, in the presence of the adipogenic cocktail DMITro, 25-HC did not induce the expression of the ABCA1 gene. In contrast, GW3965 was still able to induce the expression of ABCA1 in the presence of DMITro **(Figure 3.8)**. In the analysis

of adipogenic genes, activation of LXRs by GW3965 did not have effects similar to those of 25-HC. In contrast to 25-HC, LXR activation by GW3965 led to an increase in the expression of adipogenic genes, an indication that the anti-adipogenic effects of 25-HC are not mediated by LXRs. These findings are similar to a study performed using M2-10B4 cells where the osteogenic effects of 20S and 22R on M2 cells were found to be independent of the LXR activation since activation of LXRβ by the pharmacological agent TO-901317 did not yield effects similar to those of 20S and 22R (Kha et al., 2004).

The SREBF1/ADD1 pathway is a possible mechanism through which 25-HC exerts its anti-adipogenic effects on C3H10T1/2 cells. SREBF1/ADD1 has been shown to augment adipogenic differentiation through direct induction of PPARγ gene expression as well as through production of endogenous PPARγ ligands (Fajas et al., 1999; Kim & Spiegelman, 1996; Kim et al., 1998). Ectopic expression of SREBF1/ADD1 in 3T3-L1 and HepG2 cells was shown to induce endogenous PPARγ mRNA levels (Fajas et al., 1999). Furthermore, ectopic expression of a dominant-negative ADD1 in 3T3-L1 cell line was observed to inhibit adipocyte differentiation and expression of adipocyte-specific genes, while expression of the active form of ADD1 exhibited more lipid accumulation in the cells (Kim & Spiegelman, 1996).

In the present study, treatment of C3H10T1/2 cells with the adipogenic cocktail DMITro induced the expression of SREBF1/ADD1, and addition of 25-HC significantly inhibited the expression of SREBF1/ADD1. Since 25-HC inhibited the expression of SREBF1/ADD1 and given that expression of SREBF1/ADD1 is important in augmenting adipogenic differentiation and expression of PPARγ and the downstream adipogenic genes, it is possible that the anti-adipogenic effects of 25-HC on C3H10T1/2 cells are mediated through inhibition of SREBF1/ADD1. Although the mechanism by which 25-HC inhibits

adipogenesis and PPARγ expression has yet to be fully elucidated, this results suggest that part of the mechanism may be via the inhibition of SREBF1/ADD1 expression.

Together, our results show that 25-HC inhibits adipogenic differentiation in C3H10T1/2 cells by inhibiting accumulation of cyctoplasmic lipid droplets and expression of core adipocyte markers, PPARγ and C/EBPα. 25-HC may thus be useful in providing an intervention in excess fat accumulation associated with obesity. We also report that the anti-adipogenic effects of 25-HC in C3H10T1/2 cells are not mediated through hedgehog signalling since inhibition of this pathway by cyclopamine does not reverse the anti-adipogenic effects of 25-HC. However, it is possible that the inhibitory effects of 25-HC on adipogenic differentiation are mediated through the SREBF1/ADD1 pathway since 25-HC inhibits the expression of SREBF1/ADD1, a transcription factor that plays a role in the activation of PPARγ mRNA expression. This possibility needs to be further investigated in future.

CHAPTER FOUR

4.0 MANUSCRIPT II

Microarray analysis of gene expression profile of C3H10T1/2 cells during adipocyte differentiation and the inhibitory effects of 25 hydroxycholesterol.

4.1 ABSTRACT

Adipogenic differentiation is an important process in cell biology that involves a transcriptional network of proteins leading to formation of mature adipocytes. We conducted microarray analyses to assess the gene expression profile of C3H10T1/2 cells after 96h of adipogenic differentiation using an adipogenic cocktail consisting of Insulin, Dexamethasone, 3-Isobutyl-1-methlyxanthine and Troglitazone (DMITro). We compared the gene expression profile of non-treated (control) cells with those treated with DMITro, where out of 709 differentially expressed genes, 465 were highly expressed in the control cells and 244 were over-expressed in cells treated with the adipogenic media DMITro.

We further examined gene expression changes in response to treatment of cells with 25 hydroxycholesterol (25-HC) and compared the gene expression profile of cells treated with DMITro with those treated with DMITro+25-HC. Out of 2,204 differentially expressed genes, 276 were over-expressed in cells treated with DMITro and 1,928 were over-expressed in cells treated with DMITro+25-HC. Genes over-expressed in control cells compared to those treated with DMITro include those involved in cell growth and proliferation (APOD, CD9, POSTN), cellular assembly and organization (BMP4, CD47, AQP1) and cellular movement (CST3, EBF3, TGFB2). Genes over-expressed in DMITro compared to control cells include those involved in lipid metabolism (FABP4, PPARγ, SREBF1) and small molecule biochemistry (ADIPOQ, PLIN1 and CD36).

Genes over-expressed in DMITro compared to DMITro+ 25-HC include those involved in lipid metabolism (INSIG1, AHR, FASN), cell-to-cell signalling and interaction (CHRNB3,

MAPK13, IGHM) and small molecule biochemistry while genes over-expressed in DMITro+25-HC compared to DMITro include those involved in cell cycle (AATF, BMP4) and cellular growth and proliferation (ABCG1, CDK8, MAP2K1). These findings provide opportunities to further elucidate the mechanisms of hormonal induction and 25-HC on adipogenic differentiation and gene expression of C3H10T1/2 cells. Further gene expression studies are important for identification of new therapeutic targets for treatment of obesity and related disorders.

4.2 INTRODUCTION

Obesity is associated with an increase in adipose tissue and is a leading cause of cardiovascular diseases, cancer and various metabolic disorders such as insulin resistance (Haslam & James, 2005; Kahn & Flier, 2000). Obesity develops as a result of energy intake exceeding energy expenditure (Leibel et al., 1995). Increase in adipose tissue mass in obesity is as a result of increase in both the size (hypertrophy) and number (hyperplasia) of fat cells (Hirsch & Batchelor, 1976; Shepherd et al., 1993). Adipogenesis is thus described as the process by which mature fat cells form from undifferentiated precursor cells, and it plays an important role in both the initiation and progression of obesity (Zha et al., 2014). Adipogenic differentiation in *in vitro* cell models such as mesenchymal stem cells (MSCs) is possible through hormonal induction using a combination of insulin, dexamethasone (DEX) and 3-isobutyl-1-methyl-xanthine (IBMX) (Caprio et al., 2007; Farmer, 2006).

Hormonal stimulation of differentiation leads to major changes in cell morphology, up- or down-regulation of proteins and induction of gene expression, all which are important in adipocyte differentiation (Kratchmarova et al., 2002; Rodriguez Fernandez & Ben-Ze'ev, 1989). C3H10T1/2 cell line, established in 1973 from C3H mouse embryos (Reznikoff et al., 1973), displays differentiation characteristics similar to MSCs (Pinney & Emerson, 1989). C3H10T1/2 cells have the ability to differentiate into osteoblasts, chondrocytes or adipocytes

upon induction using an appropriate differentiation treatment (Konieczny & Emerson, 1984; Reznikoff et al., 1973; Tang et al., 2004).

Adipogenic differentiation is an important process in cell biology that is regulated by a network of transcriptional factors and proteins leading to formation of mature adipocytes (Farmer, 2006). Two transcription factors, namely PPARγ and C/EBPα, are considered important in adipogenesis as they oversee the entire terminal differentiation process. Oxysterols, which are 27-carbon oxygenated derivatives of cholesterol (Russell, 2000), are a potential strategy to regulate differentiation of mesenchymal stem cells. The oxysterols 20S, 22R and 22S have been shown to induce osteogenic differentiation in marrow stromal cells as evidenced by the induction of osteogenic differentiation markers such as osteocalcin and alkaline phosphatise activity (Kha et al., 2004).

Various analytical techniques such as quantitative real-time PCR and microarray studies have been used to study the expression profiles of transcription factors involved in adipocyte differentiation and maintenance of mature fat cells (Fu et al., 2005). The development of high-density microarray in particular, has proved a valuable tool for monitoring global gene expression profiles involving large numbers of genes, which is important in assessing the function of genes and studying causes and mechanisms of diseases (Lockhart et al., 1996). Microarray has also allowed the study of differential gene expression comparing two or more biological samples or one sample at different time points (Guo & Liao, 2000). Microarray analysis of the expression profile of 3T3-L1 fibroblasts during early stages of adipocyte differentiation has shown differential expression of genes associated with cell cycle events, where genes that block G1-to-S transition after induction of differentiation are down-regulated while genes associated with completion of the cell cycle are up-regulated (Burton et al., 2002). In human mesenchymal stem cells (hMSCs), microarray analysis during the early stages of adipogenesis was shown to reveal a unique gene expression profile of genes

encoding transcriptional regulators and signalling molecules, many of which had not been previously thought to be involved in adipogenesis (Nakamura et al., 2003). In C3H10T1/2 cell line, microarray analysis of early adipogenesis phase was able to identify up to 200 genes that are differentially expressed after 24h of adipogenic induction (Hanlon et al., 2005). However, microarray gene expression profile studies on intermediate and late stages of adipogenic differentiation in C3H10T1/2 cell line are still limited. Furthermore, there has been no study to evaluate the gene expression profile of 25-HC treatment during adipogenesis in C3H10T1/2 cells. To obtain more detailed insight into adipogenic differentiation of C3H10T1/2 cells, the microarray gene expression profile was studied at 96h post-induction of adipogenesis using DMITro. We also studied the gene expression profile upon treatment with 25-HC, which was previously identified to be an inhibitor of adipogenic differentiation in C3H10T1/2 cells.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture

C3H10T1/2 mouse embryonic stem cells were purchased from ATCC (Manassas,VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and L-glutamate (Mediatech, Inc., Manassas, VA) and incubated at 37˚C with 5% CO_2. Upon reaching confluence, the medium was changed and cells induced to differentiate into adipocytes in the presence of an adipogenic cocktail (DMITro), consisiting of 500nM DEX, 0.5mM IBMX, 20µg/ml Insulin (all from Cayman chemical company, Ann Arbor, MI, USA) + 10µM Troglitazone (Tro) (Tocris Bioscience, Ellisville, MO, USA). Inhibition of adipogenesis was induced by adding 10µM of 25-HC to DMITro treatment. The control treatment consisted of 10% FBS in DMEM. Re-treatment of cells was carried out after 48 hours (with omission of

DEX and IBMX), and incubated for another 48 hours, to give a total differentiation time of 96hours. Each treatment was carried out in 4 replicates in 6-well plates.

4.3.2 Quantitative real time PCR

Preliminary gene expression analysis by real-time PCR was carried out to assess expression of adipogenic genes, in preparation for microarray studies. Total RNA was extracted using TRIzol (Invitrogen, Burlington, ON) according to the manufacturer's instructions. This was followed by single strand cDNA synthesis by reverse transcription quantitative polymerase chain reaction (RT-PCR) analysis using a high-capacity cDNA synthesis kit following the supplier's protocol (Applied Biosystems, Burlington, ON). Quantitative real-time PCR (qRT-PCR) was performed on CFX Connect ^{TM} Real-Time PCR Detection instrument. All real time PCR samples were prepared in duplicates and gene expression data was generated using the ΔΔCt method where expression of target genes was normalized to the expression of the house keeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Pairs of primers used for the real-time PCR analysis and their sequences are presented in table 4.1

4.3.3 GeneChip microarray

After RNA extraction, four replicates of each treatment were analyzed for quality, where RNA yield of each sample was analysed using Nanodrop 2000 (Thermoscientific), while the RNA integrity was analysed using Experion automated electrophoresis system (Bio-Rad Laboratories Inc, USA). Using WT PLUS reagent kit (Affymetrix), 100ng of total RNA was subjected to first and second-strand complementary DNA (cDNA) synthesis, followed by complementary RNA (cRNA) synthesis by in vitro transcription. Second-cycle single-stranded cDNA was then synthesized by the reverse transcription of cRNA followed by fragmentation and labelling of the single-stranded cDNA, which was then hybridized on

genechips for 16h at 45˚C. The hybridized chips were washed and stained using an Affymetrix Fluidics Station 450 (Affymetrix, USA).

4.3.4 Image capturing and data analysis

The array chips were scanned using a Genechip 3000 laser confocal slide scanner (Affymetrix, USA), and the images were quantified using Affymetrix Genechip Command Console software (Affymetrix, USA). The data were then imported into Flex array software (Blazejczyk et al., 2007), where differentially expressed genes were identified using t-test procedure at a fold change greater than or equal to 2 and probability of $P < 0.05$. The list of over-expressed genes was uploaded into the Ingenuity pathway analysis (IPA) software (Qiagen), where identifiers and the relative levels of altered genes in the data set were analysed by making comparisons with molecules in the Ingenuity knowledge base.

4.3.5 Validation of microarray data using quantitative real time PCR

RNA samples that were used for the microarray were used for cDNA synthesis. Single strand cDNA was synthesised by reverse transcription quantitative polymerase chain reaction (RT-PCR) analysis using high capacity cDNA synthesis kit following the supplier's protocol (Applied Biosystems, Burlington, ON). Quantitative real-time PCR was performed on CFX Connect ^{TM} Real-Time PCR Detection instrument (Biorad) in duplicates and gene expression data was generated using the ΔΔCt method where expression of target genes was normalized to the expression of GAPDH. The values were reported as fold changes of expression of target genes in the DMITro group compared to the control group and expression of target genes in the DMITro+25-HC group compared to the DMITro group. Pairs of primers and their sequences used for array validation by real time PCR are presented in table 4.2

4.3.6 Statistical analysis

The preliminary real-time PCR results were analyzed using the General Linear Model (GLM) the Statistics Analysis System (SAS) Institute version 9.2. P values were determined using ANOVA and Fisher's protected Least significant difference (LSD) test. A probability value of $p < 0.05$ was considered significant.

