LIPIDOMICS OF MESENCHYMAL STEM CELLS UNDERGOING ADIPOGENESIS

CHEN HUIMIN
(B. Sc. (Hons.), NUS)

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Summary

Obesity is recognized as a top ten global health problem by the World Health Organisation (WHO). Dietary habits are one of the main contributing factors to obesity. As recently proven, recruitment of progenitors from the bone marrow also contributes to obesity. Thus, obesity is now considered to occur via mechanisms of hypertrophy and hyperplasia. In this *in vitro* study, we characterize lipidome changes during adipogenesis of mesenchymal stem cells (MSC) using thin layer chromatography and sensitive mass spectrometry.

The lipid profiles of MSC undergoing adipogenesis revealed that in spite of the expected increase in triacylglycerols (TAG), there is also a surprising decrease in phospholipids during adipogenesis. This decrease appears to be counterintuitive at first. During adipogenic differentiation, the cells hypertrophy (grow in size). Thus, one expects to see increased phospholipids, so as to form the larger plasma membrane required to envelope the cellular contents. However, this in turn implies that lipids perform only structural functions. Hence, our data also support a more dynamic role of lipids during cellular function.

The gene expression levels of lipins 1, 2 and 3 and phospholipases (PLA_1A, PLA_2 G4a, PLA_2 G6 and PLB) demonstrated that these proteins may be responsible for the observed decrease in phospholipids. The progressive increase in TAG and the corresponding decrease in phospholipids coupled with the upregulation of lipin 1 suggest that there is a shift in the phospholipids and TAG biosynthetic pathway that favours the synthesis of TAG. In addition, the upregulation of PLA_2 G4a and PLA_2

G6 demonstrates that the decrease in phospholipids may be due to increased hydroxylation by these enzymes.

Despite the general decrease in phospholipids, phosphatidylglycerol (PG) is the unique class of phospholipids that exhibited an overall increase. The increase in PG may indicate an increase in mitochondria, which is exemplified through the transient increase in voltage-dependent anion channel (VDAC) protein as adipogenesis progresses. In addition, there are some species of phospholipids that increased overtime. Similarly, TAG species that display progressive increase encompass similar characteristics to phospholipids types that increase overtime. Most of them are made up of monounsaturated fatty acids (MUFA). This finding suggests that there is preferential incorporation of MUFA to TAG and phospholipids and that this process is occurring via the *de novo* pathway.

In summary, lipid profiling of MSC undergoing adipogenesis presents the unique lipid fingerprints of cells at distinct differentiative stages. In-depth analysis of the abundant information acquired reveals that lipids are more than just structural and storage entities; they also play a more dynamic role in cellular functions. As a result, this yields interesting and novel observations, thus enables one to venture into unchartered boundaries of the adipogenic process.

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List of Abbreviations and acronyms

°C: Degree Celsius

15dPGJ2: 15 deoxy-$\Delta^{12,14}$-prostaglandin J_2

18s: 18S ribosomal RNA

AA: Arachidonic acid

AD: Average Deviation

ADD1: Adipocyte and Differentiation Dependent factor 1

Adipo: Adipocytes

aP2: Fatty acid binding protein

BMI: Body Mass Index

bZIP: Basic Leucine Zipper

C/EBPα: CAAT/enhancer binding protein α

C:M:W: Chloroform:Methanol:Water

CDP-DAG: Cytidine Diphosphate-DAG

CE: Collision Energy

CFU: Colony Forming Unit

CHOP-10: C/EBP homologous protein-10

CID: Collision Induced Dissociation

CKI: Cylin-dependent kinase inhibitors

cm: centimeter

CMP: Cytidine Monophosphate

COW: Correlation Optimised Warping

CREB: cAMP Response Element Binding protein

CYP4A: Microsomal ω-hydroxylase

DAG: Diacylglycerols

Dex: Dexamethasone

DGAT: acyl Co-A:DAG acyltransferase

DHA: Docosahexaenoic Acid

DMEM: Dulbecco's Modified Eagle's Medium

DMPG: 1,2-Dimyristoyl-*sn*-Glyero-3-Phosphocholine

DNA: Deoxyribonucleic acid

DP: Declustering Potentials

DPBS: Dulbecco's Phosphate Buffered Saline

EDTA: Ethylene Diaminotetraacetic Acid

ELISA: Enzyme Linked Immunosorbent Assay

EPA: Eicosapentaenoic Acid

ER: Endoplasmic Reticulum

ER: Estrogen Receptor

ESC: Embryonic Stem Cells

ESI-MS: Electrospray-Ionisation Mass Spectrometry

eV: electron volts

FA: Fatty Acid

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FSC: Forward Scatter

G3P: Glycerol-3-Phosphate

GAPDH: Glyceraldehyde Phosphate Dehydrogenase

GC-MS: Gas Chromatography Mass Spectrometry

GDP: Gross Domestic Product

GFP: Green Fluorescent Protein

GLUT4: Insulin responsive Glucose Transporter 4

GPCR: G Protein-Coupled Receptor

GVHD: Graft Versus Host Disease

HC: Hydroxycholesterol

HEFA: Hexane:Diethyl ether:Formic Acid

HMBS: Hydroxymethyl Bilane Synthase

HPRT: Hypoxanthine Guanine Phosphoribosyl Transferase I

HSC: Hematopoietic Stem Cells

IBMX: Isobutylmethylxanthine

IFN-γ: Interferon-γ

IGF: Insulin Growth Factor

Indo: Indomethacine

IP: Inositol Polyphosphates

kV: kilo volts

LD: Lipid Droplets

LPA: Lyso-phosphatidic acid

LPC: Lyso-phosphatidylcholine

LPE: Lyso-phosphatidylethanolamine

LPI: Lyso-phosphatidylinositol

LPL: Liporotein lipase

LPP: Lipid Phosphate Phosphatase

LPS: Lysophosphatidylserine

m/z: mass to charge ratio

M: molar concentration

M1: Marker 1

MAG: Monoacylglycerols

MDT mix: mixture of Monoacylglycerol, Diacylglycerol and Triacylglycerol

MGAT: acyl Co-A:MAG acyltransferase

MHC: Major Histocompatibility Complex

min: minutes

ml: mililitres

MM: Maintenance Media

mM: milimolar concentration

mm: millimeter

MRM: Multiple Reaction Monitoring

MS/MS: Tandem Mass Spectrometry

MS: Mass Spectrometry

MSC: human Mesenchymal Stem Cells

MUFA: Monounsaturated Fatty Acids

nm: nanometer

PA: Phosphotidic Acid

PAF: Platelet Activating Factor

PAP: Phosphatidic acid phosphatase

PC: Phosphatidylcholine

PCR: Polymerase Chain Reaction

PE: Phosphatidylethanolamine

PEPCK: Phosphoenol-pyruvate carboxylase

PG: Phosphatidylglycerol

PGC-1α: PPARγ coactivator-1α

PGF2α: Prostaglandin 2 α

PGP: PG-Phosphoric acid

PI: Phosphotidylinositol

PIP: Phosphoinositides

PLA1: Phospholipase A1

PLA_1A: Phosphatidylserine-specific phospholipase A_1

PLA2 G4: Phospholipase A2 Group 4

PLB: Phospholipase B

PLC: Phospholipase C

PLD: Phospholipase D

PLD1: Phospholipase D1

POPC: 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine

POPE: 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine

PPARG1: Peroxisome proliferator-activated receptor γ 1

PPARG2: Peroxisome proliferator-activated receptor γ 2

PREIS: Precursor Ion Scanning

PS: L-a-Phosphatidylserine

PS: Phosphatidylserine

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

Rpm: Revolutions per minute

RXR: Retinoid X Receptor

s: seconds

SC: Stem Cells

SCD1: Stearoyl-CoA desaturase 1

SDS: Sodium Dodecyl Sulphate

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SIM: Selected Ion Monitoring

siRNA: Small interfering RNA

SREBP: Sterol Regulatory Element Binding Protein

SSC: Side Scatter

SUCCDH: Succinate Dehydrogenase

TAE: Tris-Acetate-EDTA

TAG mix: Triacylglycerol mixture

TBST: Tris-Buffered Saline Tween 20

TGF-β3: Transforming Growth Factor-β3

TLC: Thin Layer Chromatography

TLR: Toll-like Receptor

TNF-α: Tumour Necrosis Factor-α

TZDs: Thiazolidinediones

UD: Undifferentiated MSC

v/v: volume per volume

V: volts

VDAC: Voltage-dependent Anion Channel

WHO: World Health Organisation

WT: Wild Type

ZFP: Zinc Finger Repressor Proteins

µg: microgram

µl: microlitres

INTRODUCTION

1 Introduction

1.1 Mesenchymal stem cells (MSC)

1.1.1 Definition of stem cells

Stem cells (SC) are defined functionally as cells that have the capacity to self-renew and give rise to differentiated progeny (Weissman *et al.*, 2001; Smith, 2001). Their fate choice is highly regulated by both intrinsic signals and the external microenvironment (Odorcico *et al.*, 2001).

1.1.2 Criteria of being stem cells

Essentially, stem cells need to satisfy three criteria. Firstly, they possess the ability to self renew, which is defined as having the capability to replicate in an/a unlimited or prolonged fashion, thereby maintaining the stem cell pool. There are two schools of thought for stem cells regeneration (Watt & Hogan, 2000). One, known as invariant asymmetric division, involves a stem cell undergoing asymmetric cell division to give rise to one daughter stem cell and one daughter cell that differentiates into a specific lineage (Figure 1-1A). The other theory (populational asymmetric division) describes how a stem cell undergoes cell division to form daughter cells with different fates, such as becoming daughter stem cells or daughter progenitor cells with different differentiation abilities depending on the factors they are exposed to (Figure 1-1B).

Secondly, stem cells have a certain degree of potency within them where they undergo lineage commitment and differentiate into one or more differentiated cell

types of distinct morphology and gene expression pattern. Mesenchymal stem cells (MSC) are multipotent as they are able to differentiate into more than one differentiated cell type. However, unlike the pluripotent embryonic stem cells (ESC), MSC acquire tissue specific, restricted differentiation abilities. The differentiation process begins with the cell entering a transient state of rapid proliferation. After exhausting its proliferative potential, the cell exits the proliferative cycle and enters the terminal differentiation programme (Potten *et al.*, 1979).

Lastly, stem cells have the ability to repopulate a given tissue *in vivo*. In order to do this, homing to a given tissue, via interplay of chemokines and cytokines, is necessary. Upon reaching the tissue of interest, they will respond to specific cues and differentiate into cell types of that tissue. Consequently, the differentiated cells will take on the function of that tissue. For instance, transplantation of a single murine hematopoietic stem cell (HSC) into lethally irradiated animals leads to complete reconstitution of all hematopoietic cell types. Consistent with its stem cell nature, this hematopoietic reconstitution capability is maintained with serial transplantation (Ogawa *et al.*, 1996).

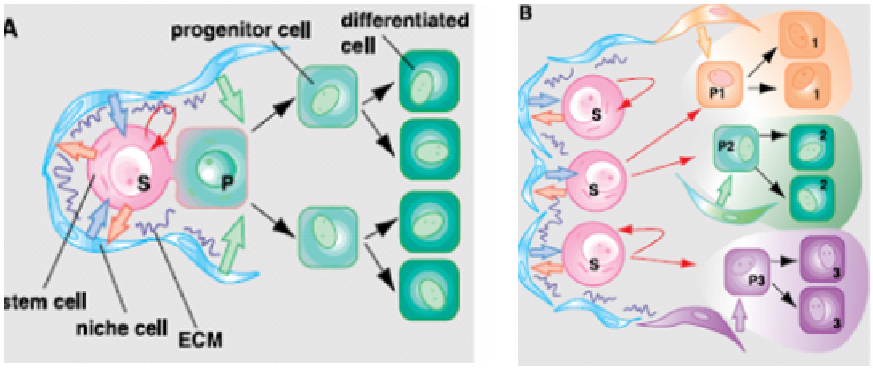


Figure 1-1: Different theories of stem cell division.

A) Invariant asymmetric division. B) Populational asymmetric division. (Adapted from Watt & Hogan, 2000)

1.1.3 Isolation of MSC

MSC were first identified by Fridenshtein in 1966 and subsequent works illustrate the ability of MSC to form fibroblast-like colonies that could give rise to adipocytes and osteoblasts *in vitro* (Fridenshtein 1982; Fridenshtein *et al.*, 1970; Fridenshtein *et al.*, 1966). Cells with MSC-liked properties have been isolated from multiple tissues such as the periosteum (Fukumoto *et al.,* 2003; O'Driscoll *et al.*, 2001; Nakahara *et al.*, 1990; Zarnett & Salter, 1989), trabecular bone (Tuli *et al.*, 2003; Noth *et al.*, 2002; Sottile *et al.*, 2002),), synovium (De Bari *et al.*, 2001), skeletal muscle (Jankowski *et al*., 2002), deciduous teeth (Miura *et al.*, 2003) and lungs (Noort *et al.*, 2002). Availability of these MSC-liked cells in a variety of adult tissues raises the question on the niche of MSC, their migration abilities and differentiation stimuli (Barry & Murphy, 2004). Nevertheless, isolation of MSC from bone marrow aspirates (Oswald *et al.*, 2004; Pittenger *et al.*, 1999) and adipose tissue (De ugarte *et al.*, 2003; Dragoo *et al.*, 2003; Wickham *et al.*, 2003; Gronthos *et al.*, 2001; Zuk *et al.*, 2001) have been the most well-studied. As tissue specimen from these areas are easily available and the techniques of isolating MSC from these tissues and *in vitro* expansion and maintenance of these cells have been well-established.