For array validation, the t-test procedure of SAS software was used to analyze qRT-PCR data, while the correlation procedure of SAS was used to assess the correlation between microarray and qRT-PCR data. A probability value of less than 0.05 (<0.05) was considered significant.

Table 4.1 List of primers and sequences used for preliminary PCR reactions

Gene	Primer sequences	Size of PCR product
GAPDH	5'-ATGGACTGTGGTCATGAGCC-3' (sense) 5'-ATTGTCAGCAATGCATCCTG-3' (antisense)	190
PPARγ	5'-TGAAACTCTGGGAGATTCTCCTG-3' (sense) 5'-CCATGGTAATTTCTTGTGAAGTGC-3' (antisense)	88
C/EBPα	5'-GGACAAGAACAGCAACGAGTACC-3' (sense) 5'-GGCGGTCATTGTCACTGGTC-3' (antisense)	146
FABP4	5'-AACACCGAGATTTCCTT-3' (sense) 5'-ACACATTCCACCACCAG-3' (antisense)	178
LPL	5'-AGGACCCCTGAAGACAC-3' (sense) 5'-GGCACCCAACTCTCATA-3' (antisense)	148

Table 4.2 List of Primers and sequences used for array validation

Gene	Primer sequences	Size of PCR product
GAPDH	5’-ATGGACTGTGGTCATGAGCC-3’ (sense)	190
	5’-ATTGTCAGCAATGCATCCTG-3’ (antisense)	
AFF3	5’-GCCGTATCCTCAAGGGTCTC-3’ (sense)	164
	5’-TGGTCAAACCTGTGCTGGTA-3’ (antisense)	
GAS1	5’-CTCCCGGCCCACTTTTGTAT-3’ (sense)	233
	5’-GTAGCACTTCGCAGCTCTGG-3’ (antisense)	
EMP1	5’-AACAAAGGGTGCCATCTCCG-3’ (sense)	171
	5’-TCTGCAACCATCCAGACGTT-3’ (antisense)	
AQP7	5’- CCTACTGACCTCTCCCCTGG-3’ (sense)	176
	5’-CTTTCAGCCTCCGTCTCACA-3’ (antisense)	
MPC1	5’- GGTCTCCCCATTGCTGCTATC-3’ (sense)	316
	5’-ATGGCCGCTTACTCATCTCG-3’ (antisense)	
RGS2	5’-TCCTGTCACTTACCAACCGC-3’ (sense)	221
	5’-CACTGCGGAGAGGAACCATT-3’ (antisense)	
RPL41	5’-GAAGTGACGACACCGAGCA-3’ (sense)	245
	5’-CAGTCCGATAGCTTGTCCCG-3’ (antisense)	
FTMT	5’-ATGACTGGGAATGCGGACTG-3’ (sense)	189
	5’-TTGTGCACGTGGTCACCTAA-3’ (antisense)	
AKNAD1	5’-GAGGAGACGCACACCACAA-3’ (sense)	158
	5’-TTAGGGTCCCGACATCACAC-3’ (antisense)	
SSTR3	5’-CTGGCTGTGCTCTGGTGGTA-3’ (sense)	138
	5’-TAGCTTGCGTCTGGTTTGGT-3’ (antisense)	
HARS	5’-GCAGAAGACTGAAGGGTGGT-3’ (sense)	164
	5’-AGGAGGCAGAGACGTGGTTA-3’ (antisense)	
MGP	5’-GCTACAACGCTGCCTACAAC-3’ (sense)	202
	5’-ACTTTCAACCCGCAGAAGGAA-3’ (antisense)	
RPL3	5’-CTCTCTATCTGCGGCGTGTG-3’ (sense)	132
	5’-GGGTCATGCCAGCCTTGTAA-3’ (antisense)	
DAPP1	5’-AGCAAAGGAACTTATGGGCAGA-3’ (sense)	232
	5’-CAGAGAGAGAGTAGAGCCCAGT-3’ (antisense)	

4.4 RESULTS

In this experiment, the gene expression profile of C3H10T1/2 cells during adipogenic differentiation was studied at 96 h after induction of differentiation using the adipogenic cocktail, DMITro. Messenger RNA expression of adipogenic genes in the different treatments was assessed by real time PCR. Cells treated with DMITro showed a higher expression of PPARγ compared to the control cells whereas treatment with DMITro+25-HC led to a significant inhibition of PPARγ expression **(Figure 4.1).** The same pattern of results was observed in the expression profiles of FABP4, LPL and C/EBPα (**Figure 4.1)**

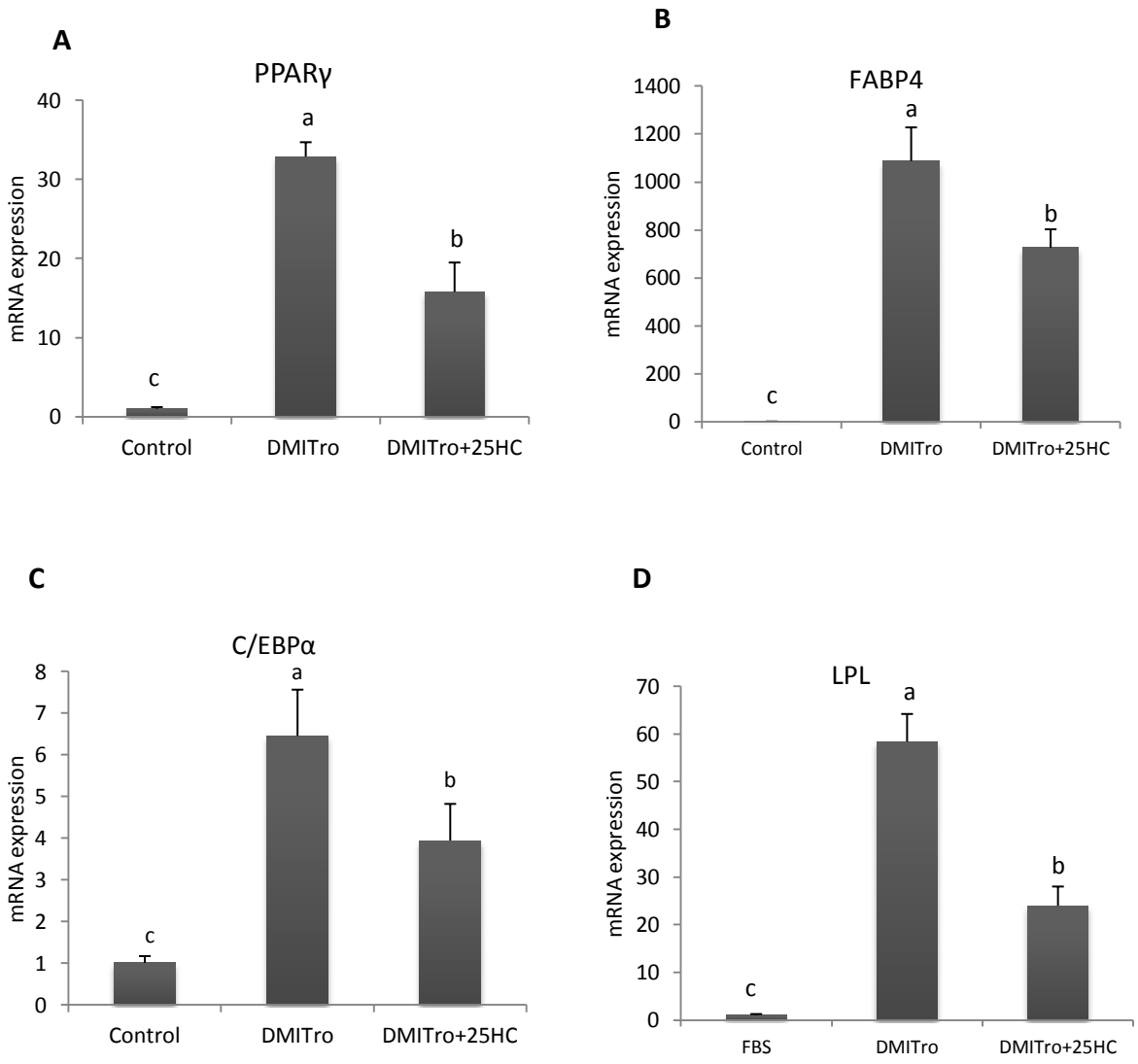

Figure 4.1. Expression of adipogenic genes, PPARγ **(A)**, FABP4 **(B),** C/EBPα **(C)** and LPL **(D)** following treatment for 96h. C3H10T1/2 cells at confluence were treated with control vehicle, an adipogenic media DMITro and DMITro+25-HC for 96h. Gene expression was measured by RT-qPCR and RNA levels were normalized to the expression of GAPDH. Fold changes were determined by the ΔΔCT method. The results show the average values of four replicates (n=4) and the SD of the means.

4.4.1 Microarray profile of C3H10T1/2 cells treated with DMITro relative to non-treated cells

From the microarray analysis, a comparison of C3H10T1/2 cells treated with DMITro and non-treated cells showed that out of 709 differentially expressed genes, 465 were over-expressed in non-treated cells and 244 were over-expressed in cells treated with DMITro.

IPA identified top networks such as cellular growth and maintenance, cellular assembly and organization, cellular function and maintenance, and cellular movement and cell morphology, associated with genes over-expressed in non-treated cells compared to DMITro treated cells. Genes identified under the category of cellular growth and proliferation include: Apolipoprotein D (APOD), CD9 molecule (CD9) and periostin, osteoblast specific factor (POSTN). Those identified under cellular assembly and organization include: bone morphogenic protein 4 (BMP4), CD47 molecule (CD47) and aquaporin 7 (AQP7). Genes identified under cellular movement include: cystatin C (CST3), early B cell factor (EBF3) and transforming growth factor beta 2 (TGFB2). Genes involved in cellular function and maintenance include: CD9 molecule (CD9), hexosaminidase A (HEXA) and secreted frizzled-related protein 1 (SFRP1), while those identified under cell morphology include: creb binding protein (CREBBP), G protein coupled receptor 126 (GPR126) and insulin-like growth factor 1 receptor (IGF1R). A functional grouping (canonical pathways) of molecules

over-expressed in non-treated cells compared to those treated with DMITro is shown in **figure 4.2**.

For genes over-expressed in DMITro compared to non-treated cells, IPA identified top networks such as lipid metabolism, molecular transport and small molecule biochemistry Genes identified under lipid metabolism include fatty acid binding protein 4 (FABP4), fatty acid synthase (FASN), PPARγ, sterol regulatory element binding transcription factor 1 (SREBF1) and adiponectin C1Q and collagen domain containing (ADIPOQ). Genes identified under molecular transport include: ATP-binding cassette, subfamily A, member 1 (ABCA1), fatty acid binding protein 5 (FABP5), resistin (RETN) and STEAP family member 4 (STEAP4), while those identified under small molecule biochemistry include ADIPOQ, perilin 1 (PLIN 1), CD36 molecule (CD36), and scavenger receptor class B, member 1 (SCARB1). A network representation of molecules associated with lipid metabolism that were over-expressed in the DMITro treated cells compared to non-treated cells is shown in **figure 4.3.** The top significantly changed gene ontology (GO) terms (molecular and cellular functions) with the proportion of molecules over-expressed in non-treated cells and those over-expressed in cells treated with DMITro are shown in **figure 4.4**

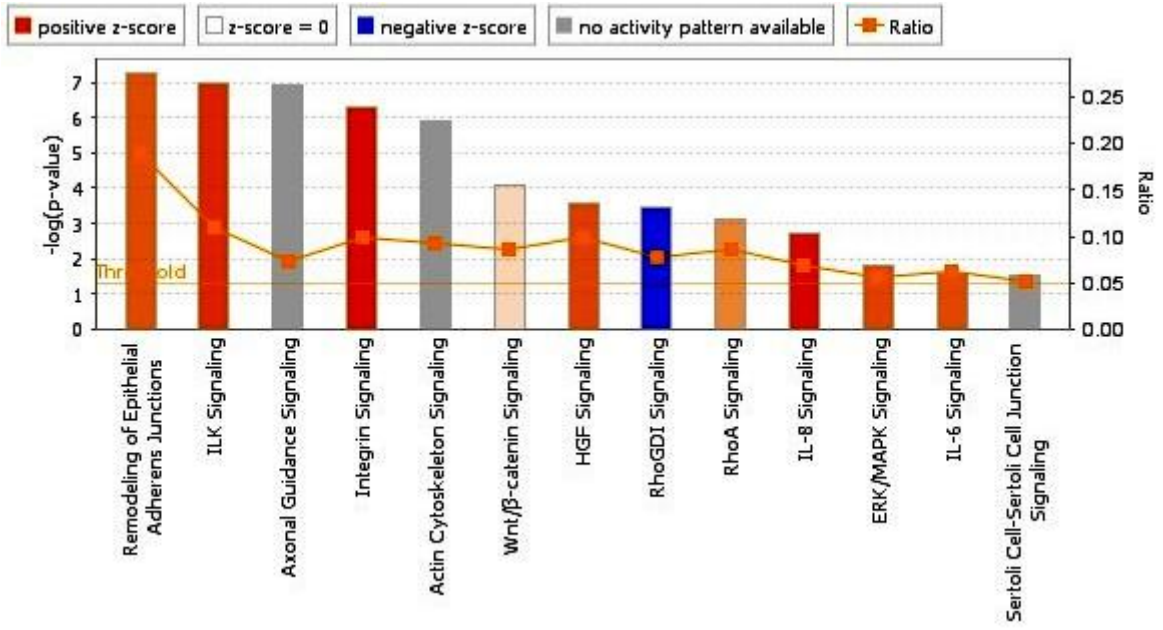

Figure 4.2. Functional grouping (canonical pathways) of molecules over-expressed in non-treated cells compared to those treated with DMITro. The significance value for the canonical pathways is calculated by Fisher's exact test right-tailed. The significance indicates the probability of association of molecules from the data set with the canonical pathway by random chance alone. The significant canonical pathways for the dataset are displayed on the x-axis. The default y-axis displays the -log of p-value which is calculated by Fisher's exact test right-tailed (the most significant groups had p value ≤ 0.05). The taller bars equate to increased significance while the bar charts are colored to indicate their activation z-scores. Orange bars predict an overall increase in the activity of the pathway while blue bars indicate a prediction of an overall decrease in activity. White bars are those with a z-score which is zero or very close to 0. Gray bars indicate pathways that are ineligible for a prediction. The orange points connected by a thin line represent the ratio, which is calculated as follows: number of genes in a given pathway that meet the cutoff criteria, divided by the total number of genes that make up that pathway and are in the reference gene set.

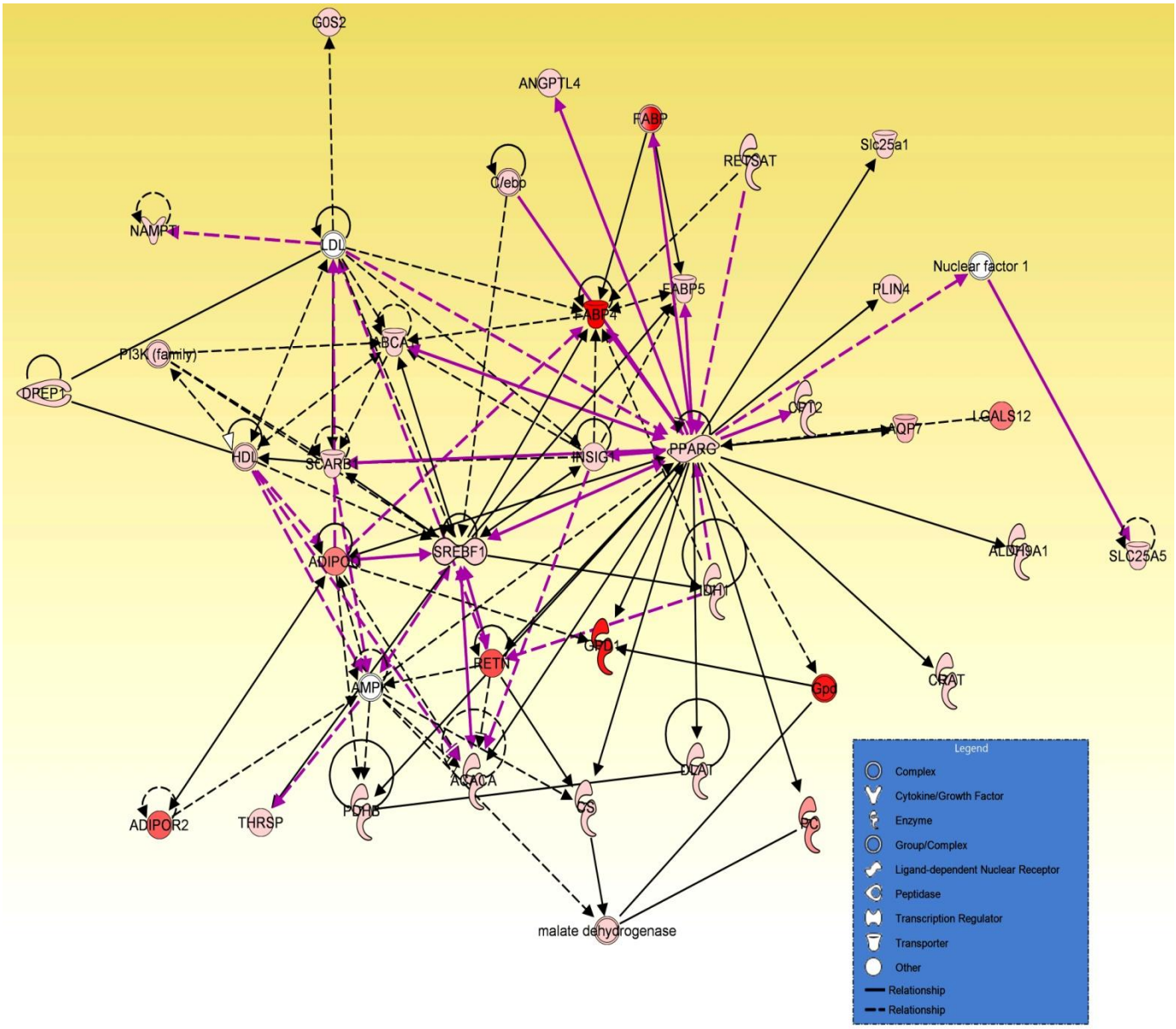

Figure 4.3. IPA network (lipid metabolism) analysis showing the interaction of molecules over-expressed in cells treated with DMITro compared to non-treated cells. Direct or indirect relationships between molecules are indicated by connecting solid or dashed lines. Over-expressed genes are indicated in red colour, with the intense red colours indicating higher fold changes. The shape of the node indicates the major function of the protein.

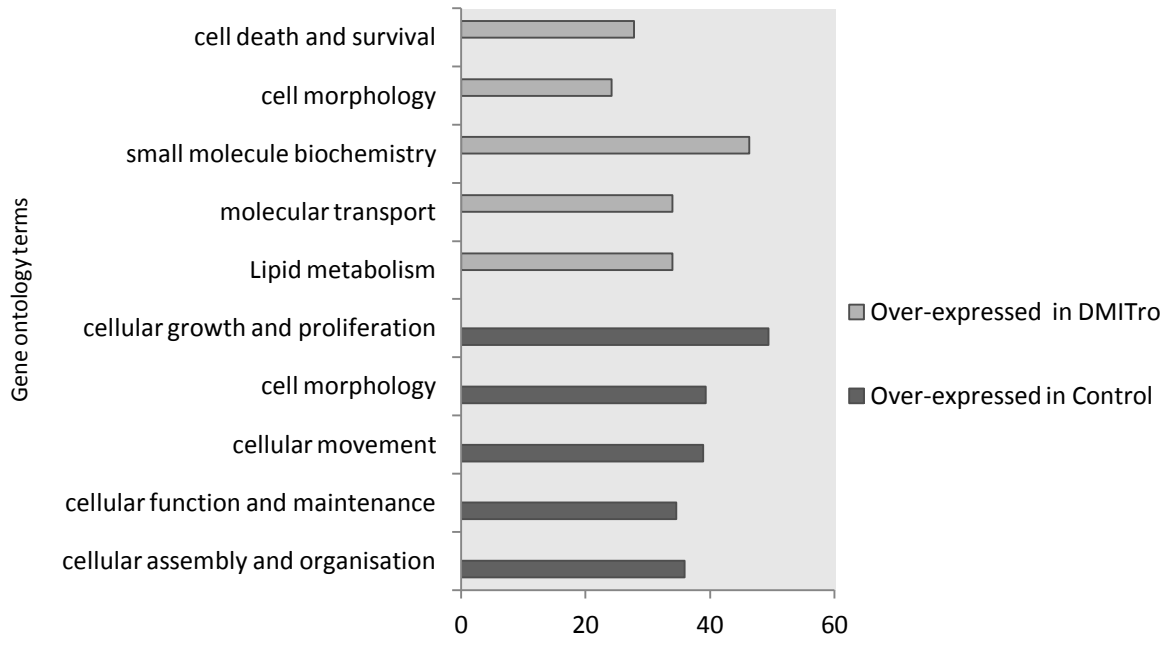

Figure 4.4. Top significantly changed GO terms (molecular and cellular functions) showing the proportion of molecules over-expressed in non-treated cells (control) and those over-expressed in DMITro treated cells. List of genes associated with a given GO term were determined by IPA at $p < 0.0001$. The p-value is determined using the right-tailed Fisher's test and measures the likelihood that the association between the number of focus molecules in a data set and a given GO term is as a result of random chance. The p-value was calculated by considering the number of focus molecules that participate in a given GO term and the total number of molecules that participate in that GO term as shown by the IPA knowledge base. To determine the number of genes/molecules in a given GO term, the number of molecules determined by IPA to be associated with that GO term was divided by the total number of molecules over-expressed in one treatment group relative to the other and multiplied by 100.

4.4.2 Microarray profile of C3H10T1/2 cells treated with DMITro alone and a combination of DMITro+25-HC

A comparison of C3H10T1/2 cells treated with DMITro and those treated with DMITro+25-HC showed that out of 2,204 differentially expressed genes, 276 were over-expressed in cells treated with DMITro and 1,928 were over-expressed in cells treated with DMITro+25-HC. IPA identified top networks such as lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism and cell-to-cell signalling and interaction, associated with genes over-expressed in DMITro compared to DMITro +25-HC. Genes identified under lipid metabolism include insulin induced gene 1 (INSIG1), aryl hydrocarbon receptor (AHR), fatty acid synthase (FASN) and FABP4. Genes identified under small molecule biochemistry include INSIG1, cytochrome P450, family 51, subfamily A, polypeptide 1 (CYP51A1), GAST and FASN. Genes identified under vitamin and mineral metabolism include CYP51A1, farnesyl diphosphate synthase (FDPS) and AHR and RETN, while those identified under cell-to-cell signalling and interaction include cholinergic receptor, nicotinic, beta-3 (CHRNB3), mitogen-activated protein kinase 13 (MAPK13) and immunoglobulin heavy constant mu (IGHM). An example of a gene network (lipid metabolism, molecular transport and small molecule biochemistry) showing the relationship between molecules over-expressed in DMITro compared with DMITro+25-HC is represented in **figure 4.5.**

For genes over-expressed in DMITro+25-HC compared to DMITro, IPA identified top networks such as cellular assembly and organization, cell cycle and cellular growth and proliferation and protein synthesis. Genes identified under protein synthesis include: cytochrome c oxidase assembly homolog 10 (COX10), epidermal growth factor receptor (EGFR) and eukaryotic translation initiation factor 3, subunit B (EIF3B). Genes identified under cell cycle include apoptosis antagonizing transcription factor (AATF), BMP4, cAMP responsive element binding protein 1 (CREB1) and growth arrest and DNA-damage-

inducible alpha (GADD45A). Those under cellular assembly and organization include: ras homolog family member A (RHOA), tumor necrosis factor, alpha-induced protein 1 (TNFAIP1) and sestrin 2 (SESN2), while those under cell growth and proliferation include: ATP-binding cassette sub-family G, member1 (ABCG1), bone morphogenic protein receptor, type 1A (BMPRIA) and epidermal growth factor receptor (EGFR). The top significantly changed gene ontology (GO) terms (molecular and cellular functions) with the proportion of molecules over-expressed DMITro and those over-expressed in cells treated with DMITro+25-HC are shown in **figure 4.8.**

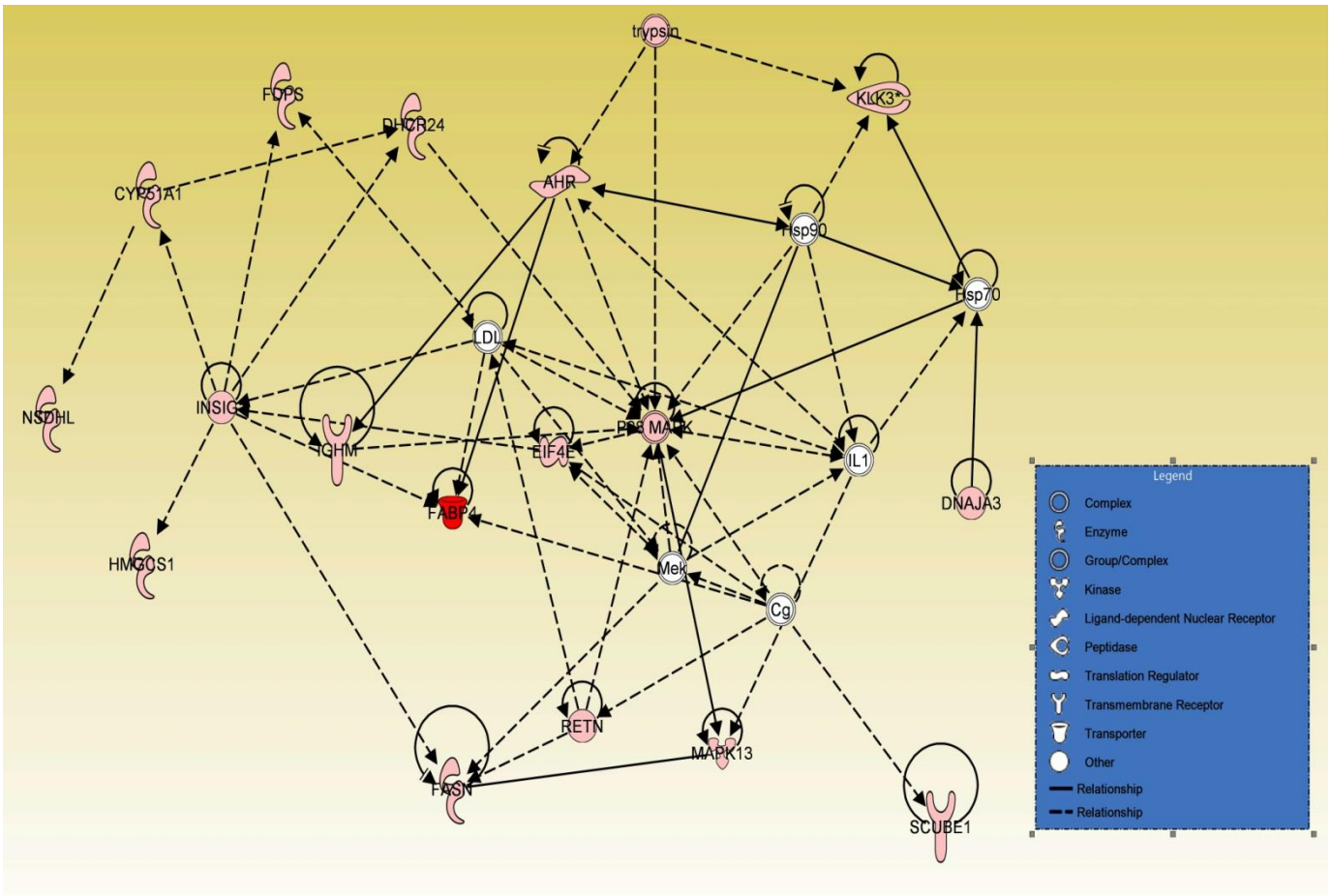

Figure 4.5 IPA network (lipid metabolism, small molecule biochemistry and molecular transport) analysis showing the interaction of molecules over-expressed in cells treated with DMITro compared to those treated with DMITro+25HC. Direct or indirect relationships

between molecules are indicated by connecting solid or dashed lines. Up-regulated genes are indicated in red/pink colour, with the intense colours indicating higher fold changes. The shape of the node indicates the major function of the protein.