The mononuclear cell fraction from either bone marrow aspirates or adipose tissue is isolated via density gradient centrifugation and plated. Non-adherent cells are removed during the subsequent passaging process. Colony forming unit assay (CFU) (Pittenger *et al.*, 1999) coupled with flow cytometric analysis based on defined antigenic determinants (Gronthos *et al.*, 2003) are performed to obtain a more homogenous population of MSC. Unlike the well-characterised HSC where there exist surface markers that can isolate HSC specifically (Wolf *et al.*, 1993; Sutherland

et al., 1989; Spangrude *et al.*, 1988), the list of antigenic MSC markers used is not as well-defined as their neighbours HSC (Pittenger & Martin, 2004; Devine, 2002). Thus, determining the tripotentiality nature (adipogenic, osteogenic and chondrogenic potential)) of MSC is an additional measure to ensure that the isolated cells are indeed MSC (Dominici *et al.*, 2006).

1.1.4 MSC functions and their potential

Adipogenic differentiation is induced by employing a combination of insulin, iso-butyl-methylxanthine (IBMX), dexamethasone (Dex) and a peroxisome proliferator-activated receptor γ (PPARγ) agonist (Pittenger *et al.*, 1999). After 7 days of adipogenic induction, lipid droplets (LD) accumulate within the cells which can be stained with lipophilic dyes consistent with the adoption of adipocyte phenotype (Ramirez-Zacarias *et al.*, 1992).

Osteogenic differentiation of MSC is performed by treating the cells with Dex, L-ascorbic acid and β-glycerophosphate (Pittenger *et al.*, 1999). Two to three weeks later, aggregates or nodules of calcium deposition are observed through Alizarin red and Von Kossa staining. Alkaline phosphatase activity also increased 4-10 folds (Jaisval *et al.*, 1997) and specific osteogenic gene markers, such as osteocalcin and osteopontin are expressed.

In chrondrogenesis, cells are centrifuged to form a "pelleted micromass" which is cultured in serum free media supplemented with transforming growth factor-β3 (TGF-β3) (Mackay *et al.*, 1998). The cell pellet develops to possess a multilayered matrix-rich morphology, whereby the extracellular domain is rich in proteoglycans

and collagen types II and IV (Muraglia *et al*., 2000). Alcian blue staining can be used to confirm the presence of proteoglycans in the cell pellets.

Besides the aforementioned three lineages, MSC also have the ability to differentiate into cardiomyocytes, skeletal myocytes and smooth muscle cells (Pittenger *et al.*, 1999; Wakitani *et al.*, 1995). In addition, MSC display some forms of plasticity (the ability of adult stem cells to acquire mature phenotypes that are different from their tissue of origin) (Grove *et al.*, 2004). Examples include MSC giving rise to cells of a neuronal phenotype, resembling astrocytes, glial cells and neuronal cells (Woodbury *et al.*, 2000; Kopen *et al.*, 1999) and MSC's ability to transdifferentiate into cell types of different embryonic dermal origin (Tocci & Forte, 2003). However, functionality of these neuronal cell types and transdifferentiated cells remains to be proven.

Apart from the multipotency of MSC, MSC also secrete an array of bioactive molecules that can have profound effects on the local microenvironment. For instance, MSC secrete cytokines that assist in the proliferation and differentiation of HSC (Azizi *et al.*, 1998; Majumdar *et al.*, 1998). In addition to the trophic effects of MSC, the presence of adhesion molecules on the surface of MSC also provide stromal support to HSC both in the *in vivo* and *in vitro* systems (Mourcin *et al.*, 2005; Kim *et al*, 2004; Maitra *et al.*, 2004; Angelopoulou *et al.*, 2003; Pittenger *et al.*, 1999). As a result, MSC can be used to promote allogenic HSC engraftment. Intravenous administration of peripheral blood progenitor cells together with MSC in a group of breast cancer patients (undergoing high dose of chemotherapy) yield rapid hematopoietic recovery as compared to the control groups (Koc *et al.*, 2000).

The trophic effects of MSC coupled with its mulitpotency display the effectiveness of MSC as a therapeutic tool for the restoration of damaged or diseased tissue (i.e. mesodermal defect repair and disease management). For instance, Young and colleagues illustrate the effectiveness of rabbit MSC in regenerating severed tendon in rabbit models (Young *et al.*, 1998). Besides this, there are reports exhibiting the promise of MSC in bringing about functional improvement of cardiac function in baboon myocardial infarction model (Tomo *et al.*, 2002; Wang *et al.*, 2000). Stamm *et al.* demonstrate that delivery of bone marrow cells into infarct zone of patients result in dramatic improvement in heart function (Stamm *et al.*, 2003). Literatures display that administration of MSC lead to specific migration to site of injury and brought about enhanced cardiac function and regeneration of bone (Shake *et al.*, 2002; Orlic *et al.*, 2001; Jackson *et al.*, 2001).

Besides this, MSC elicit immunosuppressive effects. MSC lack major histocompatibility class (MHC) II, CD40, CD40 ligand, CD80 and CD86 (Kumar *et al.*, 2008; Deans & Moseley, 2000; Tse *et al.*, 2000). Despite the expression of MHC class II when MSC are treated with interferon-γ (IFN-γ), T cells remained inactivated due to the lack of co-stimulatory molecules, such as CD80, CD86, CD40 and CD40 ligand. Consequently, anergic T cells prevail (Romieu-Mourez *et al.*, 2007; Le Blanc *et al.*, 2003). Furthermore, papers have established the abilities of MSC to disrupt the function and maturation of dendritic cells and B cells (Corcione *et al.*, 2006; Nauta *et al.*, 2006; Zhang *et al.*, 2004). Hence, MSC can be used to help reduce the incidence and severity of Graft-versus-host disease (GVHD). For example, HSC transplantation in murine models together with varying doses of MSC prevents GVHD and increases survival rate in mice (Sotiropoulou *et al.*, 2006; Chung *et al.*,

2004). Patients undergoing allogenic bone marrow transplantation along with MSC experience lower incidence of GVHD (Aggarwal & Pittenger, 2005).

1.2 Adipogenesis

1.2.1 Definition and relevance

Adipogenesis is the recruitment of precursor cells and under appropriate cues differentiate to mature fat cells (i.e. adipocytes) (Hausman *et al.*, 2001; Rosen & Spiegelman, 2000). Preadipocytes are operationally defined as cells isolated from the stromovascular fraction of fat depots that possess the ability to progress towards an adipocytic cell fate when adipogenic stimulus is provided. Adipocytes store energy in the form of triacylglycerols (TAG) and cholesterol esters that are contained inside lipid droplets composed of a neutral core enveloped by a protein coated single phospholipid layer (Martin & Parton, 2005). The ability of adipose tissue to store excess energy has been strongly selected during evolution, thus they play a vital role in energy homeostasis. Diseases such as obesity and non-insulin dependent diabetes mellitus (Type 2 diabetes) are of increasing interest due to their increasing prevalence globally (Zimmet *et al.*, 2001). With the explosion of information on the metabolic disorders linked to obesity, there is added sense of urgency to recognize the key nodal points of energy balance. Thus, understanding adipose cell development and physiology is of utmost importance.

1.2.2 Obesity and associated diseases

Obesity is a condition characterized by an abnormal or excessive accumulation of fat in the body, especially in the adipose tissue, to a magnitude that results in adverse health consequences (Spiegelman & Flier, 2001; World Health Organisation (WHO), 1995). At the moment, the gold standard for determining obesity is via body mass index (BMI), which is defined as the weight in kilograms divided by the square of

the height in metres (kg/m^2). An individual is obese when the BMI is 30 (kg/m^2) and higher. However, Asians have higher proportion of body fat as compared to Caucasians of the same age, gender and BMI (Wang *et al.*, 1994). Hence, the cut off is lowered to 25 for Asians. In Singapore, BMI is used to assess the predisposition to obesity related diseases. Individuals with BMI between 23 and 27.4 pose moderate risk, while those with 27.5 and higher are at a higher risk (Health Promotion Board, 2005).

According to the WHO, obesity has been viewed as a worldwide epidemic (WHO, 2008). Contrary to conventional belief, obesity is affecting not only the developed and affluent societies, but also emerging countries too (Monteiro *et al.*, 2007; Popkin, 2002; Wu *et al.*, 2002). The prevalence of obesity adopts a rising trend. In 1995, an estimated 200 million adults are classified as obese. By 2000, this number increased to 300 million. In 2005, WHO reports that there are at least 400 million obese adults globally and project this value to exceed 700 million by 2015 (WHO, 2008, 2003).

Obese individual have been shown to be more susceptible to diseases such as cardiovascular diseases, hypertension, stroke and certain forms of cancer. The Framingham Heart study demonstrates that with every 1 increment of the BMI, there is an increased risk of heart failure of 5% for men and 7% for women (Kenchaiah *et al.*, 2002), thus implying that the increased risk of heart failure is associated with the increase in BMI. By elevating BMI from 25 to 30 and beyond, the relative risk for hypertension increases from 1.48 to 2.23 for men and 1.70 to 2.63 for women (Wilson *et al.*, 2002). According to the North Manhattan study, subjects with greater

abdominal obesity, measured by the waist to hip ratio, experience enhanced risk in ischemic stroke and their respective odds ratio increases from 1.0 to 3.3 (Suk *et al.*, 2003). In the United States (US), an estimated 14-20% of cancer deaths are attributed to obesity (Calle *et al.*, 2003). With the emerging endocrine role of adipose tissue, adipokines and other secretory products exert profound effects on normal metabolic homeostasis (Garg, 2006), leading to the elucidation of metabolic disorders. This includes dyslipidemia, insulin resistance and Type 2 diabetes, which are collectively termed as "metabolic syndrome X", "insulin resistance syndrome" or "Reaven syndrome" (Petrie *et al.*, 1998; Reaven, 1995; Reaven, 1993). Besides the detrimental health consequences of obesity, there are also economic costs imposed on societies (Runge, 2007; Yach *et al.*, 2006). In the US, obesity accounts for 1.2 % of the gross domestic product (GDP) (US Department of Human health and services, 2001).

Increasing sedentary lifestyle and rapidly changing dietary habits, in favour of fat, caloric sweeteners and animal source food, result in major energy imbalance. The excess energy is stored as TAG in adipose tissue resulting in adipocyte hypertrophy. Hyperplasia of adipocyte is also an etiology of obesity especially in extreme form of obesity in humans and rodents (Hirsch *et al.*, 1989). It is in these morbidly obese patients that prognosis is the poorest (Bjorntorp *et al.*, 1982). Some animal studies suggest that adipocyte hyperplasia occurs later than hypertrophy and may lead to more severe and irreversible metabolic consequences (Bjorntorp *et al.*, 1974). Hyperplasia, also referred to as adipogenesis, results in the recruitment and differentiation of preadipocytes into mature adipocytes (Hausman *et al.*, 2001). *In vitro* studies have suggested that mature adipocyte secrete factors, such as tumor

necrosis factor –α (TNF- α) and insulin-growth factor (IGF) that promote hyperplasia in a paracrine manner (Avram *et al.*, 2007). Recent study has demonstrated that progenitors from the bone marrow are contributing to hyperplasia of adipocytes using GFP-labeled marrow cells (Crossno *et al.*, 2006).

1.2.3 Model for adipocytes differentiation and their relevance today

Our understanding of adipogenesis comes mainly from research conducted on the 3T3-L1 cell line, a fibroblast line derive from swiss albino mouse embryo cells (Green & Meuth, 1974). These preadipocytes differentiate into mature adipocytes under adipogenic stimuli (Student *et al.*, 1980). Although vast amounts of information regarding adipogenesis are elucidated using this cell line, 3T3-L1 has its shortcomings. Since 3T3-L1 is already committed to the adipocytic lineage, the understanding of how progenitors commit to developing into adipose tissue cannot be studied in these cells. Due to the murine origin of 3T3-L1, there may be discrepancies in adipose development between murine and human model, as suggested by literatures (Ailhaud & Hauner, 1997; Entenmann & Hauner, 1996). For instance, the need for mitotic clonal expansion prior to terminal adipogenesis is considerably controversial. It has been reported that mitotic clonal division is essential for the differentiation of 3T3-L1 to adipocytes (Tang *et al.*, 2003). Furthermore, there are several reports that reiterate the notion that mitotic clonal expansion takes precedence to differentiation (Tang *et al.*, 2003; Reichert & Eick, 1999; Yeh *et al.*, 1995). Janderova *et al.* suggested that clonal expansion is not important for terminal adipogenesis to occur in humans (Janderova *et al.*, 2003). Besides this, expression of Sterol Regulatory Element Binding Protein (SREBP) types differs between mouse and human. Murine 3T3-L1 cells express mostly

SREBP-1a, but in humans it is the ADD/SREBP-1c that is more involved in adipogenesis (Shimomura *et al.*, 1997). Although SREBPs, unlike PPARs, are not master regulators of adipogenesis, different expression of SREBP types in different species could skew the understanding of adipogenesis in humans.

Primary multipotent human cells, such as the human MSC (hMSC), can be an ideal model to learn about adipogenesis (Janderova *et al.*, 2003; Nakamura *et al.*, 2003). There are evidences demonstrating the ability of MSC differentiating to adipocytes (Baksh *et al.*, 2003; Deans & Moseley, 2000; Pittenger *et al.*, 1999) and contributing to hyperplasia of adipose tissue (Otto & Lane, 2005). Furthermore, the multipotency of MSC imply that these cells are prior to commitment to adipogenesis, thus can be used as a model for the discovery of early genes/factors that are necessary for commitment to adipogenesis, which remains elusive at the moment.