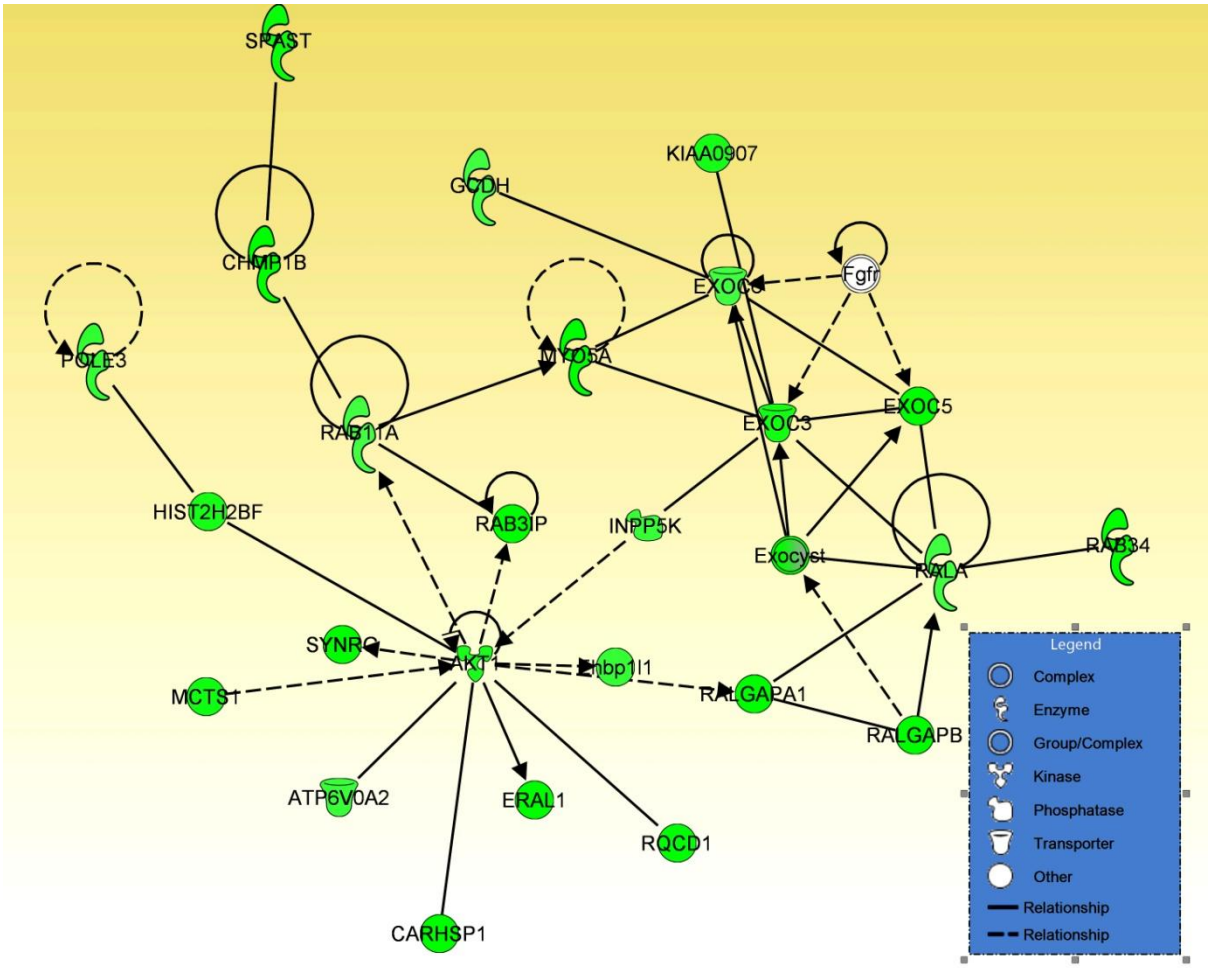

Figure 4.6. An example of a network analysis (molecular transport and cellular assembly and organization) showing the interaction of molecules over-expressed in DMITro+25-HC compared to DMITro. Direct or indirect relationships between molecules are indicated by connecting solid or dashed lines. The shape of the node indicates the major function of the protein.

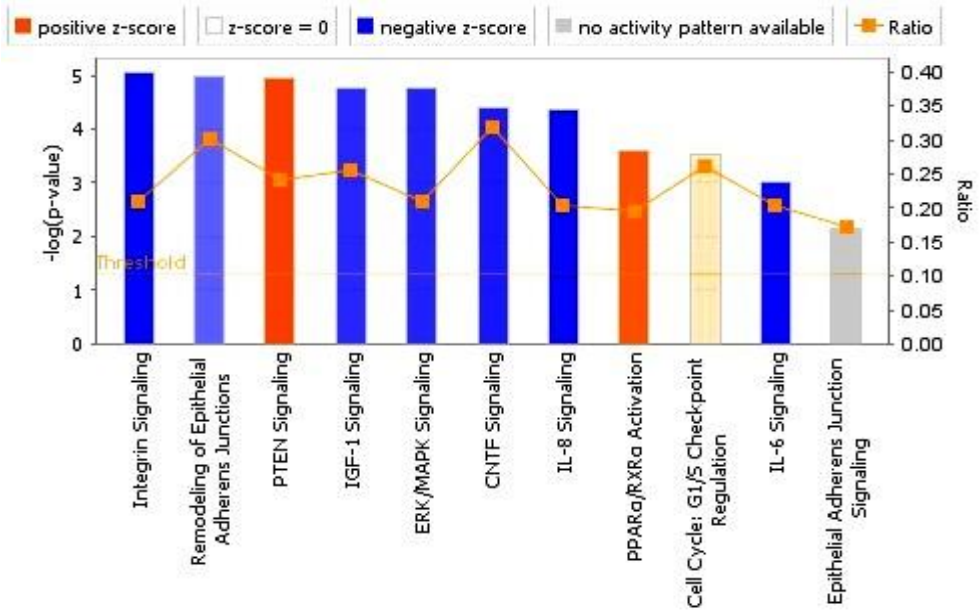

Figure 4.7. Functional grouping (canonical pathways) of molecules over-expressed in DMITro+25HC treated cells compared to those treated with DMITro. The significance value for the canonical pathways is calculated by Fisher's exact test right-tailed. The significance indicates the probability of association of molecules from the data set with the canonical pathway by random chance alone. The significant canonical pathways for the dataset are displayed on the x-axis. The default y-axis displays the -log of p-value which is calculated by Fisher's exact test right-tailed (the most significant groups had p value ≤ 0.05). The taller bars equate to increased significance while the bar charts are colored to indicate their activation z-scores. Orange bars predict an overall increase in the activity of the pathway while blue bars indicate a prediction of an overall decrease in activity. White bars are those with a z-score which is zero or very close to 0. Gray bars indicate pathways that are ineligible for a prediction. The orange points connected by a thin line represent the ratio, which is calculated by diving the number of genes in a given pathway that meet your cutoff criteria, by the total number of genes that make up that pathway and that are in the reference gene set.

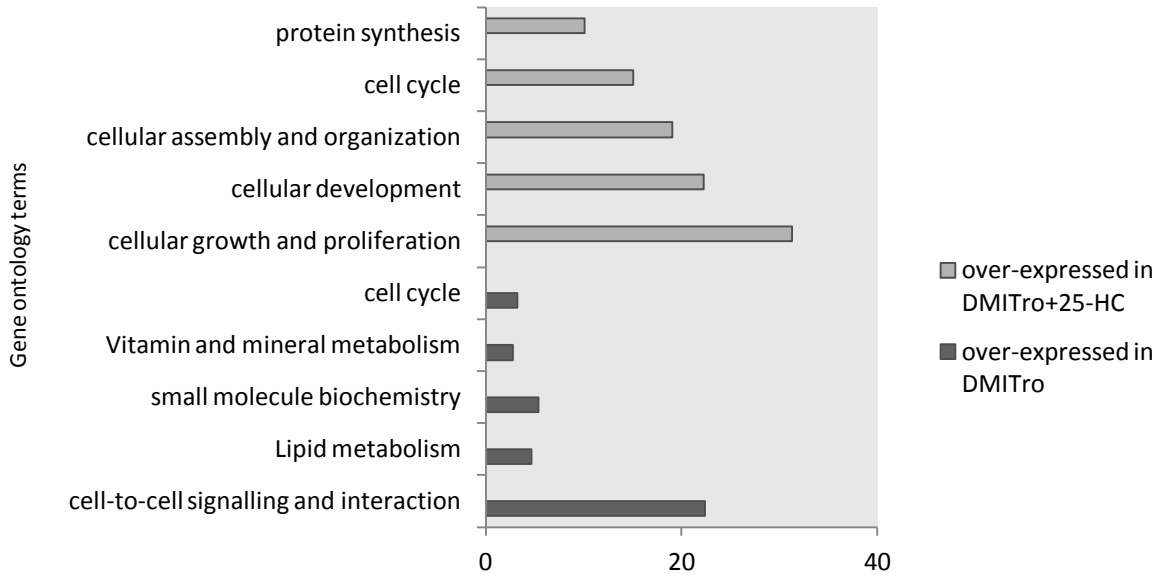

Figure 4.8. Top significantly changed GO terms (molecular and cellular functions) showing the proportion of molecules over-expressed in DMITro treated cells and those over-expressed in DMITro+25-HC treated cells. List of genes associated with a given GO term were determined by IPA at $p < 0.0001$. The p-value is determined using the right-tailed Fisher's test and measures the likelihood that the association between the number of focus molecules in a data set and a given GO term is as a result of random chance. The p-value was calculated by considering the number of focus molecules that participate in a given GO term and the total number of molecules that participate in that GO term as shown by the IPA knowledge base. To determine the number of genes/molecules in a given GO term, the number of molecules determined by IPA to be associated with that GO term was divided by the total number of molecules over-expressed in one treatment group relative to the other and multiplied by 100.

4.4.3 Validation of microarray data by quantitative real-time PCR

To validate the microarray data, genes were randomly selected from the lists of over-expressed genes and their mRNA expression analysed by real-time PCR. Fold changes between the treatments, i.e Control vs DMITro and DMITro vs DMITro+25-HC were significantly different ($p<0.05$), except for DAPP1. Correlation analysis of the PCR and microarray data showed a positive correlation ($r=0.89$ and $p<0.0001$). Microarray and PCR fold changes are shown in **Table 4.3.**

Table 4.3 Microarray and PCR fold changes and p-values.

Gene	**Fold change**		**P value**	
	RT-PCR	**Microarray**	**RT-PCR**	**Microarray**
AFF3	6.25	6.01	0.0035	0.0037
GAS1	4.3	5.0	0.0017	0.013
EMP1	3.2	2.96	0.0001	0.001
AQP7	6.52	8.4	0.001	0.0005
MPC1	7.35	10.29	0.011	0.0001
RGS2	9.18	7.58	0.0016	5.26E-07
FTMT	2.21	2.15	0.018	0.0025
AKNAD1	1.63	2.72	0.0126	0.00011
RPL41	2.39	2.60	0.0045	0.04
SSTR3	1.86	2.04	0.0001	0.006
MGP	3.1	2.17	0.0001	0.001
RPL3	2.05	2.06	0.0024	0.034
HARS	2.5	3.37	0.0005	0.001
DAPP1	1.54	3.3	0.0767	0.003

Table 4.3. Microarray and PCR fold changes and p-values. AFF3, GAS1, EMP1 were over-expressed in non-treated cells and AQP7, MPC1 and RGS2 were over-expressed in DMITro treated cells relative to each other. AKNAD1, RPL41, SSTR3 and FTMT were over-expressed in DMITro treated cells whereas MGP, RPL3, HARS and DAPP1 were over-expressed in cells treated eith DMITro+25-HC relative to each other.

4.5 DISCUSSION

Microarray is a valuable tool for monitoring global gene expression profiles, assessing the function of genes and studying pathways and mechanisms associated with diseases. (Lockhart et al., 1996). Furthermore, microarray has allowed differential gene expression studies between two or more biological samples or comparison of gene expression at different time points (Guo & Liao, 2000).

4.5.1 Effects of DMITro on adipogenic differentiation of C3H10T1/2 cells

In the present study, adipogenic differentiation of C3H10T1/2 cells was induced by DMITro. IPA identified numerous genes over-expressed in cells treated with DMITro compared to non-treated cells, most of which were associated with lipid metabolism, molecular transport and small molecule biochemistry. We focussed on molecules classified under lipid metabolism, where a total of 83 genes were up-regulated. Of these molecules, the majority consisted of genes directly involved in the accumulation and concentration of lipids, suggesting that these could be the main adipogenic events taking place at 96h post induction of adipogenic differentiation in C3H10T1/2 cells, since accumulation of lipid droplets takes place mainly during the middle and late stages of adipogenesis (Yin et al., 2014). In this study, molecules that were up-regulated in the adipogenic treatment compared to non-treated cells include genes known to be involved in adipogenesis such as PPARγ, C/EBPα and SREBF1/ADD1. These 3 genes are induced during acquisition of early adipocyte phenotype such as, the accumulation of lipid vesicles and play established roles in adipogenic differentiation (Rangwala & Lazar, 2000; Rosen et al., 2000). In particular, PPARγ, a transcription factor that is both necessary and sufficient for adipogenesis (Rosen et al., 2000), was classified as a top upstream regulator in this IPA analysis, with an overlap p value of 4.48E-42. C/EBPα is also an important transcription factor, the expression of which activates adipose-specific genes such as FABP4 and GLUT4 (MacDougald & Lane, 1995), while

SREBF1/ADD1 increases the activity of PPARγ through production of endogenous ligands that bind directly to PPARγ (Kim et al., 1998), and has also been linked to regulating insulin levels through regulation of certain genes involved in lipid biosynthesis (Kim et al., 1998). Furthermore, in 3T3-L1cells, over expression of SREBF1 leads to increased adipocyte marker expression and accumulation of lipid (Rosen et al., 2000). Other genes such as FABP4 and FABP5, which also have established roles in adipogenesis, were also upregulated in cells treated with DMITro compared to non-treated cells.

Other molecules that were over-expressed in cells treated with DMITro compared to non-treated cells include resistin (RETIN), a hormone that links obesity to diabetes by increasing insulin resistance (Steppan et al., 2001) and adiponectin, an adipocyte-derived hormone that has been shown to decrease insulin resistance by decreasing triglyceride levels in obese mice, leading to the conclusion that replenishment of adiponectin may act as an important therapeutic target for treatment of obesity and related metabolic disorders (Yamauchi et al., 2001). Various enzymes that contribute to adipogenesis were up-regulated in this analysis including hydroxyacyl-CoA dehydrogenase (HADH), INSIG1 and FASN, with functional roles in concentration of lipids. Fatty acid synthase, a key enzyme in *de novo* lipogenesis catalyzes the synthesis of saturated fatty acids such as palmitate from malonyl-CoA, and has been implicated in the long term regulation of lipid accumulation and fatty acid synthesis in the liver and adipose tissue (Volpe & Vagelos, 1976). INSIG1, on the other hand, is a key regulator of SREBPs, and is itself, regulated by PPARγ, thus providing a link between insulin sensitization, glucose homeostasis and lipid metabolism (Kast-Woelbern et al., 2004). IPA also identified an up-regulation of G0/G1 switch 2 (G0S2), which was associated with lipid/fatty acid oxidation. G0S2 has been reported to be highly expressed in adipose tissue and differentiated adipocytes; it acts as a negative regulator of adipose triglyceride lipase (ATGL), inhibiting its TAG hydrolase activity and lipolysis in adipocytes (Yang et al., 2010).

Furthermore, G0S2 is a direct target gene of PPARγ and is associated with growth arrest in 3T3-L1 cells (Zandbergen et al., 2005).

4.5.2 Effect of 25 hydroxycholesterol on adipogenesis

Oxysterols have been shown to regulate the differentiation of mesenchymal stem cells by inhibiting adipogenic differentiation and inducing osteogenic differentiation (Kha et al., 2004; Kim et al., 2007). In the present study, we report that 25-HC inhibits adipogenic differentiation and expression of adipogenic genes in C3H10T1/2 cells as demonstrated by the analysis of gene expression **(Figure 4.1)**. In the microarray analysis, we show that treatment of C3H10T1/2 cells with DMITro+25-HC leads to inhibition of expression of adipogenic genes as compared to cells treated with DMITro alone. IPA analysis of genes treated with DMITro versus those treated with DMITro+25-HC showed that 25-HC inhibited the expression of adipogenic differentiation markers such as FABP4, FASN and INSIG1, as these genes were over-expressed in DMITro treated cells compared to DMITro+25-HC treated cells. However, the microarray analysis did not show differential expression of other adipogenic genes such as PPARγ and C/EBPα between DMITro and DMITro+25-HC treated cells, although gene analysis by real-time PCR showed that these two genes were indeed up-regulated in DMITro compared to DMITro+25-HC treatment **(Figure 4.1)**. These discrepancies between microarray and real-time PCR results could be as a result of differences in microarray measures such as accuracy (the degree of conformity of the measured value to its true or actual value, sensitivity (the concentration range of target genes in which accurate measurements can be made) or specificity (the ability of a probe to provide a signal that is influenced only be the target molecule) (Draghici et al., 2006).

Furthermore, gene network analysis of molecules over-expressed in DMITro compared to DMITro+25-HC treatments showed a larger proportion of molecules involved in lipid

metabolism, an indication that 25-HC inhibits adipogenesis in C3H10T1/2 cells **(Figure 4.5)**. Molecules such as INSIG1, which is regulated by PPARγ and in turn regulates the processing of SREBPs (Kast-Woelbern et al., 2004) and IGHM, which has been found to stimulate adipocyte lipogenesis (Khokher et al., 1984) were over-expressed in DMITro treated cells compared to those treated with DMITro+25-HC **(Figure 4.5)**.

Genes over-expressed in DMITro+25-HC compared to DMITro mostly included those involved in molecular transport, cell growth and proliferation and cellular assembly and organization, including CASK, EHD1, SLC35B4, CDK16, AATF, RHOA, TNFAIP1 and GADD45A. Pathway analysis of genes over-expressed in DMITro+25-HC compared to DMITro treatments showed an involvement of these genes in signalling pathways such as integrin signalling and MAPK signalling **(Figure 4.7)**.

In conclusion, microarray analysis of C3H10T1/2 cells treated with DMITro resulted in up-regulation of molecules associated with various aspects of lipid metabolism such as lipid synthesis, lipid transport, and accumulation and storage of lipids, whereas treatment with DMITro+25-HC inhibited the expression genes associated with lipid metabolism. Further studies on the differentially expressed molecules is needed so as to determine their functions and to study their potential roles as possible targets for regulating obesity and related diseases.

5.0 GENERAL DISCUSION

As highlighted in the introduction and literature review, obesity is a global health issue that has led to increased research in the area adipogenesis and the mechanisms involved. This is important because obesity is associated with a myriad of disorders including cardiovascular diseases, cancers and type II diabetes, hence the need to find an intervention in order to curb the rising incidence rate of obesity. Various *in vitro* models of adipocyte differentiation such as mesenchymal stem cells have been employed with the aim of studying the progression and mechanisms of adipogenesis and obesity. Bone marrow-derived mesenchymal stem cells are an attractive cell source for the study of differentiation. However, these cells have been reported to show heterogeneity in colony size, colony forming rate and cell morphology, which for example, ranges from fibroblast-like, spindle-shaped cells to large and flat cells. (Bianco et al., 2001). In contrast, C3H10T1/2 cells derived from mouse embryos form homogeneous populations in culture and do not undergo spontaneous differentiation under normal cell culture conditions (Zhao et al., 2009), suggesting that they may be a good *in vitro* model of studying adipocyte differentiation. The use of oxysterols as a potential strategy to regulate differentiation of mesenchymal stem cells has been demonstrated in various studies using bone marrow stromal cells, where they have been shown to have pro-osteogenic and anti-adipogenic effects (Kha et al., 2004; Kim et al., 2007). The question that needs to be addressed therefore is whether oxysterols are able to regulate differentiation of C3H10T1/2 cells by inhibiting adipogenesis. As indicated earlier, mesenchymal stem cells are able to commit to either adipose, bone, cartilage or muscle lineages upon appropriate induction (Pinney & Emerson, 1989). The objective of this study was therefore to investigate the effects of specific oxysterols on the adipogenic differentiation of C3H10T1/2 cells. This information could prove useful in providing an intervention that would counteract the excess fat accumulation associated with obesity. Two studies were therefore conducted to: 1.) determine

the effects of specific oxysterols on the adipogenic differentiation of C3H10T1/2 cells and the mechanisms involved. 2.) To study the microarray profile of genes associated with adipogenic differentiation and inhibition of adipogenesis in C3H10T1/2 cells.

In manuscript I, C3H10T1/2 cells were treated with DMITro for six days to assess induction of adipogenesis. To assess inhibition of adipogenesis, cells were treated with DMITro+20S, 22R, 22S or 25 hydroxycholesterols. At the end of the treatment, cells were stained with oil red O stain to determine the extent of lipid accumulation during the different treatments. In addition, RNA extraction was carried out followed by qRT-PCR to determine the relative expression of adipogenic genes. As reported in manuscript I, treatment of cells with DMITro resulted in a significant accumulation of lipid droplets compared to the control, as shown in the oil red O pictures. Addition of 25-HC inhibited formation of lipid droplets while addition of 20S, 22S and 22R did not. Furthermore, gene expression analysis showed that 25-HC significantly inhibited the expression of PPARγ, C/EBPAα, LPL and FABP4 **(Figure 3.3)**. In contrast, 20S, 22R and 22S did not inhibit the expression of PPARγ. 22S did not significantly inhibit any of the tested adipogenic genes, suggesting that it may not have anti-adipogenic effects on C3H10T1/2 cells. From this study, 25-HC proved to be the most potent oxysterol in inhibiting adipogenesis and expression of adipogenic genes.

In manuscript I, a study on the role of hedgehog signalling on the anti-adipogenic effects of 25-HC in C3H10T1/2 cells showed that the inhibitory effects are independent of hedgehog signalling. This was demonstrated by the action of cyclopamine, whereby treatment of cells with DMITro+cyclopamine did not reverse the anti-adipogenic effects of 25-HC. These findings are consistent with a study carried out using murine 3T3-L1 cells where inhibition of Hh signalling using cyclopamine did not induce adipogenesis or expression of adipogenic differentiation markers (Cousin et al., 2006).

As reported in manuscript I, the anti-adipogenic effects of 25-HC in C3H10T1/2 cells could be mediated in part by the ADD1/SREBF1 pathway. This is because 25-HC inhibits the expression of ADD1/SREBF1, a transcription factor that has been implicated to play a role in the induction of expression of PPARγ (Fajas et al., 1999; Kim & Spiegelman, 1996).

In manuscript II, a microarray analysis was carried out to assess the gene expression pattern in none-treated, DMITro treated and DMITro+25-HC treated cells. Statistical analysis of initial PCR results showed an increase in expression of adipogenic genes namely PPARγ, C/EBPα, FABP4 and LPL upon treatment with DMITro and a significant decrease in expression of these genes upon treatment with DMITro+25-HC **(Figure 4.1).** These results are consistent with those observed in manuscript I. In the microarray analysis, a comparison of non-treated and DMITro treated cells showed 709 differentially expressed genes while a comparison of cells treated with DMITro and those treated with DMITro+25-HC showed a total of 2,204 differentially expressed genes. Genes more highly expressed in control cells compared to those treated with DMITro include those involved in cellular assembly and organization (BMP4, CD47, AQP1), cellular movement (CST3, EBF3, TGFB2) and cell-to-cell signalling and interaction (VCAM1, GAS6, GPR39). Genes over-expressed in DMITro compared to control cells include those involved in lipid metabolism (FABP4, PPARG, SREBF1) and small molecule biochemistry (ADIPOQ, PLIN1 and CD36). In the comparison of differentially expressed genes in the control group and DMITro group, it is important to note that genes that were highly expressed in the control group were down-regulated in the DMITro group and *vice versa*. The adipogenic gene, C/EBPα, which has been shown to induce the activation of a number of adipocyte specific genes such as SCD, PEPCK, FABP4 and GLUT4 (Christy et al., 1989; Park et al., 1993; Yeh et al., 1995) was also upregulated in DMITro treated cells compared to non-treated cells.

Genes over-expressed in DMITro compared to DMITro+ 25-HC treated cells include those involved in lipid metabolism (INSIG1, AHR, FASN, FABP4) and cell-to-cell signalling and interaction (CHRNB3, MAPK13, IGHM). Genes over-expressed in DMITro+25-HC compared to DMITro include those involved in cell cycle such as: AATF, BMP4, CREB1, RHOA, pre-mRNA processing factor 4 (PRPF4), growth arrest-specific 6 (GAS6), single stranded DNA binding protein 2 (SSBP2), interferon gamma inducible protein 16 (IFI16), GATA bibdibg protein 6 (GATA6) and Kruppel like factor 6 (KLF6). Those involved in cellular growth and proliferation include ABCG1, CDK8, MAP2K1, activating transcription factor 6 (ATF6), forkhead box O3 (FOXO3), ets variant 1 (ETV1) and SMAD family member 4 (SMAD 4). The functions of these genes in relation to inhibition of adipogenic differentiation is not established, hence the need for more studies to determine their roles in adipogenesis and obsity. It is also important to note that the genes that were over-expressed in the DMITro group were down-regulated in the DMITro+25-HC group and vice versa.