1.2.4 Events involved in adipogenesis

1.2.4.1 General overview of adipocyte development programme

Much of our understanding on adipogenesis is based on 3T3-L1. Although using a human model, such as hMSC, may be more appropriate, the ability of MSC to differentiate down the adipogenic lineage is demonstrated by Pittenger *et al.* in 1999. Due to its recent introduction, insufficient knowledge on their complex biological system and difficulty in isolating homogenous population of MSC, MSC is not extensively used to study adipogenesis. Thus, subsequent description on adipogenesis revolves round 3T3-L1. In an *in vitro* system, adipogenesis is initiated through the exposure of confluent 3T3-L1 cultures to adipogenic cocktail containing

isobutylmethylxanthine (IBMX) (a cAMP elevating agent), dexamethasone (Dex) (a glucocorticoid hormone) and insulin (Rosen *et al.*, 2000; Lane *et al.*, 1999; Darlington *et al.*, 1998). There are four major events governing adipocyte differentiation – commitment, growth arrest, mitotic clonal expansion, terminal differentiation.

Commitment is the process by which stem cells from the vascular stroma respond to signals to undergo determination to the adipocytic lineage. It has been proposed that factors secreted by mature adipocytes signal the recruitment of cells to undergo adipogenesis (Marques *et al.*, 1998; Considine *et al.*, 1996; Lau *et al.*, 1990). Wnt signaling regulates bone mass through its ability to promote osteogenesis and inhibit adipogenesis (Bennett *et al.*, 2005). In addition, Wnt-10b is highly expressed in preadipocytes and is decreased upon differentiation (Ross *et al.*, 2000). This implies that Wnt signaling may be involved in the early phase of adipogenesis (Ross *et al.*, 2000). Nevertheless, there is little information on the commitment process of adipogenesis and adipocyte-specific commitment factors remain to be discovered.

Growth arrest occurs twice throughout the adipocyte development process and is brought about by contact inhibition (Fajas, 2003). Once before mitotic clonal expansion, while the other occurs prior to terminal differentiation (Scott *et al.*, 1982). Literature has illustrated that there is significant increase in cyclin-dependent kinase inhibitors (CKI), p21 and p27, during the first mitotic arrest. Similarly, p18, a type of CKI, is elevated greatly at the second growth arrest (Morrison & Farmer, 1999). The same report documents the role of PPARγ in regulating the expression of CKI, thus

implying the relationship between mitotic arrest and differentiation (Morrison & Farmer, 1999).

Upon receiving appropriate combination of mitogenic and adipogenic signals, the cells synchronously undergo multiple rounds of DNA replication and cell doubling (i.e. mitotic clonal expansion). It is believed that during DNA replication, the changes made to chromatin structure allow for easy access of transcription factors to regions of their binding sites. This in turns enable the upregulation of 834 genes and downregulation of 877 genes necessary for adipogenesis, thus resulting in the adipogenic phenotype (Lefterova *et al.*, 2008; MacDougald & Lane, 1995). Although several reports reiterate the notion that mitotic clonal expansion takes precedence to differentiation (Tang *et al.*, 2003; Reichert & Eick, 1999; Yeh *et al.*, 1995), there are some that illustrate the non-essentiality of clonal expansion (Liu *et al.*, 2002; Qiu *et al.*, 2001; Entenmann & Hauner, 1996). Such anomaly may be the result of cells being initiated for differentiation at a phase beyond mitotic division (Fajas, 2003; Gregoire *et al.*, 1998).

Following clonal expansion, cells undergo a second growth arrest, termed G_D (Scott *et al.*, 1982). This marks the point of no return where cells are committed and determined to undergo adipogenesis (Otto & Lane, 2005).

1.2.4.2 Transcriptional control

Adipocyte differentiation involves tightly regulated gene expression events. In order to combat diseases that are related to adipogenesis (e.g. obesity), understanding the underlying transcriptional control is of utmost importance. The predominant players

are the peroxisome proliferator-activated receptors (PPAR), followed by the CCAAT enhancer binding proteins (C/EBP), then the sterol regulatory element binding proteins (SREBP). Other transcriptional factors will not be discussed.

Peroxisome Proliferator-Activated Receptors (PPAR)

Peroxisome Proliferator-Activated Receptors (PPARs) belong to the superfamily of the steroid/thyroid nuclear hormone receptor (Mangelsdorf *et al.*, 1995). PPARs form heterodimers with Retinoid X Receptor (RXR) (Tontonoz *et al.*, 1994) and in turn bind to a response element that regulates transcriptional activities pertaining to lipid metabolism, anti-inflammatory response, atherosclerosis development and progression (Michalik & Wahi, 1999). Presently, three PPAR family members have been identified: PPARα, PPARβ (also known as PPARδ) and PPARγ (Schoonjans *et al.*, 1996; Dreyer *et al.*, 1992).

PPARα is mostly expressed in brown adipose tissue, liver, kidney, duodenum, heart and skeletal muscle (Braissant *et al.*, 1996). It is responsible for fatty acid catabolism through regulating the production of acyl-coenzyme A oxidase, carnitine palmitoyl transferase and microsomal ω-hydroxylase (CYP4A6) (Kroetz *et al.*, 1998; Mascaro *et al.*, 1998).

Relatively little is known about PPARβ/δ despite its ubiquitous expression in almost all tissues, except adipose tissue, and at a higher amount than PPARα and PPARγ (Braissant *et al.*, 1996). Nevertheless, stimulated PPARβ is involved in embryo implantation, myelination, lipid metabolism and adiposity (Barak *et al.*, 2002; Peters *et al.*, 2000).

PPARγ is predominantly found in adipose tissue, but is also expressed in monocytes, macrophages, smooth muscle cells and endothelium (Wang *et al.*, 2002). There are four mRNA isoforms (PPARγ1, 2, 3 and 4) created by alternative promoter usage and alternative splicing at the 5' end of the gene. However, only PPARγ1 and 2 can be expressed as proteins (Fajas *et al.*, 1997). PPARγ1 is expressed at low levels in many cell types including adipocytes (Shockley *et al.*, 2007; Fajas *et al.*, 1997), while PPARγ2 is highly and exclusively expressed in adipose tissue (Tontonoz *et al.*, 1994; Braissant *et al.*, 1996). The additional 30 residues in PPARγ2 may have assisted in the transcription activation function, thus increasing the expression of adipogenic genes by 5 to 10 folds (Werman *et al.*, 1997; Zhu *et al.*, 1995).

Through gain and loss-of-function experiments, reports have illustrated the importance of PPARγ2 in adipogenesis. For instance, when PPARγ is expressed in non-adipogenic, fibroblastic cells or myoblastic cells co-expressing C/EBPα, high-affinity selective PPARγ agonists, such as thiazolidinediones (TZDs) are able to result in strong adipogenic response in these cells (Hu *et al.*, 1995; Sandouk *et al.*, 1993; Kletzien *et al.*, 1992). In addition, through the use of zinc finger repressor proteins (ZFPs), such as ZFP54, PPARγ knockdowns are generated. Re-expression of PPARγ2, but not PPARγ1, reactivates adipogenesis in these knockdown cells (Ren *et al.*, 2002). Other than genetic studies, the use of pharmacological inhibitors also complemented the above described results (Gurnell *et al.*, 2000; Wright *et al.*, 2000).

CCAAT Enhancer Binding Protein (C/EBP)

CCAAT enhancer binding proteins (C/EBP) belong to the basic leucine zipper (bZIP) family of transcription factors. They contain a highly conserved domain at the C-terminus which is responsible for the dimerisation of proteins and binding to DNA. They act as either homo- or hetero-dimers with other family members (Lekstrom-Himes & Xanthopoulos, 1998). Their distribution is not only limited to the adipose tissue (Lekstrom-Himes & Xanthopoulos, 1998), but also to tissues that metabolize lipid and cholesterol-related compounds, such as the liver (Gregoire *et al.*, 1998). There are a total of six members, namely C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε and C/EBPζ (Ron & Habener, 1992; Cao *et al.*, 1991; Williams *et al.*, 1991; Akira *et al.*, 1990; Change *et al.*, 1990; Descombes *et al.*, 1990; Poli *et al.*, 1990; Roman *et al.*, 1990). They all share substantial sequence homology in the C-terminal 55-65 amino acid residues, which contain the bZIP domain (Hurst, 1995). Cellular differentiation, control of metabolism, inflammation and cellular proliferation are some of C/EBP functions. Adipose tissue expresses C/EBPα, C/EBPβ, C/EBPδ and C/EBPζ.

C/EBPα comprises of three isoforms of sizes 30, 40 and 42kDa (Lin *et al.*, 1993). These are generated due to the presence of multiple in-frame AUG start sites. The 42kDa protein is the most potent inducer of adipogenesis and mitotic blocker. Ectopic expression of C/EBPα and C/EBPβ in 3T3-L1 cells results in adipogenesis in the absence of adipogenic hormones (Freytag *et al.*, 1994; Lin *et al.*, 1994). On the other hand, expression of antisense C/EBPα RNA in 3T3-L1 cells inhibits adipogenesis (Lin & Lane, 1992). C/EBPα-deficient mice display dramatically

reduced adipose tissue levels (Wang *et al.*, 1995). These evidences address the ability of C/EBPα to engage in adipogenesis.

Similarly, C/EBPβ also consists of three isoforms generated from alternative translation via multiple in-frame AUG start sites (Lin *et al.*, 1993). Ectopic expression of C/EBPβ in 3T3-L1 preadipocytes is sufficient to bring about adipogenesis in the absence of hormone inducers (Yeh *et al.,* 1995). When similar experiment is done in NIH 3T3 fibroblasts, adipogenesis also prevails, however, in the presence of adipogenic cocktail (Wu *et al.*, 1995).

On the other hand, no adipogenesis results when C/EBPδ is overexpressed in 3T3 L1 and NIH 3T3 fibroblasts in the absence and presence of hormonal inducers respectively (Wu *et al.*, 1995; Yeh *et al.*, 1995). Literatures point towards C/EBPβ playing a larger and more important role in adipogenesis than C/EBPδ. Nonetheless, in the presence of adipogenic inducers, overexpression of C/EBPδ in 3T3 L1 expedites adipogenesis (Frevtag *et al.*, 1994; Lin & Lane, 1994). This is due to C/EBPβ preferentially forming heterodimers with C/EBPδ to result in greater transcriptional activity, despite the ability of C/EBPβ to homodimerise (Lane *et al.*, 1999; Cao *et al.*, 1991; Christy *et al.*, 1991). When both C/EBPβ and C/EBPδ are deficient in embryonic fibroblasts, adipogenesis fails to initiate in the presence of hormonal stimulus (Tanaka *et al.*, 1997). This implies the importance of both transcription factors for adipogenesis.

C/EBPζ, also known as C/EBP homologous protein-10 (CHOP-10), possesses sequence similarity with the other C/EBPs in the DNA binding and dimerisation

domain. However, its basic region is different from that with other C/EBPs. It does not form homodimers; rather it avidly forms heterodimers with other C/EBPs and it lacks the ability to bind to classical C/EBP-binding DNA elements (Ron & Habener, 1992). It is absent under normal conditions and only synthesized when the cells are under cellular stress (e.g. glucose deprivation of cells). Ectopic expression of C/EBPζ in 3T3 L1 cells inhibits adipogenesis by interfering with C/EBPα and C/EBPβ expression and function (Tang *et al.*, 2000; Batchvarova *et al.*, 1995). C/EBPζ deficient mice display greater adiposity than the control mice (Ariyama *et al.*, 2007). Thus, this implies the negative role C/EBPζ plays in regulating adipogenesis.

Sterol Regulatory Element Binding Protein (SREBP)

This group of proteins belongs to the basic helix-loop-helix-leucine zipper transcription factor family that regulates the transcription of genes essential to cholesterol and fatty acid metabolism (Horton *et al.*, 2002). The identified members are SREBP-1a, SREBP-1c and SREBP-2. Adipocyte and differentiation-dependent factor 1 (ADD1), found in mice, is homologous to SREBP-1c found in humans (Tontonoz *et al.*, 1993). SREBP-1a and SREBP-1c are derived from the alternative splicing of the same gene, while SREBP-2 is transcribed from a different gene (Hua *et al.*, 1995). SREBPs are expressed as membrane-bound precursor protein in the endoplasmic reticulum (ER). Upon proteolytic cleavage, various SREBPs are released and subsequently translocate into the nucleus to bind to sterol response element and bring about the expression of target genes (Horton *et al.*, 2002). SREBP-1a is a strong activator of all SREBPs. SREBP-1c expresses genes related to the fatty acid metabolism and TAG synthesis via binding to E-box motif (CANNTG) instead of binding to the sterol response element (Kim & Spiegelman, 1996; Kim *et al.*,

1995). SREBP-2 enhances cholesterol synthesis. More emphasis will be placed on SREBP-1c due to its homology to ADD1 and its importance in adipogenic differentiation.

Overexpression of SREBP-1c induces adipogenesis in NIH 3T3 fibroblasts in the presence of PPARγ activators (Kim & Spiegelman, 1996). Despite this, SREBP-1c knockout mice exhibit normal adipose depot (Shimano *et al.*, 1997). The authors speculate that this may be due to the compensatory effects of SREBP-2, though present at low amounts. Formulation of knockout mice that lack both SREBP-1c and SREBP-2 can be useful for the study of this phenomenon. Nevertheless, evidences imply the importance of SREBP-1c during adipogenesis, especially during the initial phase of differentiation.

1.2.4.3 Adipogenic transcriptional cascade

An overview of the adipogenic transcriptional cascade based on findings using 3T3 L1 is presented in Figure 1-2. To reiterate, the adipogenic cocktail contains insulin, IBMX and Dex. IBMX (a cAMP elevating agent) and insulin activate cAMP response element binding protein (CREB) (Klemm *et al.*, 1998). In turn, phosphorylated CREB activates C/EBPβ (Zhang *et al.*, 2004a; Niehof *et al.*, 1997). Early in differentiation, C/EBPβ expression in preadipocytes increases transiently. By late differentiation, its expression level decreases by 50% (Gregoire *et al.*, 1998). Since mitotic clonal expansion is necessary during the early phase of adipogenesis and C/EBPβ is endogenously expressed during the same period of time, there is likelihood that C/EBPβ plays a role in mitotic expansion. There is evidence that C/EBPβ (-/-) mouse embryonic fibroblasts cannot undergo mitosis (Tang *et al.*,

2003), thus implying the function of C/EBPβ in mitotic division and promoting proliferation. Phosphorylation of C/EBPβ activates its DNA binding function, which is quintessential in mitotic clonal expansion (Tang *et al.*, 2005). However, this mechanism is still not well understood.