Although LPL was significantly induced in DMITro treated cells and significantly inhibited in cells treated with DMITro+25-HC as shown in the real time PCR analysis in both manuscript I **(Figure 3.5)** and manuscript II **(Figure 4.1)**, it was not expressed in the microarray results, probably because it may not have been incorporated in the gene chips used for the array and therefore was undetected. The findings from this microarray analysis add insight to the effects of hormonal induction and 25-HC on adipogenic differentiation and gene expression in C3H10T1/2 cells. The roles of most of the differentially expressed genes are not known, hence the importance of further analysis of these genes so as to determine their role in adipogenesis.

In conclusion, 25-HC was seen to inhibit adipogenesis in the two studies, demonstrating its potential for use in treatment of obesity and related disorders.

6.0 SUMMARY AND CONCLUSIONS

i. 25-HC significantly inhibited adipogenic differentiation in C3H10T1/2 cells by inhibiting lipid droplet formation and expression of the main adipogenic transcripts.

ii. 25-HC was able to significantly inhibit the expression of PPARγ, C/EBPα, LPL and FABP4 as early as 48hr after treatment.

iii. 20S, 22R, 22S did not significantly inhibit the expression of PPARγ in C3H10T1/2 cells.

iv. The anti-adipogenic effects of 25-HC on C3H10T1/2 cells were not mediated by hedgehog signalling.

v. The SREBF1/ADD1 pathway was shown to play a role in the anti-adipogenic effects of 25-HC.

vi. Microarray analysis of gene expression between non-treated cells and those treated with the adipogenic media, DMITro, showed a total of 709 differentially expressed genes whereby 465 genes were more highly expressed in non-treated cells and 244 genes were over-expressed in cells treated with DMITro.

vii. Microarray analysis between cells treated with DMITro and those treated with DMITro+25-HC showed a total of 2,204 differentially expressed genes. Of these, 276 genes were over-expressed in cells treated with DMITro and 1,928 genes were over-expressed in cells treated with DMITro+25-HC.

viii. Genes over-expressed in non-treated cells compared to cells treated with DMITro were found to be involved mainly in cell events such as cellular assembly and organization, cellular movement and cell-to-cell signalling and interaction.

ix. Genes over-expressed in cells treated with DMITro compared to non-treated cells were found to be involved mainly in lipid metabolism, molecular transport and small molecule biochemistry.

x. Genes over-expressed in DMITro compared to DMITro+25-HC treated cells were mainly found to be involved in lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism and cell-to-cell signalling and interaction.

xi. Genes over-expressed in cells treated with DMITro+25-HC compared to those treated with DMITro were found to be involved in cell cycle, cellular growth and differentiation, molecular transport and cell growth and proliferation.

xii. These differentially expressed genes can be further studied individually to determine their roles in adipogenesis.

7.0 FUTURE STUDIES

In the present study, we showed that 25-HC inhibits adipogenic differentiation in C3H10T1/2 cells, demonstrating that it could potentially be used in treatment of obesity and overweight. We also showed that the inhibitory effects of 25-HC on adipogenesis are mediated in part by the SREBF1/ADD1 pathway. However, further investigation on this topic is important, so as to enhance understanding of its anti-adipogenic properties. From what I have observed in this thesi project, this should include:

i. A further investigation of the mechanism of action of 25-HC on adipogenesis and whether more than one mechanisms is involved in mediating its effects.
ii. A further study of the individual genes that were differentially expressed in the microarray to determine their role in adipogenesis. A total of 1,928 genes were over-expressed in cells treated with DMITro+25-HC compared to those treated with DMITro. Although these genes were mostly associated with cell events such as cell cycle and molecular transport, their specific roles in relation to the anti-adipogenic effects of 25-HC are unknown.
iii. An in vivo study of the anti-adipogenic effects of 25-HC using mouse models would be a good way of further understanding the effects of 25-HC and other biochemical pathways that are affected.
iv. A ‘proof of concept’ experiment that will confirm 25-HC as a potential therapeutic for obesity. This would involve treatment of cells that already have fat droplets with 25-HC to confirm that the treatment reduces fat droplets and expression of adipocyte genes.

8.0 REFERENCES

Amemiya-Kudo, M., Shimano, H., Yoshikawa, T., Yahagi, N., Hasty, A. H., Okazaki, H., and Yamada, N. (2000). Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene. *The Journal of Biological Chemistry, 275*(40), 31078-31085

Bain, D. L., Heneghan, A. F., Connaghan-Jones, K. D., & Miura, M. T. (2007). Nuclear receptor structure: Implications for function. *Annual Review of Physiology, 69*, 201-220.

Banerjee, S. S., Feinberg, M. W., Watanabe, M., Gray, S., Haspel, R. L., Denkinger, D. J., and Jain, M. K. (2003). The kruppel-like factor KLF2 inhibits peroxisome proliferator-activated receptor-gamma expression and adipogenesis. *The Journal of Biological Chemistry, 278*(4), 2581-2584.

Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., and Evans, R. M. (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. *Molecular Cell, 4*(4), 585-595.

Bastie, C., Luquet, S., Holst, D., Jehl-Pietri, C., & Grimaldi, P. A. (2000). Alterations of peroxisome proliferator-activated receptor delta activity affect fatty acid-controlled adipose differentiation. *The Journal of Biological Chemistry, 275*(49)

Bianco, P., Riminucci, M., Gronthos, S., & Robey, P. G. (2001). Bone marrow stromal stem cells: Nature, biology, and potential applications. *Stem Cells (Dayton, Ohio), 19*(3)

Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H., & McKnight, S. L. (1989). Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes & Development, 3*(8), 1146-1156.

Birsoy, K., Chen, Z., & Friedman, J. (2008). Transcriptional regulation of adipogenesis by KLF4. *Cell Metabolism, 7*(4), 339-347.

Bishop-Bailey, D., & Wray, J. (2003). Peroxisome proliferator-activated receptors: A critical review on endogenous pathways for ligand generation. *Prostaglandins & Other Lipid Mediators, 71*(1-2), 1-22.

Bitgood, M. J., Shen, L., & McMahon, A. P. (1996). Sertoli cell signaling by desert hedgehog regulates the male germline. *Current Biology : CB, 6*(3), 298-304.

Bjorkhem, I., & Diczfalusy, U. (2002). Oxysterols: Friends, foes, or just fellow passengers? *Arteriosclerosis, Thrombosis, and Vascular Biology, 22*(5), 734-742.

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., & Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): Tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology, 137*(1), 354-366.

Brown, A. J., & Jessup, W. (1999). Oxysterols and atherosclerosis. *Atherosclerosis, 142*(1), 1-28.

Bruder, S. P., Jaiswal, N., & Haynesworth, S. E. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *Journal of Cellular Biochemistry, 64*(2), 278-294.

Burton, G. R., Guan, Y., Nagarajan, R., & McGehee, R. E.,Jr. (2002). Microarray analysis of gene expression during early adipocyte differentiation. *Gene, 293*(1-2), 21-31.

Cao, Z., Umek, R. M., & McKnight, S. L. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes & Development, 5*(9), 1538-1552.

Caplan, A. I. (1994). The mesengenic process. *Clinics in Plastic Surgery, 21*(3), 429-435.

Caplan, A. I., & Bruder, S. P. (2001). Mesenchymal stem cells: Building blocks for molecular medicine in the 21st century. *Trends in Molecular Medicine, 7*(6), 259-264.

Caprio, M., Feve, B., Claes, A., Viengchareun, S., Lombes, M., & Zennaro, M. C. (2007). Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 21*(9), 2185-2194.

Chamberlain, G., Fox, J., Ashton, B., & Middleton, J. (2007). Concise review: Mesenchymal stem cells: Their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells (Dayton, Ohio), 25*(11), 2739-2749.

Chan, G. K., & Duque, G. (2002). Age-related bone loss: Old bone, new facts. *Gerontology, 48*(2), 62-71.

Chandra, V., Huang, P., Hamuro, Y., Raghuram, S., Wang, Y., Burris, T. P., & Rastinejad, F. (2008). Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. *Nature, 456*(7220), 350-356.

Chen, D., Zhao, M., & Mundy, G. R. (2004). Bone morphogenetic proteins. *Growth Factors (Chur, Switzerland), 22*(4), 233-241.

Chen, J. K., Taipale, J., Cooper, M. K., & Beachy, P. A. (2002). Inhibition of hedgehog signaling by direct binding of cyclopamine to smoothened. *Genes & Development, 16*(21), 2743-2748.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., & Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. *Nature, 383*(6599), 407-413.

Choy, L., & Derynck, R. (2003). Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *The Journal of Biological Chemistry, 278*(11), 9609-9619.

Christy, R. J., Kaestner, K. H., Geiman, D. E., & Lane, M. D. (1991). CCAAT/enhancer binding protein gene promoter: Binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proceedings of the National Academy of Sciences of the United States of America, 88*(6), 2593-2597.

Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., & Lane, M. D. (1989). Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes & Development, 3*(9), 1323-1335.

Cohen, M. M.,Jr. (2003). The hedgehog signaling network. *American Journal of Medical Genetics.Part A, 123A*(1), 5-28.

Couillard, C., Mauriege, P., Imbeault, P., Prud'homme, D., Nadeau, A., Tremblay, A., . . . Despres, J. P. (2000). Hyperleptinemia is more closely associated with adipose cell

hypertrophy than with adipose tissue hyperplasia. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 24*(6), 782-788.

Cousin, W., Dani, C., & Peraldi, P. (2006). Inhibition of the anti-adipogenic hedgehog signaling pathway by cyclopamine does not trigger adipocyte differentiation. *Biochemical and Biophysical Research Communications, 349*(2), 799-803.

Curbing the obesity epidemic. (2006). *Lancet, 367*(9522), 1549.

Dani, C., Amri, E. Z., Bertrand, B., Enerback, S., Bjursell, G., Grimaldi, P., & Ailhaud, G. (1990). Expression and regulation of pOb24 and lipoprotein lipase genes during adipose conversion. *Journal of Cellular Biochemistry, 43*(2), 103-110.

Darlington, G. J., Ross, S. E., & MacDougald, O. A. (1998). The role of C/EBP genes in adipocyte differentiation. *The Journal of Biological Chemistry, 273*(46), 30057-30060.

Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell, 51*(6), 987-1000.

DeBose-Boyd, R. A., Ou, J., Goldstein, J. L., & Brown, M. S. (2001). Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proceedings of the National Academy of Sciences of the United States of America, 98*(4), 1477-1482.

Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocrine Reviews, 20*(5), 649-688.

Draghici, S., Khatri, P., Eklund, A. C., & Szallasi, Z. (2006). Reliability and reproducibility issues in DNA microarray measurements. *Trends in Genetics : TIG, 22*(2), 101-109.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., & Wahli, W. (1992). Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell, 68*(5), 879-887.

Dyer, M. A., Farrington, S. M., Mohn, D., Munday, J. R., & Baron, M. H. (2001). Indian hedgehog activates hematopoiesis and vasculogenesis and can respecify prospective neurectodermal cell fate in the mouse embryo. *Development (Cambridge, England), 128*(10), 1717-1730.

Edwards, P. A., Kast, H. R., & Anisfeld, A. M. (2002). BAREing it all: The adoption of LXR and FXR and their roles in lipid homeostasis. *Journal of Lipid Research, 43*(1), 2-12.

Edwards, P. A., Kennedy, M. A., & Mak, P. A. (2002). LXRs; oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. *Vascular Pharmacology, 38*(4), 249-256.

El-Jack, A. K., Hamm, J. K., Pilch, P. F., & Farmer, S. R. (1999). Reconstitution of insulin-sensitive glucose transport in fibroblasts requires expression of both PPARgamma and C/EBPalpha. *The Journal of Biological Chemistry, 274*(12), 7946-7951.

Fajas, L., Schoonjans, K., Gelman, L., Kim, J. B., Najib, J., Martin, G., and Auwerx, J. (1999). Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: Implications for adipocyte differentiation and metabolism. *Molecular and Cellular Biology, 19*(8), 5495-5503.

Farmer, S. R. (2006). Transcriptional control of adipocyte formation. *Cell Metabolism, 4*(4), 263-273.

Farmer, S. R. (2008). Molecular determinants of brown adipocyte formation and function. *Genes & Development, 22*(10), 1269-1275.

Fontaine, C., Cousin, W., Plaisant, M., Dani, C., & Peraldi, P. (2008). Hedgehog signaling alters adipocyte maturation of human mesenchymal stem cells. *Stem Cells (Dayton, Ohio), 26*(4), 1037-1046. doi:10.1634/stemcells.2007-0974

Fu, M., Sun, T., Bookout, A. L., Downes, M., Yu, R. T., Evans, R. M., & Mangelsdorf, D. J. (2005). A nuclear receptor atlas: 3T3-L1 adipogenesis. *Molecular Endocrinology (Baltimore, Md.), 19*(10), 2437-2450

Gesta, S., Tseng, Y. H., & Kahn, C. R. (2007). Developmental origin of fat: Tracking obesity to its source. *Cell, 131*(2), 242-256.

Green, H., & Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture. II. factors affecting the adipose conversion. *Cell, 5*(1), 19-27.

Green, H., & Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *Journal of Cellular Physiology, 101*(1), 169-171.

Green, H., & Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell, 3*(2), 127-133.

Gregoire, F. M., Smas, C. M., & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews, 78*(3), 783-809.

Guo, X., & Liao, K. (2000). Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation. *Gene, 251*(1), 45-53.

Gupta, R. K., Arany, Z., Seale, P., Mepani, R. J., Ye, L., Conroe, H. M., & Spiegelman, B. M. (2010). Transcriptional control of preadipocyte determination by Zfp423. *Nature, 464*(7288), 619-623.