Although C/EBPβ has the ability to homodimerise, heterodimerisation with C/EBPδ results in greater transcriptional activity (Lane *et al.*, 1999; Cao *et al.*, 1991; Christy *et al.*, 1991). C/EBPδ is expressed in preadipocytes. Similar to C/EBPβ, its level increases transiently in early differentiation. However, by late differentiation, its level drops to almost undetectable range (Gregoire *et al.*, 1998). Hence, like C/EBPβ, C/EBPδ may also be responsible for the clonal expansion prior to terminal differentiation. Since glucocorticoid has been shown to increase the expression of C/EBPδ (Cao *et al.*, 1991), Dex, a synthetic glucocorticoid that is used during the adipogenic process, is responsible for the increase in C/EBPδ.

Endogenous expression of C/EBPβ and C/EBPδ precedes PPARγ and the ectopic expression of C/EBPβ and C/EBPδ in NIH 3T3 fibroblasts leads to expression of PPARγ (Wu *et al.*, 1996). Heterodimer C/EBPβ -C/EBPδ in turn bring about the expression of PPARγ. The resultant PPARγ heterodimerises with RXR and is the predominant factor that promote adipogenesis through the expression of hundreds of genes responsible for the elucidation of the adipocyte phenotype (Farmer 2005; Rosen *et al.*, 2000). In addition, the complex helps to induce the expression of C/EBPα.

C/EBPα is anti-mitotic (Umek *et al.*, 1991) and its expression does not increase until the end of clonal expansion (Lekstrom-Himes & Xanthopoulos, 1998; Hendricks & Darlington, 1995). The expression of C/EBPα occurs prior to the expression of most adipocyte-specific genes (e.g. fatty acid binding protein (aP2), stearoyl-CoA desaturase-1 (SCD1), insulin-responsive glucose transporter (GLUT4), phosphoenol-pyruvate carboxykinase (PEPCK), leptin and insulin receptors). Findings have shown that expression of C/EBPα is low in preadipocytes (Cao *et al.*, 1991) and MSC (unpublished data), but high in terminally differentiated cells (Antonson & Xanthopoulos, 1995). Therefore, this suggests the involvement of C/EBPα in the termination of mitotic clonal expansion and its role as an initiator of terminal differentiation. In 3T3-L1 preadipocytes, the transcription factor, Sp1, represses the C/EBPα promoter. When cAMP levels rise (due to the presence of IBMX), Sp1 expression is transiently down-regulated and allows C/EBPα activating transcription factors (i.e. C/EBPβ and C/EBPδ) to transactivate C/EBPα (Tang *et al.*, 1999). Subsequently, the synergistic actions of PPARγ and C/EBPα result in adipogenic gene expression. It is likely that binding sites for C/EBP proteins and PPAR-RXR complex exist upstream of adipogenic genes. Recently, Lefterova and colleagues demonstrate the colocalisation of C/EBPα at more than 90% of PPARγ-binding sites using chromatic immunoprecipitation and that the absence of both transcription factors leads to decrease of common target genes (Lefterova *et al.*, 2008). Hence, this further substantiates the notion that PPARγ and C/EBPα act in a concerted manner to bring about adipogenesis.

In order to maintain the adipogenic process, C/EBPα has the ability to autoregulate its activation in a species-specific manner, through interaction with a site present in

its proximal promoter region (Timchenk *et al.*, 1995). Besides this, the presence of a positive feedback loop between PPARγ and C/EBPα mutually reinforces the expression of PPARγ and C/EBPα, thereby sustaining the adipogenic phenotype.

In some of the genetic studies, expression level of C/EBPα and PPARγ remain normal despite deficiency in C/EBPβ and C/EBPδ in mice (Tanaka *et al.*, 1997). This implies that there are other factors regulating the expression of these transcription factors, such as SREBP-1c. SREBP-1c increases during the first twenty-four hours of adipogenic induction (Kim & Spiegelman, 1996). Due to the ability of SREBP-1c to bind to the E-box motif present in the PPARγ promoter, transcriptional activation of PPARγ results (Fajas *et al.*, 1999). The activated PPARγ in turn induce the expression of C/EBPα and elucidate the expression of adipogenic genes, thus the adipogenic phenotype.

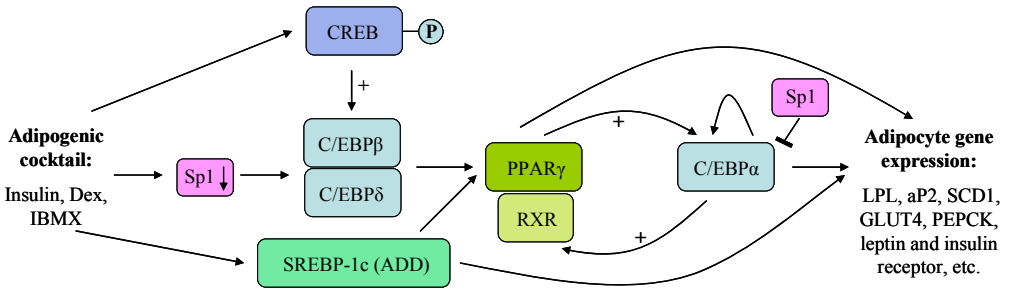


Figure 1-2: Adipogenic transcriptional cascade.

1.3 Lipids

1.3.1 Definitions

Lipids are "hydrophobic or amphiphatic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters and/or carbocation-based condensation of isoprene units" (Fahy *et al.*, 2005).

1.3.2 Lipid classifications

Lipids can be categorized into groups annotated by their chemically functional backbone. Glycerolipids and glycerophospholipids, herein referred to as phospholipids, will be described in greater detail.

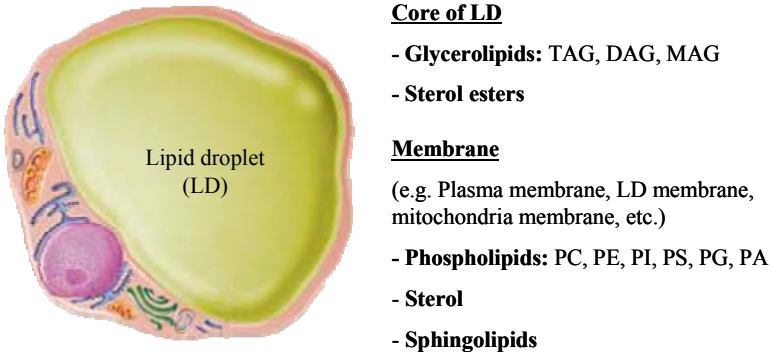


Figure 1-3: Composition of lipids in an adipocyte.

TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols
PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PG: phosphatidylglycerol; PA: phosphatidic acid
(Modified from http://i239.photobucket.com/albums/ff20/michaelwong75/fatcell.jpg)

Different parts of a cell are composed of different classes of lipids (Figure 1-3). Lipid droplet consists of a core of neutral lipids, such as triacylglycerols (TAG) and sterol esters, surrounded by a monolayer of phospholipids and associated proteins (Martin

& Parton, 2005). Constituents of lipid membranes are made up of mainly the phospholipids, sterols, such as cholesterol, and sphingolipids.

Phosphatidylcholine (PC)

More than 50% of total phospholipids in the eukaryotic membrane belong to the class of phosphatidylcholine (PC) (Van meer *et al.*, 2008; Kent, 2005). Due to the presence of one *cis*-unsaturated fatty acyl chain at either the *sn*-1 or *sn*-2 positions, it allows PC to be liquid at room temperature. With the asymmetrical distribution of phospholipids in the plasma membrane, PC occupies a higher proportion on the outer leaflet on the lipid bilayer. In addition, it tends to exist in the diacyl form, with a small group of them in the alkylacyl and alkenylacyl forms. These alkylacyl and alkenylacyl forms will be explained in greater detail in the subsequent section of ***Ether lipids and plasmalogens***. PC is generally known for its structural function. Besides this, it also acts as a component of pulmonary surfactant and is involved in signal transduction (McDermott *et al.*, 2004; Exton, 1994).

Phosphatidylethanolamine (PE)

This is the second most abundant phospholipids, after PC. It constitutes 20-50% of total phospholipids in the eukaryotic system (Vance, 2008). A large proportion resides in the brain (Vance, 2008). Like the PC, it also exists in diacyl, alkyacyl and alkenylacyl forms. Similar to all other phospholipids, it also performs structural functions. It is the presence of both PC and PE that provides curvature stress on the membrane, which assists fission, fusion and budding (Marsh, 2007; Dowhan & Bogdanov, 2002). Furthermore, PE also participates in the disassembly of the contractile ring during cytokinesis of mammalian cells (Emoto *et al.*, 1997) and is

involved in the hepatic lipoprotein secretion (Agren *et al.*, 2005; Hamilton & Fielding, 1989).

Phosphatidylserine (PS)

Phosphatidylserine comprises about 10-20% of all phospholipids in the plasma membrane and the endoplasmic reticulum. PS is located entirely in the inner leaflet of the plasma membrane of cells (Vance, 2008) and is a precursor to the biosynthesis of PE. During apoptosis, PS moves from the inner leaflet to the outer leaflet of the cell. The presence of PS on the surface of the cell is recognized by macrophages and related scavenger cells, thereby bringing about the removal of apoptotic cells. Based on this mechanism, PS can be used as an indicator for the clearance of apoptotic cells (Balasubramanian *et al.*, 2007; Fadok *et al.*, 2001; Fadok *et al.*, 1992). In addition, PS also plays a role in the blood clotting process (Zwaal *et al.*, 2004; Schroit & Zwaal, 1991; Bevers *et al.*, 1982). The expression of PS on the surface of activated platelets interacts with factor VII-a tissue factor complex. This in turn activates the proteolytic activity of the protein, thus commencing the blood clotting cascade. PS also acts as co-factor for many signalling proteins, such as the protein kinase C (Bittova *et al.*, 2001; Nishizuka, 1992) and neutral sphingomyelinase (Tomiuk *et al.*, 2000).

Phosphatidylinositol (PI)

Phosphatidylinositol (PI) constitutes about 10% of the cellullar lipid repertoire (Pendaries *et al.*, 2003). It is a primary source for arachidonic acid, which is esterified at the *sn*-2 position. With arachidonic acid being a precursor to eicosanoid production, PI is intertwined into the eicosanoid pathway. Many of the inositol derivatives, such as the phosphoinositides (PIPs) and inositol polyphosphates (IPs),

are synthesized from PI. The function of PI and its metabolites include the glycolipid anchoring of proteins (Shields & Arvan, 1999), signal transduction (Odom *et al.*, 2001; Carman & Henry, 1999; Henry & Patton-Vogt, 1998; Greenberg & Lopes, 1996), exportation of mRNA from the nucleus (Odom *et al.*, 2000; Saiardi *et al.*, 2000; Shears, 1996) and vesicle trafficking (Martin, 2001; Czech, 2000).

Phosphatidylglycerol (PG)

Although PG is ubiquitous in all organisms, it is present at minute amounts of about 1-2%. Despite the rarity of PG, it constitutes almost up to 5% of total phospholipid in lung surfactant (Harwood, 1987). It is highly essential for the normal functioning of the lungs (Poelma *et al.*, 2005). Besides its residence in lung surfactant, it is also found in mitochondria (Dowan, 1997). Its exact role in mitochondria remains elusive.

Phosphatidic Acid (PA)

PA is the simplest phospholipid in terms of structure and plays a crucial role in glycerolipid and phospholipid biosynthesis. Besides this, it regulates cell growth and proliferation through its role as a mitogenic activator of the mTOR signalling pathway (Chen, 2004; Foster & Xu, 2003). Also, it engages in vesicle budding at the Golgi, excocytosis and plasma membrane endocytosis (Jenkins & Frohman, 2005). There is evidence to show that PA has the secretory function. Huang *et al.* illustrated that phospholipase D1 (PLD1)-produced PA that is present on the granule membrane, possesses the ability to secrete insulin from pancreatic β cells (Huang *et al.*, 2005). Furthermore, PA can bind to p47 component of NADPH oxidase complex, which in turn produces surperoxides and results in respiratory burst (Palicz

et al., 2001). Lastly, PA modulates cytoskeletal rearrangement. Some literature suggested that PA binds to actin fibers directly to bring about actin reorganization (Su *et al.*, 2006; Komati *et al.*, 2005; O'Luanaigh *et al.*, 2002; Kam & Exton, 2001; Anderson *et al.*, 1999).

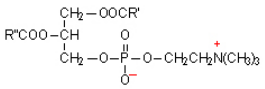
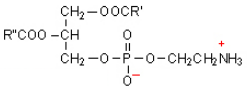
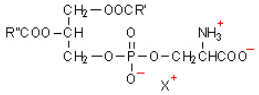
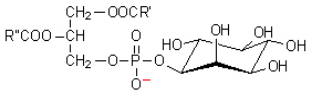
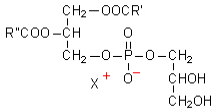
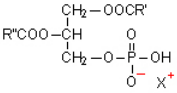
Phospholipid class	Phospholipid structure
Phosphatidylcholine (PC)	
Phosphatidylethanolamine (PE)	
Phosphatidylserine (PS)	
Phosphatidylinositol (PI)	
Phosphatidylglycerol (PG)	
Phosphatidic acid (PA)	

Table 1-1: Structures of phospholipids.

(Modified from http://www.lipidlibrary.co.uk)

Ether lipids and plasmalogens

Ether lipids are lipids with ether-linked alkyl chain at the *sn*-1 position instead of the usual ester-linked fatty acid (Figure 1-5). Plasmalogens are a subset of ether lipids. They contain a *cis* bond on the alkyl chain adjacent to the ether bond, forming a "vinyl-ether linkage". Plasmalogens were first identified by Feulgen and Voit in 1924 (reviewed in Nagan & Zoeller, 2001). Later, scientists realised there are more plasmalogens than there are ether-linked phospholipids. PC and PE make up the

majority of plasmologens and ether-linked lipids. About 70% of plasmalogens possess an ethanolamine headgroup (Horrocks *et al.*, 1982). Nevertheless, ether-linked PI and PS are also present in eukaryotic cells, but at much lesser extent (about 0.2% of total phospholipid mass) (Nagan & Zoeller, 2001). Plasmalogens function as a reservoir for polyunsaturated fatty acids (Tamby *et al.*, 1996; MacDonald & Sprecher, 1991; Akoh & Chapkin, 1990; Ford & Gross, 1989; Chilton & Murphy, 1986; Sugiura *et al.*, 1987; Blank *et al.*, 1973). They can also act as antioxidants. Studies have shown the vulnerability of the vinyl-ether linkage in plasmalogen to reactive oxygen species (ROS) (Hahnel *et al.*, 1999; Zoeller *et al.*, 1999 ; Hagar *et al.*, 1996; Jurgens *et al.*, 1995; Engelmann *et al.*, 1994; Hoefler *et al.*, 1991; Gatt & Osmundsen, 1988; Morand *et al.*, 1988; Zoeller *et al.*,1988). When ROS preferentially attack the unique linkage, this spares the neighbouring molecules from oxidative damage, thus reducing the chance of oxidative degradation on these compounds. Lastly, plasmalogens are involved in membrane biogenesis and fusion. Gremo and colleagues have illustrated the rapid vesicular events undergone by membranes high in plasmalogen composition (Gremo *et al.*, 1985).

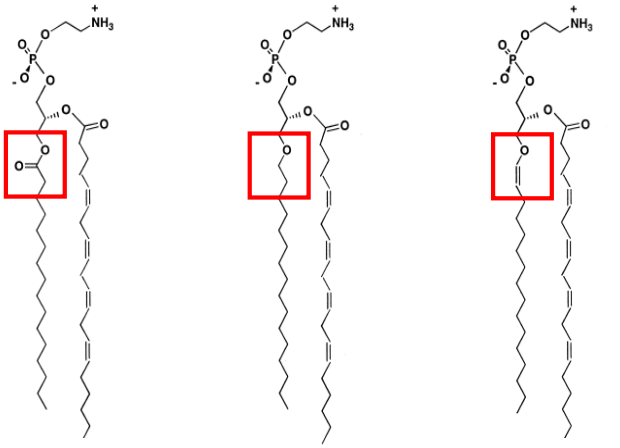


Figure 1-4: Structure of ether lipid and plasmalogen – using PE as an example.
(Adapted from Nagan & Zoeller, 2001)

1.3.3 Functional properties of lipids

The classical view of lipids is that they serve as energy storage in the form of lipid droplets. Besides being an efficient storage of energy reserves, lipid droplets also provide a source of fatty acids and sterol components for membrane biogenesis (Van meer *et al.*, 2008), a function that has been conserved from prokaryotes to eukaryotes (Waltermann & Steinbuchel, 2005; Murphy, 2001).

In addition to energy storage, lipids are also constituents of lipid membranes. Polar lipids, which are amphiphatic, allow hydrophobic regions to associate themselves together and hydrophilic moieties to interact with each other and water. This offers the physical basis of lipid membrane formation. Consequently, presence of lipid membranes enable the segregation of internal components from the external environment (Van meer *et al.*, 2008), compartmentalizing the cell. Different chemical reactions can occur in their own niche, thereby increasing their efficiency.

Furthermore, with the formation of the membranes, fission, fusion and budding will be possible and are necessary for cell division and trafficking.

Lipids have also acquired the ability to act as signalling molecules that lead to various cellular functions, such as cell growth, death and migration. Sphingolipids possess an emerging role in cell signalling, growth and death (Gomez-Munoz, 2006; Merril *et al.*, 1997; Spiegel & Merrill, 1996). Degradation of lipids, such as the phosphatidylinositol-4,5-bisphosphate, yields an array of molecules that serve as signalling molecules (Wenk, 2005). Some of these molecules can initiate signaling cascades that result in the release of calcium from the endoplasmic reticulum (Berridge, 1987, 1984). Also, oxidized products of cholesterol, such as 22 (*R*)-hydroxycholesterol (HC), 24 (*S*)-HC, 27-HC and 24 (*S*), 25-HC, act as ligands for liver X receptors α and β. At physiological concentrations, they can prevent development of atherosclerosis in animal models (Tontonoz & Mangelsdorf, 2003; Tangirala *et al.*, 2002).

Lipids also play vital roles in inflammatory, algesic and pyrogenic cascades. The release of arachidonic acid from glycerophospholipids by phospholipase action leads to the synthesis of eicosanoids. Its derivatives have been well studied for their involvement in inflammatory process (Balazy, 2004). Funk *et al.*, in their review on prostaglandins and leukotrienes, have illustrated the actions of prostaglandins in eliciting inflammatory, algesic and pyrogenic response, depending on the location of action; leukotrienes in causing allergic inflammation (Funk *et al.*, 2001).

Lastly, lipids also have immunomodulatory function. In terms of pathogen recognition, some Toll-like receptors (TLR) are able to recognize lipid compounds

(Akira & Takeda, 2004; Poltorak *et al.*, 1998). For instance, TLR-2 is able to recognize glycoinositolphospholipids and glycolipids (Coelho *et al.*, 2002; Opitz *et al.*, 2001). Moreover, various classes of lipid have been discovered to be able to bind to CD1 receptors and evoke a T-cell response (Sieling *et al.*, 1995; Porcelli *et al.*, 1989). Most amazingly, some bacteria have perfected the ability to evade host immune system through the shedding of bacterial lipids, thereby invading and replicating in host successfully (Rhoades *et al.*, 2003).

1.4 Relationship between lipids, MSC and adipogenesis

1.4.1 Effects of lipids on adipogenesis

It is only recently that there is emerging literatures on how lipids affect adipogenesis in MSC. Most works on lipids modulating the adipogenic pathway are carried out in 3T3-L1 cells *in vitro*. Despite this, inference from 3T3-L1 works can provide insight on types of lipids that can regulate the development of adipocytes in MSC. This section unfolds the various classes of lipids having an effect on adipogenesis.

Different forms of sterols have varying effects on adipogenesis. Oxysterols have been shown to inhibit adipogenesis in MSC. Exogenous addition of 20(*S*)-hydroxycholesterol (20S) to mouse MSC inhibits troglitazone-induced adipocyte

formation (Kim *et al.*, 2007). In addition, 22 (*R*)-hydroxycholesterol (22R), 22 (*S*)-hydroxycholesterol (22S) and 20S also inhibit troglitazone-induced adipogenesis in the following order of 20S ≥ 22S > 22R in mouse MSC (Kha *et al.*, 2004). It has recently been reviewed that oxysterols act as novel activators of the hedgehog signalling pathway, thereby inhibiting adipogenesis and promoting osteogenesis (Eaton, 2008; Dwyer *et al.*, 2007). On the other hand, supplementation of another form of sterol, 17-β estradiol, enhances adipogenesis in human MSC by acting through estrogen receptor (ER) α (Hong *et al.*, 2006).

15-deoxy-$\Delta^{12,\ 14}$-prostaglandin J_2 (15dPGJ2) promotes adipogenesis (Mazid *et al.*, 2006). Based on their in-house enzyme-linked immunosorbent assay (ELISA), they discovered that adipocytes differentiated from 3T3-L1 secrete 15dPGJ2. Furthermore, the accumulation of lipid droplets in the adipocytes correlates with the increasing amounts of secreted 15dPGJ2. When cycoloxygenase inhibitors (aspirin and indomethacine) are added, no formation of lipid droplets is observed. This phenomenon is reversed when prostaglandin D_2 (precursor of 15dPGJ2) is added exogenously. They propose that this may be due to 15dPGJ2's PPARγ agonist status that allows it to bind to PPARγ and results in the expression of adipogenic genes, thus adipogenesis prevails. Troglitazone, a well-known specific PPARγ agonist, also exhibits similar effect when used under the same conditions.

Conversely, another member of the prostaglandin family, prostaglandin 2α (PGF2α), inhibits adipogenesis in 3T3-L1 during early differentiation (Liu & Clipstone, 2007). PGF2α does not affect the expression and activity of C/EBPβ, thus implying that it probably acts after the first mitotic clonal expansion in the early phase of

adipogenesis. The paper suggests that PGF2α may have acted through the calcineurin-dependent mechanism that inhibits the expression of essential transcription factors, C/EBPα and PPARγ, thus preventing adipogenesis (Liu & Clipstone, 2007).

Fatty acids (FA) can be pro- or anti-adipogenic depending on their carbon chain lengths, degree of unsaturation and location of double bonds. Medium-chain length FA, such as octanoic acid (FA 8:0) and decanoic acid (FA 10:0), increase lipid accumulation in 3T3-L1, with decanoic acid (FA 10:0) being a stronger inducer (Yang *et al.*, 2008). On the other hand, ω-3 polyunsaturated FA, such as docosahexaenoic acid (DHA; FA 22:6) and eicosapentaenoic acid (EPA; FA 20:5), inhibits adipogenesis through suppression of adipogenic gene expression in 3T3-L1 (Lee *et al.*, 2008; Kim *et al.*, 2006; Madsen *et al.*, 2005). Conversely, ω-6 polyunsaturated FA, such as linoleic acid (FA 18:2), enhances lipid droplet formation within human preadipocytes (Madsen *et al.*, 2005; Hutley *et al.*, 2003). Arachidonic acid (FA 20:4), another ω-6 polyunsaturated FA, does not directly affect adipogenesis. Rather, its metabolites, prostaglandins have dual effects on adipogenesis (Liu & Clipstone, 2007; Mazid *et al.*, 2006).

Lastly, lysophosphatidic acid (LPA) has been demonstrated to stifle adipogenesis in both mouse and human preadipocytes (Simon *et al.*, 2005). LPA binding to LPA_1 receptor present on preadipocytes reduces the expression and activity of PPARγ, preventing the ensuing expression of adipogenic genes, such as fatty acid binding protein (aP2), stearoyl-CoA desaturase-1 (SCD1), insulin-responsive glucose

transporter (GLUT4), phosphoenol-pyruvate carboxykinase (PEPCK), leptin and insulin receptors.

Although these are probable lipids that can modulate adipogenesis, they take place in preadipocytes, which are committed to adipogenesis. Exogenous addition of lipids to MSC would be interesting as this would provide some insight as to whether these lipids can enable commitment to adipogenesis and ultimately terminal differentiation.

1.4.2 How MSC can contribute to obesity

Obesity can be brought about by adipocyte hypertrophy (increase in size of adipocyte) followed by adipocyte hyperplasia (increase in number of adipocytes). The enlargement of adipocyte occurs early in obesity and levels off, while the increase in number of adipocytes persists throughout obesity (Bjorntorp 1974). This implies that once adipocytes reach its maximum size, other factors may be activated thus contributing further to obesity. Studies have suggested that mature adipocytes, as an endocrine organ, can secrete factors that signal preadipocyte proliferation and differentiation. For instance, conditioned media from adipocytes or hyperptrophic white adipose tissue result in adipogenesis of preadipocytes (Marques *et al.*, 1998). In addition, mature adipocyte secretes tumor necrosis factor –α (TNF- α) and insulin-growth factor (IGF) that can promote hyperplasia in a paracrine manner (Avram *et al.*, 2007). In addition, a recent report demonstrates that progenitors of white adipocytes reside in the adipose vasculature. The authors propose that the adipose vasculature forms the niche that provides suitable signals for adipocyte development (Tang *et al.*, 2008). Furthermore, there is evidence illustrating that bone-marrow

derived preadipocytes can migrate and contribute to fat deposits using GFP-labeled bone marrow cells (Crossno *et al.*, 2006). These substantiate the notion that MSC and preadipocytes, under the appropriate stimuli, can differentiate and lead to hyperplasia of adipocytes and ultimately obesity.

1.4.3 Lipidomics

Lipidomics is a branch of metabolomics that adopts a systems-level approach in studying all lipids, their interacting partners and their functions within the biological system (Watson, 2006; Wenk, 2005). However, this alone is not enough. Through integration of genomics and proteomics, a more wholesome overview of how lipids function in a biological system can be achieved. Since genomics and proteomics data on MSC are already available (Jeong *et al.*, 2007; Park *et al.*, 2007; Lee *et al.*, 2006; Silva *et al.*, 2003), metabolomics is the remaining piece required to unlock the mystery of MSC. This aspect of the –omics genre provides information on the downstream effects of gene and protein regulation, thus presents the link to biological state of the system (Goodacre et al., 2004). To bring us one step closer to understanding the biology of MSC, we will first look at lipidomics of MSC.

Lipids used to be known for their membrane forming and energy storing abilities. To date, they are also involved in cell signaling, inflammatory actions and immunomodulatory functions. With the immense combinatory structural diversity of lipids, improved analytical methods are required. Mass spectrometry can be the solution as it is acutely sensitive, efficient and of high throughput. For instance, crude lipid extract of biological fluid or tissue, even at minute amounts, when introduced into the mass spectrometer will lead to the derivation of a "fingerprint" of

the biological substance. By employing this method, subtle changes in the lipid composition of cells can be detected (Milne *et al.*, 2006; Ivanova *et al.*, 2004).