Hanley, K., Ng, D. C., He, S. S., Lau, P., Min, K., Elias, P. M., & Feingold, K. R. (2000). Oxysterols induce differentiation in human keratinocytes and increase ap-1-dependent involucrin transcription. *The Journal of Investigative Dermatology, 114*(3), 545-553.

Hanlon, P. R., Cimafranca, M. A., Liu, X., Cho, Y. C., & Jefcoate, C. R. (2005). Microarray analysis of early adipogenesis in C3H10T1/2 cells: Cooperative inhibitory effects of growth factors and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology and Applied Pharmacology, 207*(1), 39-58.

Haslam, D. W., & James, W. P. (2005). Obesity. *Lancet, 366*(9492), 1197-1209.

Hayden, J. M., Brachova, L., Higgins, K., Obermiller, L., Sevanian, A., Khandrika, S., & Reaven, P. D. (2002). Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol. *Journal of Lipid Research, 43*(1), 26-35.

Hirsch, J., & Batchelor, B. (1976). Adipose tissue cellularity in human obesity. *Clinics in Endocrinology and Metabolism, 5*(2), 299-311.

Hooper, J. E., & Scott, M. P. (2005). Communicating with hedgehogs. *Nature Reviews.Molecular Cell Biology, 6*(4), 306-317.

Huang, Y., Das, A. K., Yang, Q. Y., Zhu, M. J., & Du, M. (2012). Zfp423 promotes adipogenic differentiation of bovine stromal vascular cells. *PloS One, 7*(10), e47496.

Huelsken, J., & Behrens, J. (2002). The wnt signalling pathway. *Journal of Cell Science, 115*(Pt 21), 3977-3978.

Ingham, P. W., & McMahon, A. P. (2001). Hedgehog signaling in animal development: Paradigms and principles. *Genes & Development, 15*(23), 3059-3087.

James, A. W. (2013). Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. *Scientifica, 2013*, 684736.

James, W. P. (2008). The epidemiology of obesity: The size of the problem. *Journal of Internal Medicine, 263*(4), 336-352.

Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R., & Mangelsdorf, D. J. (1996). An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature, 383*(6602), 728-731.

Joseph, S. B., Laffitte, B. A., Patel, P. H., Watson, M. A., Matsukuma, K. E., Walczak, R., & Tontonoz, P. (2002). Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *The Journal of Biological Chemistry, 277*(13).

Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *The Journal of Clinical Investigation, 106*(4), 473-481.

Kast-Woelbern, H. R., Dana, S. L., Cesario, R. M., Sun, L., de Grandpre, L. Y., Brooks, M. E., & Leibowitz, M. D. (2004). Rosiglitazone induction of insig-1 in white adipose tissue reveals a novel interplay of peroxisome proliferator-activated receptor gamma and sterol

regulatory element-binding protein in the regulation of adipogenesis. *The Journal of Biological Chemistry, 279*(23), 23908-23915.

Kersten, S. (2002). Peroxisome proliferator activated receptors and obesity. *European Journal of Pharmacology, 440*(2-3), 223-234.

Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *The Journal of Clinical Investigation, 103*(11), 1489-1498.

Kha, H. T., Basseri, B., Shouhed, D., Richardson, J., Tetradis, S., Hahn, T. J., & Parhami, F. (2004). Oxysterols regulate differentiation of mesenchymal stem cells: Pro-bone and anti-fat. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research, 19*(5), 830-840.

Khokher, M. A., Woods, R. J., & Dandona, P. (1984). Human immunoglobulin M stimulates adipocyte lipogenesis. *Metabolism: Clinical and Experimental, 33*(3), 208-211.

Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., & Spiegelman, B. M. (1998). Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *The Journal of Clinical Investigation, 101*(1), 1-9.

Kim, J. B., & Spiegelman, B. M. (1996). ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes & Development, 10*(9), 1096-1107.

Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C., & Spiegelman, B. M. (1995). Dual DNA binding specificity of ADD1/SREBP1 controlled

by a single amino acid in the basic helix-loop-helix domain. *Molecular and Cellular Biology, 15*(5), 2582-2588.

Kim, J. B., Wright, H. M., Wright, M., & Spiegelman, B. M. (1998). ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand. *Proceedings of the National Academy of Sciences of the United States of America, 95*(8), 4333-4337.

Kim, W., Kim, M., & Jho, E. H. (2013). Wnt/beta-catenin signalling: From plasma membrane to nucleus. *The Biochemical Journal, 450*(1), 9-21.

Kim, W. K., Meliton, V., Amantea, C. M., Hahn, T. J., & Parhami, F. (2007). 20(S)-hydroxycholesterol inhibits PPARgamma expression and adipogenic differentiation of bone marrow stromal cells through a hedgehog-dependent mechanism. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research, 22*(11), 1711-1719.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., & Evans, R. M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proceedings of the National Academy of Sciences of the United States of America, 91*(15), 7355-7359.

Konieczny, S. F., & Emerson, C. P.,Jr. (1984). 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: Evidence for regulatory genes controlling determination. *Cell, 38*(3), 791-800.

Kratchmarova, I., Kalume, D. E., Blagoev, B., Scherer, P. E., Podtelejnikov, A. V., Molina, H., & Pandey, A. (2002). A proteomic approach for identification of secreted proteins

during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Molecular & Cellular Proteomics : MCP, 1*(3), 213-222.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., & Kadowaki, T. (1999). PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Molecular Cell, 4*(4), 597-609.

Kwack, S. J., Kwon, O., Kim, H. S., Kim, S. S., Kim, S. H., Sohn, K. H., & Park, K. L. (2002). Comparative evaluation of alkylphenolic compounds on estrogenic activity in vitro and in vivo. *Journal of Toxicology and Environmental Health.Part A, 65*(5-6), 419-431.

Lecka-Czernik, B., Moerman, E. J., Grant, D. F., Lehmann, J. M., Manolagas, S. C., & Jilka, R. L. (2002). Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. *Endocrinology, 143*(6), 2376-2384.

Lefterova, M. I., Haakonsson, A. K., Lazar, M. A., & Mandrup, S. (2014). PPARgamma and the global map of adipogenesis and beyond. *Trends in Endocrinology and Metabolism: TEM, 25*(6), 293-302.

Lefterova, M. I., & Lazar, M. A. (2009). New developments in adipogenesis. *Trends in Endocrinology and Metabolism: TEM, 20*(3), 107-114.

Lehmann, J. M., Kliewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J. L., & Willson, T. M. (1997). Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *The Journal of Biological Chemistry, 272*(6), 3137-3140.

Lehrke, M., & Lazar, M. A. (2005). The many faces of PPARgamma. *Cell, 123*(6), 993-999.

Leibel, R. L., Rosenbaum, M., & Hirsch, J. (1995). Changes in energy expenditure resulting from altered body weight. *The New England Journal of Medicine, 332*(10), 621-628.

Levi, B., & Longaker, M. T. (2011). Concise review: Adipose-derived stromal cells for skeletal regenerative medicine. *Stem Cells (Dayton, Ohio), 29*(4), 576-582.

Lin, F. T., MacDougald, O. A., Diehl, A. M., & Lane, M. D. (1993). A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: Transcriptional activator lacking antimitotic activity. *Proceedings of the National Academy of Sciences of the United States of America, 90*(20), 9606-9610.

Linhart, H. G., Ishimura-Oka, K., DeMayo, F., Kibe, T., Repka, D., Poindexter, B., & .Darlington, G. J. (2001). C/EBPalpha is required for differentiation of white, but not brown, adipose tissue. *Proceedings of the National Academy of Sciences of the United States of America, 98*(22), 12532-12537.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., & Brown, E. L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology, 14*(13), 1675-1680.

MacDougald, O. A., & Lane, M. D. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Annual Review of Biochemistry, 64*, 345-373.

Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O., & Pittenger, M. F. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Engineering, 4*(4), 415-428.

Massague, J., Seoane, J., & Wotton, D. (2005). Smad transcription factors. *Genes & Development, 19*(23), 2783-2810.

McMahon, A. P., Ingham, P. W., & Tabin, C. J. (2003). Developmental roles and clinical significance of hedgehog signaling. *Current Topics in Developmental Biology, 53*, 1-114.

Miyawaki, J., Kamei, S., Sakayama, K., Yamamoto, H., & Masuno, H. (2008). 4-tert-octylphenol regulates the differentiation of C3H10T1/2 cells into osteoblast and adipocyte lineages. *Toxicological Sciences : An Official Journal of the Society of Toxicology, 102*(1), 82-88.

Nakamura, T., Shiojima, S., Hirai, Y., Iwama, T., Tsuruzoe, N., Hirasawa, A., & Tsujimoto, G. (2003). Temporal gene expression changes during adipogenesis in human mesenchymal stem cells. *Biochemical and Biophysical Research Communications, 303*(1), 306-312.

Nawrocki, A. R., Rajala, M. W., Tomas, E., Pajvani, U. B., Saha, A. K., Trumbauer, M. E., & Scherer, P. E. (2006). Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *The Journal of Biological Chemistry, 281*(5), 2654-2660.

Nemali, M. R., Usuda, N., Reddy, M. K., Oyasu, K., Hashimoto, T., Osumi, T., & Reddy, J. K. (1988). Comparison of constitutive and inducible levels of expression of peroxisomal beta-oxidation and catalase genes in liver and extrahepatic tissues of rat. *Cancer Research, 48*(18), 5316-5324.

Nieuwenhuis, E., & Hui, C. C. (2005). Hedgehog signaling and congenital malformations. *Clinical Genetics, 67*(3), 193-208.

Nusslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in drosophila. *Nature, 287*(5785), 795-801.

Nuttall, M. E., & Gimble, J. M. (2000). Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis? *Bone, 27*(2), 177-184.

Obesity: Preventing and managing the global epidemic. report of a WHO consultation. (2000). *World Health Organization Technical Report Series, 894*, i-xii, 1-253.

Oishi, Y., Manabe, I., Tobe, K., Tsushima, K., Shindo, T., Fujiu, K., & Nagai, R. (2005). Kruppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation. *Cell Metabolism, 1*(1), 27-39

Okuno, A., Tamemoto, H., Tobe, K., Ueki, K., Mori, Y., Iwamoto, K., & Kadowaki, T. (1998). Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese zucker rats. *The Journal of Clinical Investigation, 101*(6), 1354-1361.

Oliveira, F. S., Bellesini, L. S., Defino, H. L., da Silva Herrero, C. F., Beloti, M. M., & Rosa, A. L. (2012). Hedgehog signaling and osteoblast gene expression are regulated by purmorphamine in human mesenchymal stem cells. *Journal of Cellular Biochemistry, 113*(1), 204-208.

Olkkonen, V. M., Beaslas, O., & Nissila, E. (2012). Oxysterols and their cellular effectors. *Biomolecules, 2*(1), 76-103.

Pantoja, C., Huff, J. T., & Yamamoto, K. R. (2008). Glucocorticoid signaling defines a novel commitment state during adipogenesis in vitro. *Molecular Biology of the Cell, 19*(10), 4032-4041.

Parhami, F., Jackson, S. M., Tintut, Y., Le, V., Balucan, J. P., Territo, M., & Demer, L. L. (1999). Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research, 14*(12), 2067-2078.

Park, E. A., Gurney, A. L., Nizielski, S. E., Hakimi, P., Cao, Z., Moorman, A., & Hanson, R. W. (1993). Relative roles of CCAAT/enhancer-binding protein beta and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *The Journal of Biological Chemistry, 268*(1), 613-619.

Pathi, S., Pagan-Westphal, S., Baker, D. P., Garber, E. A., Rayhorn, P., Bumcrot, D., & Williams, K. P. (2001). Comparative biological responses to human sonic, indian, and desert hedgehog. *Mechanisms of Development, 106*(1-2), 107-117.

Peet, D. J., Janowski, B. A., & Mangelsdorf, D. J. (1998). The LXRs: A new class of oxysterol receptors. *Current Opinion in Genetics & Development, 8*(5), 571-575.

Pei, H., Yao, Y., Yang, Y., Liao, K., & Wu, J. R. (2011). Kruppel-like factor KLF9 regulates PPARgamma transactivation at the middle stage of adipogenesis. *Cell Death and Differentiation, 18*(2), 315-327.

Pinney, D. F., & Emerson, C. P.,Jr. (1989). 10T1/2 cells: An in vitro model for molecular genetic analysis of mesodermal determination and differentiation. *Environmental Health Perspectives, 80*, 221-227.

Plaisant, M., Fontaine, C., Cousin, W., Rochet, N., Dani, C., & Peraldi, P. (2009). Activation of hedgehog signaling inhibits osteoblast differentiation of human mesenchymal stem cells. *Stem Cells (Dayton, Ohio), 27*(3), 703-713.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science (New York, N.Y.), 276*(5309), 71-74.

Rahimi, N., Tremblay, E., McAdam, L., Roberts, A., & Elliott, B. (1998). Autocrine secretion of TGF-beta 1 and TGF-beta 2 by pre-adipocytes and adipocytes: A potent negative regulator of adipocyte differentiation and proliferation of mammary carcinoma cells. *In Vitro Cellular & Developmental Biology.Animal, 34*(5), 412-420.

Rangwala, S. M., & Lazar, M. A. (2000). Transcriptional control of adipogenesis. *Annual Review of Nutrition, 20*, 535-559.

Rawadi, G., Vayssiere, B., Dunn, F., Baron, R., & Roman-Roman, S. (2003). BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a wnt autocrine loop. *Journal of Bone and Mineral Research : The Official Journal of the American Society for Bone and Mineral Research, 18*(10), 1842-1853.