Understanding the lipid profile and identifying the lipid changes between undifferentiated and differentiated MSC enables the targeting of probable lipids involved in adipogenesis. For instance, after determining distinct lipid changes, metabolic pathways related to these lipids can be recognized. Subsequently, one can hypothesize links between the metabolic pathway of interest and the adipogenic pathway and from there uncover areas where intervention may attenuate adipogenesis, and thus provide an alternative solution to obesity. Since there are evidences to show that lipids can modulate adipogenesis and MSC can play a role in obesity through hyperplasia, acquiring the lipid profile will take us one step closer to comprehending adipocyte biology and in turn able to combat obesity more efficiently.

1.5 Hypothesis

When MSC undergo adipogenesis, the resultant adipocytes possess unique lipid signatures/profiles different from their predecessors, which can serve as possible markers to distinguish the different stages of differentiation.

1.6 Objectives

1) To validate the adipogenic status of terminally differentiated MSC via histochemistry, real time polymerase chain reaction (PCR) and fluorescence activated cell sorting (FACS)

2) To determine phospholipid and TAG changes between MSC and MSC derived adipocytes using thin layer chromatography (TLC) and mass spectrometry (MS) approaches
3) To understand the observed lipid changes through real time PCR and immunoblotting

1.7 Workflow

MSC cultures are expanded to the predetermined optimized passage five (P5) before adipogenic differentiation takes place. Adipogenic inducers, namely insulin, dexamethasone (Dex), indomethacine (Indo) and isobutymethylxanthine (IBMX), are exogenously added to cultures at optimized concentrations (refer to Materials and Methods – 2.1.1).

Based on prior optimization work, LD starts to form after seven days of adipogenic induction. In order to compare between the early and late stges of differentiation, weekly timepoints were set up. Four timepoints are chosen during adipogenic differentiation protocol (day0, day7, day14 and day21) (Figure 1-6) for the determination of changes in cellular lipid profile. Pairwise comparisons are made between undifferentiated cells (UD) and differentiated cells (Adipo) at these selected timepoints.

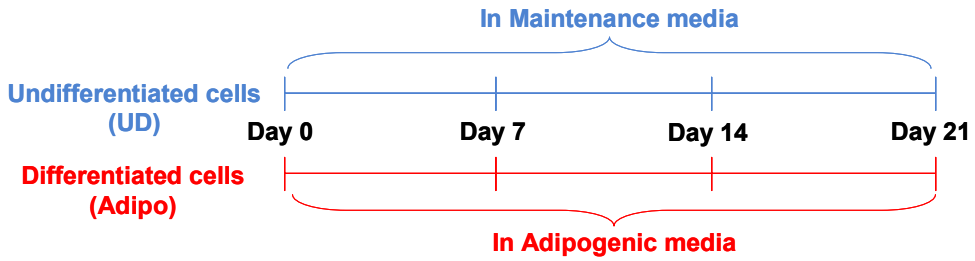


Figure 1-5: Experimental timepoints.

Day 0 denotes the start of adipogenic differentiation
Day 7, 14 and 21 referred to 7, 14 and 21 days after adipogenic induction respectively

The workflow comprises of two parts (Figure 1-7). In the first part (Part I), triplicate samples collected at each timepoint are subjected to two sections – validation of adipogenesis and characterization of lipids. In order to determine that MSC undergo adipogenesis, fluorescence activated cell sorting (FACS) to quantitate the extent of differentiation, histochemical staining of lipid droplets (LD) using Oil Red O solution, real time polymerase chain reaction (real time PCR) for adipogenic gene expression analysis and simple observation using phase contrast microscope for visualization of LD are performed. Various thin layer chromatography (TLC) and mass spectrometry (MS) techniques are employed to profile lipid changes when MSC differentiates into adipocytes. Mainly two TLC methods, Hexane:Diethyl ether:Formic acid (HEFA) and Chloroform:Methanol:Water (CMW), are adopted. HEFA and CMW allow for the resolution of neutral and polar lipids respectively. In order to dwell deeper into the changes observed from the TLC, single scan MS is used to obtain an unbiased lipid profile. Tandem MS and precursor ion scan (PREIS) elucidate the identity of lipid species, while multiple reaction monitoring (MRM) enable the quantitation of lipid changes observed.

After analyzing the lipid profiles, certain enzymes seem to suggest their involvement in the lipid changes observed. The second part (Part II) of the workflow is designed as an attempt to explain these changes. Real time PCR is used to determine the relative gene expression of the enzymes of interest. However, gene expression of these enzymes does not translate to their phenotypic function. Immunoblotting serves as an independent assay to validate the gene expression analysis. Materials and methods used are discussed in detail in chapter 2.

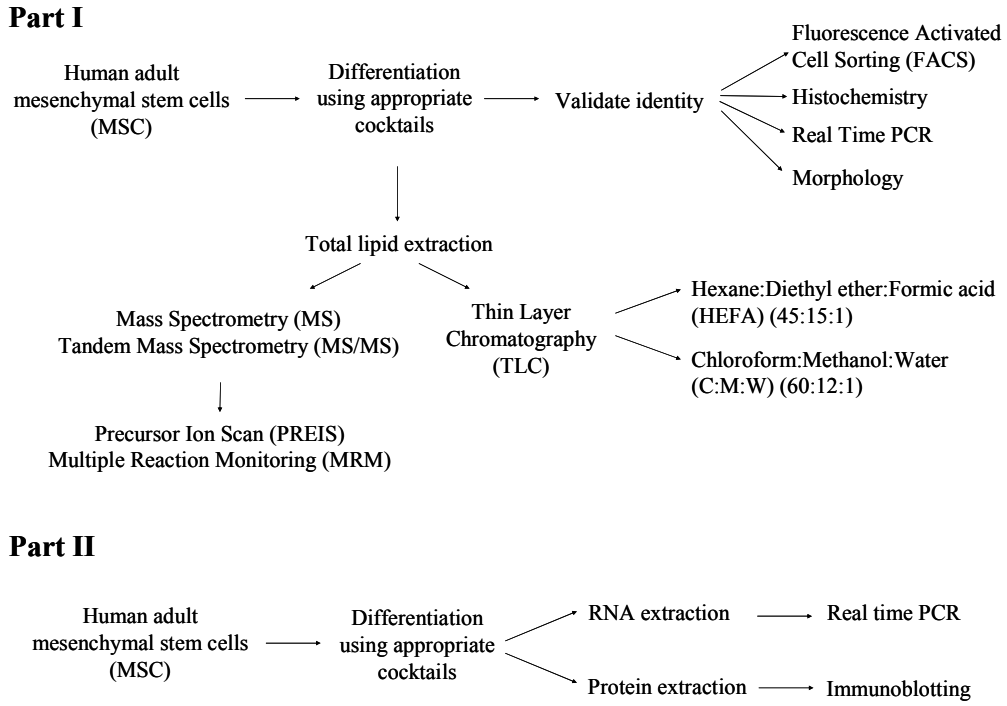


Figure 1-6: Outline of workflow.

MATERIALS AND METHODS

2 Materials and Methods

2.1 Tissue culture

2.1.1 Adipogenesis

Human mesenchymal stem cells (MSC) purchased from Cambrex (East Rutherford, NJ) were maintained in Dulbecco's modified Eagle's medium (DMEM – 1000mg/ml glucose; Sigma-Aldrich. St Louis, MO), 10% (v/v) fetal bovine serum (FBS) (Lonza. Bassel, Switzerland), 2mM of L-glutamine (Gibco-Invitrogen. Carlsbad, CA), 100units/ml of penicillin (Gibco-Invitrogen. Carlsbad, CA) and 100μg/ml of streptomycin (Gibco-Invitrogen. Carlsbad, CA) (maintenance media). Cells were passaged at 80% confluence via trypsinisation (0.125% Trypsin/Versene in Dulbecco's Phosphate Buffered Saline without calcium chloride and magnesium chloride (DPBS)) (Sigma-Aldrich. St Louis, MO). Cells were counted via hemocytometer and seeded 5000 cells/cm^2. For adipogenic differentiation, hMSC were seeded at 18000 cells/cm^2. Upon reaching 100% confluence, maintenance media was switched to adipogenic media (maintenance media containing 4500mg/ml glucose, 10μg/ml Insulin (Sigma-Aldrich. St Louis, MO), 115μg/ml 3-Isobutyl-1-methylxanthine (Sigma-Aldrich. St Louis, MO), 1μM Dexamethasone (Sigma-Aldrich. St Louis, MO) and 20μM Indomethazine (Sigma-Aldrich. St Louis, MO) for the next 21 days. Media were changed twice a week. All culture incubations were performed in a humidified 37°C, 5% CO_2 incubator (Sanyo Electric Co. Osaka, Japan. Japan)

2.2 Oil Red O staining

Oil Red O staining was used to identify lipid droplet formation within cells. *In situ*, adherent cells were washed with DPBS (Sigma-Aldrich. St Louis, MO) and fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich. St Louis, MO) for 1 hour at room temperature. The fixative was removed and cells were washed with water. The cells were stained with filtered 0.36% (w/v) Oil Red O solution (Sigma-Aldrich. St Louis, MO) in 60% (v/v) isopropanol (BDH – Merck. Whitehouse Station, NJ) for 1hour at room temperature. The cells were washed with 60% (v/v) isopropanol twice followed by 5 washes with water. Next, cells were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich. St Louis, MO) for 5 minutes at room temperature (RT) followed by 3 washes with water. Images were captured using a dissecting microscope (Olympus SZX12. Tokyo, Japan) in 5 random fields at the different magnifications.

2.3 Fluorescence Activated Cell Sorting (FACS)

To quantitate the percentage of MSC that contained lipid droplets, Nile Red staining of cells and flow cytometry were employed (BD FACSCalibur (Biomed Diagnostics. White City, OR)). Briefly, cells were harvested via trypsinisation and following centrifugation (400 x g, 5mins RT), cell pellet was resuspended in DPBS. However, for day 21 adipocyte samples, in addition to the above method, region between the cell pellet and floating adipocytes was also aspirated. The final cell suspension and samples from between the cell pellet and floating adipocytes were subsequently stained with 1μg/ml of Nile Red solution (Sigma-Aldrich. St Louis, MO). Excitation and emission at different wavelengths reflect Nile red interactions with either neutral or polar lipids (Greenspan & Fowler, 1985). Using FACS, cells detected on the FL2

channel are those containing neutral lipids. Firstly, cells were gated in a pre-determined forward scatter (FSC) and side scatter (SSC) region. Next, cells within the gated region and detected on FL2 were counted and marked by marker 1 (M1). As a result, the number of MSC containing lipid droplets was revealed. All data in histograms has been gated on the same FSC and SSC region.

2.4 Gene expression

2.4.1 RNA extraction

Cells were washed with DPBS and lysed in 1ml of TRIzol® reagent (Invitrogen. Carlsbad, CA). Cell lysates were incubated at room temperature (RT) for 5 minutes before adding 200µl of 100% chloroform and mixing well. Following 2 minutes incubation at room temperature, samples were centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous phase was collected and 500µl of 100% isopropanol was added. The samples were incubated at -20°C for 20 minutes and the RNA pelleted via centrifugation (13200g for 15 minutes at 4°C). The supernatant was aspirated and the RNA pellet washed with 1ml of 75% (v/v) ethanol and re-pelleted (13200g for 15 minutes at 4°C). The supernatant was aspirated and the pellet air-dried before re-suspending in RNAse/DNAse free water, containing 1/10 (v/v) RNAseOUT (Invitrogen. Carlsbad, CA). Concentrations of all RNA samples were determined by measuring absorbance at wavelengths of 260nm and 280nm using the Spectrometer (NanoDrop®. Thermo Scientific. Waltham, MA). It was assumed that an absorbance of 1 at 260nm was equivalent to 40µg/ml RNA and 33µg/ml of oligonucleotides. The ratio of absorbance at these two wavelengths was used to estimate the purity of the RNA. To check the integrity of the RNA, electrophoresis of 1% (w/v) TAE gel (1g

agarose powder (Invitrogen. Carlsbad, CA) in 100ml 1X TAE (Tris base (Sigma-Aldrich. St Louis, MO), Glacial acetic acid (BDH – Merck. Whitehouse Station, NJ) and 0.5M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich. St Louis, MO) pH8.0) buffer) containing 0.5 mg/ml of ethidium bromide (Sigma-Aldrich. St Louis, MO Aldrich) at 120V for 25 minutes was carried out. Images of the bands were visualized via ultraviolet light using Gel documentation system (VLChemiSmart 3000. France).

2.4.2 DNA digestion

1µg of total RNA was treated with 1µl of RQ1 RNase-Free DNase I (Promega. Fitchburg, WI) and 1µl of RQ1 DNase 10X reaction buffer (Promega. Fitchburg, WI) and topped up with DNase/RNase free water to yield a total reaction volume of 10µl. After incubating at 37°C for 40 minutes, digestion was curbed using 1µl of RQ1 DNase STOP solution (Promega. Fitchburg, WI) and incubated at 65°C for 10 minutes. Negative control for the subsequent Polymerase Chain Reaction (PCR) was collected by aspirating 1µl from the above mixture and added to 19µl of 10mM Tris pH 8.5 (Sigma-Aldrich. St Louis, MO). The above protocol was carried out for all samples in duplicates.

2.4.3 Reverse transcription

To 1µg of DNA digested- total RNA , 1.5µl of 10mM dNTPs mix (Invitrogen. Carlsbad, CA), 1.5µl of 250ng/µl of random hexamers (Invitrogen. Carlsbad, CA) and 7µl of DNase/RNase Free water were added. They were then heated at 65°C for 5 minutes, After a quick chill on ice, 6µl of 5X modified First Strand buffer

(Invitrogen. Carlsbad, CA), 1.5µl of 0.1M of dithiothreitol (DTT, Invitrogen. Carlsbad, CA), 1µl of 40units/µl of RNAseOUT and 1.5µl of Superscript™ III (Invitrogen. Carlsbad, CA) were added. The samples were incubated at 25°C for 10 minutes, followed by 120 minutes at 50°C. Finally, reverse transcription was inactivated by heating at 70°C for 15 minutes. Upon completion, duplicate samples were pooled and diluted in 400µl of 10mM Tris pH 8.5.

2.4.4 Polymerase Chain Reaction (PCR)

Verification of DNA digestion was carried out via standard PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems. Foster City, CA). 5µl of 10X PCR buffer (Applied Biosystems. Foster City, CA), 5µl of 25nM Magnesium chloride (Applied Biosystems. Foster City, CA), 2µl of 10mM dNTPs, 4µl of 5µM GAPDH primer pair (Appendix 1), 0.25µl of 5 units/µl AmpliTaq Gold DNA polymerase, 31.75µl of DNase/RNase Free water and 2µl of template (DNase-treated RNA or cDNA). Thermal cycling consisted of a 10 minutes heat activation step at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 30 seconds. A final 10 minutes extension time at 72°C concluded the PCR reaction. This was performed in a thermal cycler (Thermo Electron Corporation. Waltham, MA).

2.4.5 Real time PCR

2X SYBR® Green PCR master mix (Applied Biosystems. Foster City, CA) was used to carry out real time PCR. In short, 5µl of cDNA was added to 10µl of 2X SYBR® Green PCR master mix followed by 5 µl of 2µM primer pairs (100µM forward and

100µ M reverse, with the final amounts being diluted to 2µM). A resultant final reaction volume of 20µl was yielded. All reactions were performed in duplicates in Corbett Research Rotor Gene™ reaction tubes (Corbett Research. Sydney, Australia).

The PCR cycling parameters included an activation of 15 minutes at 95°C, followed by 45 cycles of denaturation of 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. Fluorescence data was recorded at the end of each extension step. To conclude each run, a DNA melt profile was run from 72°C to 95°C with a ramp of 1°C every 5 seconds. Fluorescence data was recorded continuously during the melt profile and allowed the identification of specific amplicon production or primer dimers. The second derivative analysis (d^2F/dT^2) of the melt curve showed a single peak for specific amplicons and a non-specific or broad peak identified PCR artefacts. A single run quantified and determined the level of expression of the following genes in undifferentiated and differentiated cells at different timepoints: 5 presumptive housekeeping genes (β-actin, GAPDH, HMBS, HPRT and 18s rRNA; sequence refer to Appendix 1) and 15 genes of interest (PPARG1, PPARG2, C/EBPα, C/EBPδ, aP2, LPL, PLA1, PLA2-G4a, PLA2-G6, PLB, Lipin1, Lipin2 and Lipin3, LPPa, LPPb; sequence refer to Appendix 1)

C_T values were imported into Microsoft Excel. The relative expression of the 5 housekeeping genes was examined by the C_T method (Vandesompele *et al.*, 2002). Assuming equal amplification values, gene expression were normalized against every other gene. All 5 housekeeping genes were selected and their expression normalized by determining their geometric mean (Vandesompele *et al.*, 2002). Gene expression values were normalized against this geometric mean and allowed

comparison between undifferentiated and differentiated cells at various timepoints. A 2.5 fold change in expression relative to undifferentiated cells was deemed significant because of the acute sensitivity of real time PCR which resulted in frequently observed fluctuations in gene expression of up to 50% despite using 5 housekeeping genes to normalize data.

2.5 DNA quantification

In order to normalize results obtained from mass spectrometry, total DNA was extracted from cells and quantitated using Pico Green (Molecular Probes P7589 Quant-iT PicoGreen dsDNA kit. Invitrogen. Carlsbad, CA). Briefly, cells were scrapped in ice cold DNAse/RNAse free water (Gibco-Invitrogen. Carlsbad, CA). The cell lysates underwent 3 freeze/thaw cycles and later spun down at 10000rpm for 2 minutes to pellet cell debris.

In a standard 96 well flat bottom culture dish, DNA standards and samples were added in a final volume of 50µl. A Standard curve (in duplicate) ranging from 2µg/ml to 0.03125 µg/ml was obtained by serial diluting lambda DNA. Samples were analysed in triplicate following a predetermined optimal 1:4 dilution in TE. Finally, PicoGreen solution was added in a 1:1 ratio to all wells, and incubated for 5 mins at RT in the dark. Fluorescence was read at 495/515nm using Wallac Victor$_{TM}^{3}$ multilabel counter (Perkin Elmer. Foster City, CA). Standard curve with R^2 value of 0.99 or greater was used for the determination of DNA concentration in samples. Average deviation of 5% or less was considered acceptable within triplicates.

2.6 Lipids

2.6.1 Lipid standards

For phospholipids analysis, the following were used as standards. Phosphatidic acid with C20 fatty acyl chains (diarachidonoyl PA, 40:8-PA), C14-phosphatidylserine (dimyristoyl PS, 28:0-PS), C14-phosphatidyglycerol (dimyristoyl PG, 28:0-PG), C14-phosphatdiylethanolamine (dimyristoyl PE, 28:0-PE), C14-phosphtidylcholine (dimyritoyl PC, 28:0-PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylinositol with C8 fatty acyl chains (dioctanoyl PI, 16:0-PI) was obtained from Echelon Biosciences, Inc. (Salt Lake City, UT). The internal standards were solubilized in chloroform at a stock concentration of 10 μg/μl.

2.6.2 Total lipid extraction

Undifferentiated MSC and MSC-differentiated adipocytes were harvested at 4 timepoints, namely day0 (point of time when MSC reached 100% confluence and adipogenic induction was to begin), day 7, day 14 and day 21 post-adipogenic induction. Modified Bligh and Dyers phospholipid extraction was carried out (Bligh & Dyer, 1959). Briefly, cells were washed in ice-cold DPBS. Following total aspiration of DPBS, cells were scraped in 400μl of ice-cold 100% methanol (Merck. Whitehouse Station, NJ) on a cold plate (Thermo Shandon. Pittsburgh, PA) and collected into eppendorf tubes. 200μl of ice-cold 100% chloroform (BDH – Merck. Whitehouse Station, NJ) was added to cell suspension and vortexed for 1minute before 10 minutes incubation on ice. Next, 300μl of 100% chloroform was added, followed by 450μl of MiliQ water. This was subsequently vortexed for 2 minutes and incubated in ice for 1 minute. The samples were centrifuged at 20000g for 5 minutes

at 4°C to bring about phase separation. The lower organic phase was collected and transferred into a clean fresh microfuge tube (Axygen. Union City, CA). Re-extraction was performed with 600µl of 100% chloroform. Extracted lipids were dried down using the SpeedVac (Thermo Electron Corporation. Waltham, MA) and stored at -80°C.

2.7 Thin Layer Chromatography (TLC)

Two solvent systems were used to resolve polar and neutral lipids.

For polar lipid resolution, chloroform:methanol:water (60:12:1, v/v) system was used. A Silica gel 60 (Merck. Whitehouse Station, NJ) TLC plate was heated at 105°C for 15 minutes. An allowance of 1.5cm from the base, 1cm from the sides and 2 cm from the top were determined before samples (day 0, day 21 undifferentiated and day 21 differentiated samples) and standards (1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine (POPE), 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC), L-a-Phosphatidylserine (PS), natural plant, soybean Phosphatidylinositol (PI), 1,2-Dimyristoyl-*sn*-Glyero-3-Phosphocholine (DMPG), 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphatidic acid (POPA) and cholesterol – Avanti® polar lipids, Inc. Alabaster, AL) were deposited. Each spot was 1cm apart and 2µg of standards were used. Each of the dried down lipids samples was resuspended in 200µl of chloroform:methanol (1:1, v/v), of which 50µl was transferred into a fresh tube and dried down via the SpeedVac. 5µl of chloroform:methanol (1:1, v/v) was used to solubilise the newly dried down lipids and spotted on to the plate. When all required standards and samples were spotted, the plate was left to dry before placing it in the chamber for migration. Once the solvent had reached the 2cm mark (from the top), the plate was removed and left to

dry. The migrated lipids were visualized by placing the plate in a tank saturated with iodine vapour.

For neutral lipid resolution, hexane:diethyl ether:formic acid (45:5:1, v/v) system was used. The above described method was carried out except that the standards used were as follows cholesterol, TAG mix (consisting of glyceryl tridecanoate, glyceryl tridodecanoate, glyceryl trimyristate, glyceryl trioctanoate and tripalmitin purchase as a mixture from Sigma-Aldrich. St Louis, MO), MDT mix (consisting of 1,3-Diolein, 1,2-Dioleoyl-rac-glycerol, Triolein and Monoolein purchased as a mixture from Sigma-Aldrich. St Louis, MO), C17-ceramide (Avanti® polar lipids, Inc. Alabaster, AL), oleic acid (Sigma-Aldrich. St Louis, MO) and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphoethanolamine (POPE).

Standard curve of each lipid class ranging from 10mg/ml to 0.3125mg/ml was obtained by serial diluting each of the lipid standard described above. Subsequently, appropriate lipid standards and their diluted counterparts were resolved in the appropriate systems. The resulting spots were analysed via densitometric scanning of iodined stained plates using the NIH ImageJ software. Standard curve with R^2 value of 0.98 or greater was used for the determination of lipid concentration in samples. Average deviation of 5% or less was considered acceptable within triplicates. The final computed lipid concentration was normalized to their corresponding total DNA amounts.

2.8 Mass spectrometry (MS)

2.8.1 Single scan MS

Dried down lipid samples were resuspended in 200µl of chloroform:methanol (1:1, v/v). Debris were pelleted through centrifugation at 14000 g at 4°C for 10 minutes. 100µl of sample was aspirated from the top and introduced into the Waters Micromass Q-Tof Micro (Waters Corp. Milford, MA) mass spectrometer, in which electrospray ionization mass spectrometry (ESI-MS) was to be performed. The samples were directly infused using a Harvard syringe pump at a flow rate of 10µl/min. The capillary voltage and sample cone voltage were both maintained at 3.0kV and 50V respectively, while the source temperature was kept at 80°C and the nano-flow gas pressure at 0.7 bar. The mass spectrum acquired was in the range of mass-to-charge ratio (m/z) of 400 to 1200 in the negative ion mode, with an acquisition time of 5 minutes and scan duration of 1 second.

2.8.2 Tandem MS (MS/MS)

To acquire the identity of individual molecular species, tandem mass spectrometry (MS/MS) using the Waters Micromass Q-Tof Micro (Waters Corp. Milford, MA) mass spectrometer was performed. Lipid samples were introduced into the machine in the same way as for the single scan MS, using a Harvard syringe pump at a flow rate of 10µl/min. Similarly, the parameters used for single scan MS were also used for tandem MS as well. However, unlike the single scan MS, MS/MS was carried out individually for each m/z value. For instance, MS/MS for m/z value X comprise of pre-setting the first cell with X, followed by acquiring the mass spectrum from m/z of 50 to X + 50 in the negative mode, with an acquisition time of 5 minutes and scan

duration of 1 second. A range of collision energy from 25 to 40eV is used to achieve desired fragmentation.

2.8.3 Precursor Ion Scanning (PREIS) and Multiple Reaction Monitoring (MRM)

The identification of lipid molecular species was carried out via precursor ion scanning (PREIS) and quantification by multiple reaction monitoring (MRM), with both using the Applied Biosystems 4000 Q-Trap mass spectrometer (Applied Biosystems. Foster City, CA, Foster City, CA). The HPLC system, consisting of Agilent 1100 Thermo Autosampler and an Agilent 1100 LC Binary Pump, was used to provide the mobile phase (chloroform:methanol (1:1, v/v)) and to introduce the samples into the machine. Flow rate differed between the modes used. In the positive mode, flow rate was set at 250µl/min over 1.5 minutes. In the negative mode, flow rate was at 200µl/min over 2 minutes. The ion spray voltage was set at -4500 V for the negative mode and 5500V for the positive mode. Temperature was set at 250°C. Nitrogen was used as curtain gas (value of 20) and collision gas was set to high. These settings were applied to both PREIS and MRM.

Prior to MRM, PREIS was carried out to determine the precursor ion of interest by allowing all ions to pass through the first quadrupole, Q1, and into the collision cell, Q2, where they underwent collision-induced dissociation (CID). In the third quadrupole, Q3, structure specific product ion characteristic was set in accordance to Appendix 2.

In MRM, the first quadrupole, Q1, was set to allow precursor ion of interest to pass through and enter the collision cell, Q2, where they were subjected to CID. In the

third quadrupole, Q3, structure specific product ion characteristic of the lipid of interest were set to pass and detected. For both experiments, individual ion dissociation pathway was optimized with regard to collision energy (CE) to minimize variations in relative ion abundance due to differences in rates of dissociation. MRM transitions and their corresponding declustering potentials (DP) and collision energies (CE), listed in Appendix 3, were established for the quantification of phospholipids. Triacylglycerols (TAG) were quantified by the selected ion monitoring (SIM) method. The difference between MRM and SIM was that the former had a pre-set Q1 and Q3 m/z value; while the latter had only pre-determined Q3 m/z values.

2.9 Western blot

2.9.1 Protein extraction

Four timepoints were set up for protein harvest, namely day 0, day 7, day 14 and day 21. At each timepoint, triplicate samples of undifferentiated MSC and adipocytes were washed with DPBS and DPBS was totally aspirated. Cells were lysed in 250µl of RIPA buffer, comprised of 1% (v/v) Triton X-100 (Sigma-Aldrich. St Louis, MO), 150mM sodium chloride (NaCl (BDH – Merck. Whitehouse Station, NJ), 10mM Tris pH7.4, 2mM EDTA (Sigma-Aldrich. St Louis, MO), 0.5% (w/v) Igepal (NP40) (Sigma-Aldrich. St Louis, MO), 0.1% (w/v) sodium dodecyl sulphate (SDS) (Merck. Whitehouse Station, NJ) and protease inhibitor cocktail at 1:100 dilution (Sigma-Aldrich. St Louis, MO). Subsequently, cell lysates were centrifuged at 10000g for 10 minutes at 4°C. Supernatant (for adipocyte samples, supernatant below the floating adipocyte layer) were collected and stored at -20°C for later use.