Ren, D., Collingwood, T. N., Rebar, E. J., Wolffe, A. P., & Camp, H. S. (2002). PPARgamma knockdown by engineered transcription factors: Exogenous PPARgamma2 but not PPARgamma1 reactivates adipogenesis. *Genes & Development, 16*(1), 27-32.

Reusch, J. E., Colton, L. A., & Klemm, D. J. (2000). CREB activation induces adipogenesis in 3T3-L1 cells. *Molecular and Cellular Biology, 20*(3), 1008-1020.

Reznikoff, C. A., Brankow, D. W., & Heidelberger, C. (1973). Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Research, 33*(12), 3231-3238.

Rodriguez Fernandez, J. L., & Ben-Ze'ev, A. (1989). Regulation of fibronectin, integrin and cytoskeleton expression in differentiating adipocytes: Inhibition by extracellular matrix and polylysine. *Differentiation; Research in Biological Diversity, 42*(2), 65-74.

Rosen, E. D., Hsu, C. H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., & Spiegelman, B. M. (2002). C/EBPalpha induces adipogenesis through PPARgamma: A unified pathway. *Genes & Development, 16*(1), 22-26.

Rosen, E. D., & MacDougald, O. A. (2006). Adipocyte differentiation from the inside out. *Nature Reviews.Molecular Cell Biology, 7*(12), 885-896.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., & Mortensen, R. M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Molecular Cell, 4*(4), 611-617.

Rosen, E. D., & Spiegelman, B. M. (2006). Adipocytes as regulators of energy balance and glucose homeostasis. *Nature, 444*(7121), 847-853.

Rosen, E. D., Walkey, C. J., Puigserver, P., & Spiegelman, B. M. (2000). Transcriptional regulation of adipogenesis. *Genes & Development, 14*(11), 1293-1307.

Ross, S. E., Erickson, R. L., Gerin, I., DeRose, P. M., Bajnok, L., Longo, K. A., & MacDougald, O. A. (2002). Microarray analyses during adipogenesis: Understanding the effects of wnt signaling on adipogenesis and the roles of liver X receptor alpha in adipocyte metabolism. *Molecular and Cellular Biology, 22*(16), 5989-5999.

Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., & MacDougald, O. A. (2000). Inhibition of adipogenesis by wnt signaling. *Science (New York, N.Y.), 289*(5481), 950-953.

Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L., & Therond, P. P. (2003). Stability and association of smoothened, Costal2 and fused with cubitus interruptus are regulated by hedgehog. *Nature Cell Biology, 5*(10), 907-913.

Russell, D. W. (2000). Oxysterol biosynthetic enzymes. *Biochimica Et Biophysica Acta, 1529*(1-3), 126-135.

Samulin, J., Berget, I., Lien, S., & Sundvold, H. (2008). Differential gene expression of fatty acid binding proteins during porcine adipogenesis. *Comparative Biochemistry and Physiology.Part B, Biochemistry & Molecular Biology, 151*(2), 147-152.

Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., & Kahn, B. B. (1993). Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *The Journal of Biological Chemistry, 268*(30), 22243-22246.

Shimoike, T., Yanase, T., Umeda, F., Ichino, I., Takayanagi, R., & Nawata, H. (1998). Subcutaneous or visceral adipose tissue expression of the PPARgamma gene is not altered in the fatty (fa/fa) zucker rat. *Metabolism: Clinical and Experimental, 47*(12), 1494-1498.

Siersbaek, R., Nielsen, R., & Mandrup, S. (2010). PPARgamma in adipocyte differentiation and metabolism--novel insights from genome-wide studies. *FEBS Letters, 584*(15), 3242-3249.

Sinha, S., & Chen, J. K. (2006). Purmorphamine activates the hedgehog pathway by targeting smoothened. *Nature Chemical Biology, 2*(1), 29-30.

Spinella-Jaegle, S., Rawadi, G., Kawai, S., Gallea, S., Faucheu, C., Mollat, P., & Roman-Roman, S. (2001). Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *Journal of Cell Science, 114*(Pt 11), 2085-2094.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., & Lazar, M. A. (2001). The hormone resistin links obesity to diabetes. *Nature, 409*(6818), 307-312.

Student, A. K., Hsu, R. Y., & Lane, M. D. (1980). Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *The Journal of Biological Chemistry, 255*(10), 4745-4750.

Suga, K., Saitoh, M., Fukushima, S., Takahashi, K., Nara, H., Yasuda, S., & Miyata, K. (2001). Interleukin-11 induces osteoblast differentiation and acts synergistically with bone morphogenetic protein-2 in C3H10T1/2 cells. *Journal of Interferon & Cytokine Research : The Official Journal of the International Society for Interferon and Cytokine Research, 21*(9), 695-707.

Suh, J. M., Gao, X., McKay, J., McKay, R., Salo, Z., & Graff, J. M. (2006). Hedgehog signaling plays a conserved role in inhibiting fat formation. *Cell Metabolism, 3*(1), 25-34.

Summers, S. A., Yin, V. P., Whiteman, E. L., Garza, L. A., Cho, H., Tuttle, R. L., & Birnbaum, M. J. (1999). Signaling pathways mediating insulin-stimulated glucose transport. *Annals of the New York Academy of Sciences, 892*, 169-186.

Tanaka, T., Yoshida, N., Kishimoto, T., & Akira, S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *The EMBO Journal, 16*(24), 7432-7443.

Tang, Q. Q., Otto, T. C., & Lane, M. D. (2003). CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 100*(3), 850-855.

Tang, Q. Q., Otto, T. C., & Lane, M. D. (2003). Mitotic clonal expansion: A synchronous process required for adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 100*(1), 44-49. doi

Tang, Q. Q., Otto, T. C., & Lane, M. D. (2004). Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proceedings of the National Academy of Sciences of the United States of America, 101*(26), 9607-9611.

Tong, Q., Dalgin, G., Xu, H., Ting, C. N., Leiden, J. M., & Hotamisligil, G. S. (2000). Function of GATA transcription factors in preadipocyte-adipocyte transition. *Science (New York, N.Y.), 290*(5489), 134-138.

Tong, Q., & Hotamisligil, G. S. (2001). Molecular mechanisms of adipocyte differentiation. *Reviews in Endocrine & Metabolic Disorders, 2*(4), 349-355.

Tong, Q., Tsai, J., Tan, G., Dalgin, G., & Hotamisligil, G. S. (2005). Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation. *Molecular and Cellular Biology, 25*(2), 706-715.

Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., & Spiegelman, B. M. (1994). mPPAR gamma 2: Tissue-specific regulator of an adipocyte enhancer. *Genes & Development, 8*(10), 1224-1234.

Tontonoz, P., Hu, E., & Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell, 79*(7), 1147-1156.

Tontonoz, P., Kim, J. B., Graves, R. A., & Spiegelman, B. M. (1993). ADD1: A novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Molecular and Cellular Biology, 13*(8), 4753-4759.

Tontonoz, P., & Spiegelman, B. M. (2008). Fat and beyond: The diverse biology of PPARgamma. *Annual Review of Biochemistry, 77*, 289-312.

Valenti, M. T., Garbin, U., Pasini, A., Zanatta, M., Stranieri, C., Manfro, S., & Dalle Carbonare, L. (2011). Role of ox-PAPCs in the differentiation of mesenchymal stem cells (MSCs) and Runx2 and PPARgamma2 expression in MSCs-like of osteoporotic patients. *PloS One, 6*(6), e20363.

Van Reyk, D. M., Brown, A. J., Hult'en, L. M., Dean, R. T., & Jessup, W. (2006). Oxysterols in biological systems: Sources, metabolism and pathophysiological relevance. *Redox Report : Communications in Free Radical Research, 11*(6), 255-262.

Varjosalo, M., & Taipale, J. (2008). Hedgehog: Functions and mechanisms. *Genes & Development, 22*(18), 2454-2472.

Vidal-Puig, A., Jimenez-Linan, M., Lowell, B. B., Hamann, A., Hu, E., Spiegelman, B., & Moller, D. E. (1996). Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *The Journal of Clinical Investigation, 97*(11), 2553-2561.

Vidal-Puig, A. J., Considine, R. V., Jimenez-Linan, M., Werman, A., Pories, W. J., Caro, J. F., & Flier, J. S. (1997). Peroxisome proliferator-activated receptor gene expression in human tissues. effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *The Journal of Clinical Investigation, 99*(10), 2416-2422.

Volpe, J. J., & Vagelos, P. R. (1976). Mechanisms and regulation of biosynthesis of saturated fatty acids. *Physiological Reviews, 56*(2), 339-417.

Von Bubnoff, A., & Cho, K. W. (2001). Intracellular BMP signaling regulation in vertebrates: Pathway or network? *Developmental Biology, 239*(1), 1-14.

Vu, D., Ong, J. M., Clemens, T. L., & Kern, P. A. (1996). 1,25-dihydroxyvitamin D induces lipoprotein lipase expression in 3T3-L1 cells in association with adipocyte differentiation. *Endocrinology, 137*(5), 1540-1544.

Wahli, W. (2002). Peroxisome proliferator-activated receptors (PPARs): From metabolic control to epidermal wound healing. *Swiss Medical Weekly, 132*(7-8), 83-91.

Waki, H., Nakamura, M., Yamauchi, T., Wakabayashi, K., Yu, J., Hirose-Yotsuya, L., & Kadowaki, T. (2011). Global mapping of cell type-specific open chromatin by FAIRE-seq reveals the regulatory role of the NFI family in adipocyte differentiation. *PLoS Genetics, 7*(10), e1002311.

Wang, E. A., Israel, D. I., Kelly, S., & Luxenberg, D. P. (1993). Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors (Chur, Switzerland), 9*(1), 57-71.

Weintraub, H. (1993). The MyoD family and myogenesis: Redundancy, networks, and thresholds. *Cell, 75*(7), 1241-1244.

White, U. A., & Stephens, J. M. (2010). Transcriptional factors that promote formation of white adipose tissue. *Molecular and Cellular Endocrinology, 318*(1-2), 10-14.

Willson, T. M., Lambert, M. H., & Kliewer, S. A. (2001). Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annual Review of Biochemistry, 70*, 341-367.

World Health Organization (WHO) Media Centre (2013): obesity and overweight, Geneva, Switzerland.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., & Wang, E. A. (1988). Novel regulators of bone formation: Molecular clones and activities. *Science (New York, N.Y.), 242*(4885), 1528-1534.

Wu, X., Walker, J., Zhang, J., Ding, S., & Schultz, P. G. (2004). Purmorphamine induces osteogenesis by activation of the hedgehog signaling pathway. *Chemistry & Biology, 11*(9), 1229-1238.

Wu, Z., Bucher, N. L., & Farmer, S. R. (1996). Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Molecular and Cellular Biology, 16*(8), 4128-4136.

Wu, Z., Rosen, E. D., Brun, R., Hauser, S., Adelmant, G., Troy, A. E., & Spiegelman, B. M. (1999). Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Molecular Cell, 3*(2), 151-158.

Wu, Z., Xie, Y., Bucher, N. L., & Farmer, S. R. (1995). Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes & Development, 9*(19), 2350-2363.

Xing, H., Northrop, J. P., Grove, J. R., Kilpatrick, K. E., Su, J. L., & Ringold, G. M. (1997). TNF alpha-mediated inhibition and reversal of adipocyte differentiation is accompanied by suppressed expression of PPARgamma without effects on pref-1 expression. *Endocrinology, 138*(7), 2776-2783.

Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., & Kadowaki, T. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine, 7*(8), 941-946.

Yang, X., Lu, X., Lombes, M., Rha, G. B., Chi, Y. I., Guerin, T. M., & Liu, J. (2010). The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metabolism, 11*(3), 194-205.

Yeh, W. C., Cao, Z., Classon, M., & McKnight, S. L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes & Development, 9*(2), 168-181.

Yin, C., Xiao, Y., Zhang, W., Xu, E., Liu, W., Yi, X., & Chang, M. (2014). DNA microarray analysis of genes differentially expressed in adipocyte differentiation. *Journal of Biosciences, 39*(3), 415-423.

Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., & Brown, M. S. (1993). SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell, 75*(1), 187-197.

Zandbergen, F., Mandard, S., Escher, P., Tan, N. S., Patsouris, D., Jatkoe, T., & Kersten, S. (2005). The G0/G1 switch gene 2 is a novel PPAR target gene. *The Biochemical Journal, 392*(Pt 2), 313-324.

Zha, W., Edin, M. L., Vendrov, K. C., Schuck, R. N., Lih, F. B., Jat, J. L., & Lee, C. R. (2014). Functional characterization of cytochrome P450-derived epoxyeicosatrienoic acids in adipogenesis and obesity. *Journal of Lipid Research, 55*(10), 2124-2136.

Zhang, X., Yang, M., Lin, L., Chen, P., Ma, K. T., Zhou, C. Y., & Ao, Y. F. (2006). Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose--derived stem cells in vitro and in vivo. *Calcified Tissue International, 79*(3), 169-178.

Zhao, L., Li, G., Chan, K. M., Wang, Y., & Tang, P. F. (2009). Comparison of multipotent differentiation potentials of murine primary bone marrow stromal cells and mesenchymal stem cell line C3H10T1/2. *Calcified Tissue International, 84*(1), 56-64.

Zhu, Y., Qi, C., Korenberg, J. R., Chen, X. N., Noya, D., Rao, M. S., & Reddy, J. K. (1995). Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPAR gamma) gene: Alternative promoter use and different splicing yield two mPPAR gamma isoforms. *Proceedings of the National Academy of Sciences of the United States of America, 92*(17), 7921-7925.

Zur Nieden, N. I., Kempka, G., Rancourt, D. E., & Ahr, H. J. (2005). Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: Effect of cofactors on differentiating lineages. *BMC Developmental Biology, 5*, 1.