2.9.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein concentration was determined using bicinchoninic acid (BCA) protein assay kit (Pierce-Thermo Scientific. Waltham, MA) as stated in the provided instruction manual. 2X Laemmli sample buffer (Sigma-Aldrich. St Louis, MO) was added to 20µg of protein. Finally, protein samples were boiled at 100°C for 5 minutes. All of the prepared protein samples and 10µl of dual-colour protein ladder (BIO-RAD. Hercules, CA) were separated on NuPAGE 4-12% Bis-Tris gel (Invitrogen. Carlsbad, CA) in 1X MOPS buffer (Invitrogen. Carlsbad, CA) and 500µl of NuPAGE antioxidant (Invitrogen. Carlsbad, CA) at 200V for 1 hour.

2.9.3 Membrane transfer

Upon the completion of gel electrophoresis, the gel, nitrocellulose membrane (BIO-RAD. Hercules, CA), filter papers (Whatman plc. Maidstone, UK) and fiber pads were soaked in transfer buffer (2.5g/l Tris-Base (Sigma-Aldrich. St Louis, MO), 11.26g/l glycine (BIO-RAD. Hercules, CA) and 20% (v/v) methanol) prior to the assembly of the gel and membrane "sandwich" (Figure X). Briefly, the various items were placed in the following order: white side of the transfer cassette - fiber pad - filter paper - nitrocellulose membrane – gel – filter paper – fiber pad – black side of the transfer cassette. Subsequently, the transfer was carried out at 100V for 1.5 hours at RT. After the transfer, the membrane was stained with Ponceau S solution (Sigma-Aldrich. St Louis, MO) to determine the efficiency of the transfer. When the desired protein bands were observed, membrane was cut at the appropriate protein size and

destained in 1X Tris-buffered saline Tween-20 (TBST: 4.84g/l Tris-Base, 16g/l NaCle and 0.2% Tween-20 (Sigma-Aldrich. St Louis, MO)) for 5 minutes on a shaker at RT.

2.9.4 Immunoblotting

Membrane was blocked in using 5% (w/v) non fat dry milk (Anlene. New Zealand) in 1X TBST for 1 hour on a shaker. The membrane was incubated with primary antibody diluted in 5% milk TBST at the appropriate dilution factor (Table 2-1) overnight with gentle shaking at 4°C. The membrane was washed 5 times in TBST for 5 minutes each on a shaker at RT. The relevant secondary antibody was also diluted in 5% milk TBST at the recommended dilution factor (Table 2-1) and incubated with the membrane for 2 hours at RT. The membrane was washed 6 times in TBST for 5 minutes each on a shaker at RT. Following this, pat-dried membrane was dipped into a solution containing Super Signal® West Pico stable peroxide and Luminol / Enhancer solutions (both from Pierce-Thermo Scientific. Waltham, MA) in the ratio of 1:1. Finally, in the dark room, the membrane was exposed by placing a Hyperfilm™ MP (Amersham Biosciences. Piscataway, NJ) on top of it in the Hypercassette™ (Amersham Biosciences. Piscataway, NJ) for 1 minute followed by development of the film using the Kodak X-OMAT 2000 film processor (Kodak. Rochester, NY).

Primary Antibodies	Dilution Factor
Rabbit polyclonal to VDAC (Cell signalling. Beverly, MA)	1:1000
Mouse Monoclonal to Beta Actin (IgG1k) (Chemicon. Temecula, CA)	1:10000

Secondary Antibodies	Dilution Factor
Goat polyclonal to Rabbit IgG (Jackson Immunoresearch Laboratories Inc. Baltimore, MD); conjugated to HRP	1:10000
Goat polyclonal to Mouse IgG (Jackson Immunoresearch Laboratories Inc. Baltimore, MD); conjugated to HRP	1:10000

Table 2-1: Primary and secondary antibodies used and their dilution factors.

2.9.5 Re-blotting

For re-probing of membrane with other primary antibodies, the above membrane was first washed once in TBST followed by incubation in Restore™ western blot stripping buffer (Pierce-Thermo Scientific. Waltham, MA) for 15 minutes at 37ºC with gentle shaking. The membrane was washed again and re-blocked, and re-probed as described in *2.9.4 Immunoblotting*.

2.10 Data analysis

2.10.1 Single scan MS

Lipid chromatograms were combined to generate combined spectra (Figure 2-1) and a corresponding spectrum list using MassLynx 4.0 (Waters Corp. Milford, MA). The data in plain text files were loaded into Matlab (The MathWorks Inc. Natick, MA) for alignment of spectra using correlation-optimised warping (COW) (Nielsen *et al.*, 1998). It was a pre-processing method that obtained precise alignment of normalized mass spectrometry (MS) spectra from replicate samples. For averaging of spectra from replicate independent samples, each ion intensity was normalized to the sum of all ion intensities and the normalized data of each replicate was warped against a

reference set. After aligning the peaks, the intensity values of individual m/z were then averaged to obtain one mean spectrum representative of the replicates. To compare between different experimental conditions, the mean spectrum for one experimental condition (MSC derived adipocytes (Adipo)) was warped against the mean spectrum for the control condition (undifferentiated MSC (UD)). After alignment, relative differences in the lipid compositions of the two conditions can be computed by simple arithmetic division and represented in the form of ratios on a logarithm scale ($\log_{10}$ ratios of adipocyte is to MSC). Consequently, unbiased lipid profile changes of MSC differentiation were achieved.

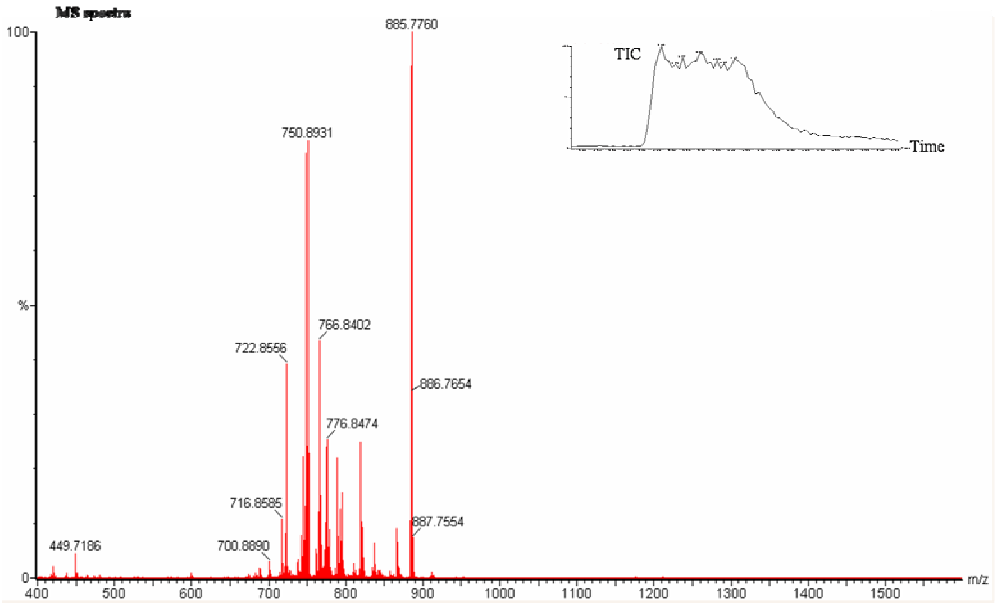


Figure 2-1: Combined mass spectrometry (MS) spectra obtained from Masslynx software.

Insert represents lipid chromatogram.

2.10.2 MRM

Similarly, lipid chromatograms were combined to generate combined spectra and its corresponding spectrum list using Analyst 1.4.2 software (Applied Biosystems. Foster City, CA, Foster City, CA). The intensities of individual ions were compared with their corresponding internal standard species in order to obtain their analogous concentrations. This was subsequently normalized to their respective DNA amounts. Finally, the mean concentration of each ion was tabulated and the results were expressed as relative content of differentiated MSC (Adipo) compared with that of undifferentiated MSC (UD).

2.10.3 Statistical analysis

Comparison of the undifferentiated MSC (UD) and differentiated MSC (Adipo) was performed using the mean of at least three independent biological replicates ± standard deviation (SD) from individual samples. Statistical significance between them was determined using Student's *t* –test, with significance level set at $p < 0.05$. In addition, one way ANOVA followed by Post Hoc tests, Bonferroni and Tukey, were also carried out to determine the statistical significance within each condition (UD and Adipo) over three timepoints, day 7, day 14 and day 21.

RESULTS

3 Results

3.1 Validation of adipogenesis

3.1.1 Morphological characterization

Adipogenic differentiation is characterized by the development of lipid droplets (LD) within the cell cytoplasm. MSC in the presence of adipogenic cocktail, thereafter denotes as Adipo, will develop LD (Pittenger *et al.*, 1999) (Figure 3-1). The number of cells with LD increases proportionally with duration of MSC exposure to the hormonal cocktail. In addition, larger LD are observed in day 21 adipogenic cultures as compared to those from day 14 and day 7. This implies that the LD could have either fused together as adipogenesis progresses or they may have grown overtime. As for the undifferentiated MSC (UD), their morphology remains relatively the same over all three timepoints. Due to the overconfluence of cultures, which is required for adipogenesis to take place, the fibroblastic morphology of MSC is not clearly distinguishable.

In order to histologically prove that the observed LD are indeed LD constituting neutral lipid, Oil Red O (and hematoxylin) staining is employed (Figure 3-2). Oil Red O stains neutral lipids red (Pearse, 1968), while hematoxylin stains chromatin blue (Mayer, 1903). As expected, all LD are stained red. The low magnification images illustrate the extent of Oil Red O staining employed as measure of differentiation. The greater intensity of Oil Red O staining in the day 21 adipogenic culture is a culmination of a greater proportion of cells containing LD and an increase in the size of the LD, thereby validating our light microscopy findings. In the UD, there is an absence of red staining. This shows that there is a lack of LD

observed in UD, this entails that there is no spontaneous differentiation in these cultures and they thus remain in their undifferentiated state.

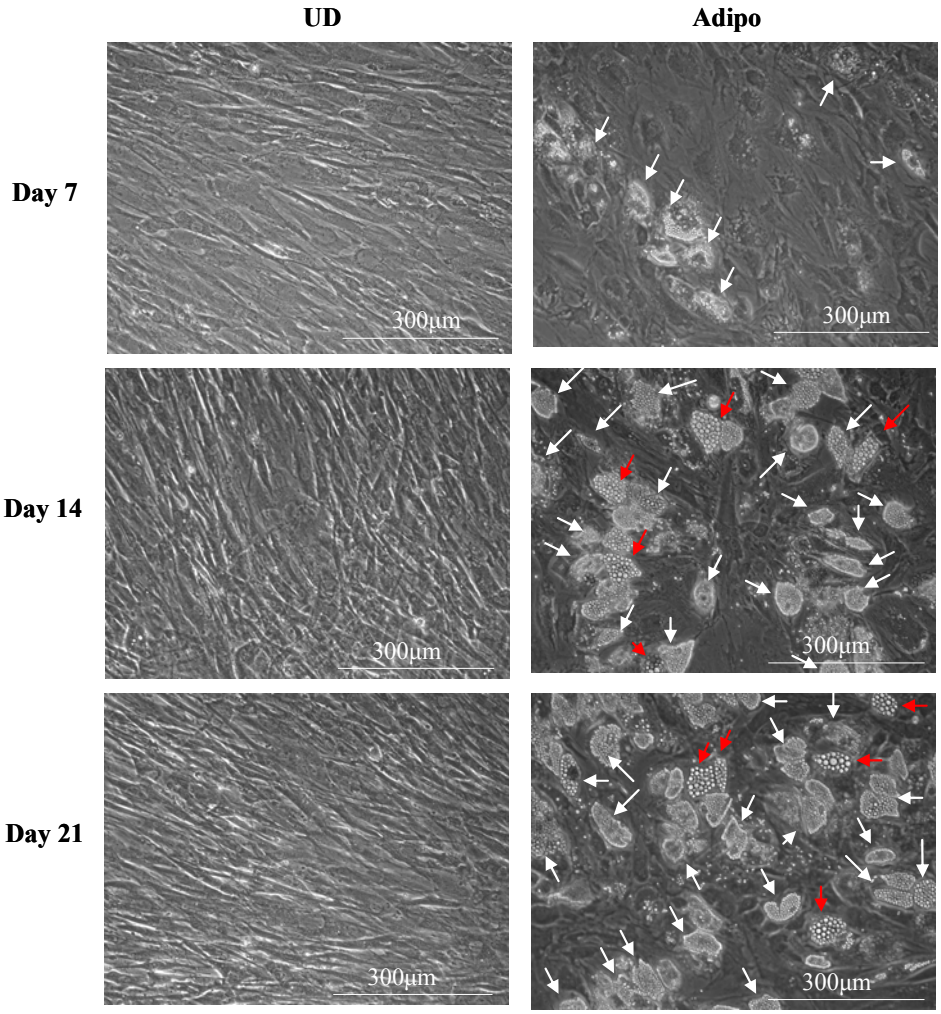


Figure 3-1: Morphological observations of undifferentiated MSC (UD) and adipocytes (Adipo) at day 7, day 14 and day 21.

UD: undifferentiated MSC; Adipo: differentiated MSC, adipocytes; Lipid Droplets: LD. White arrows point small LD, red arrows point large LD.

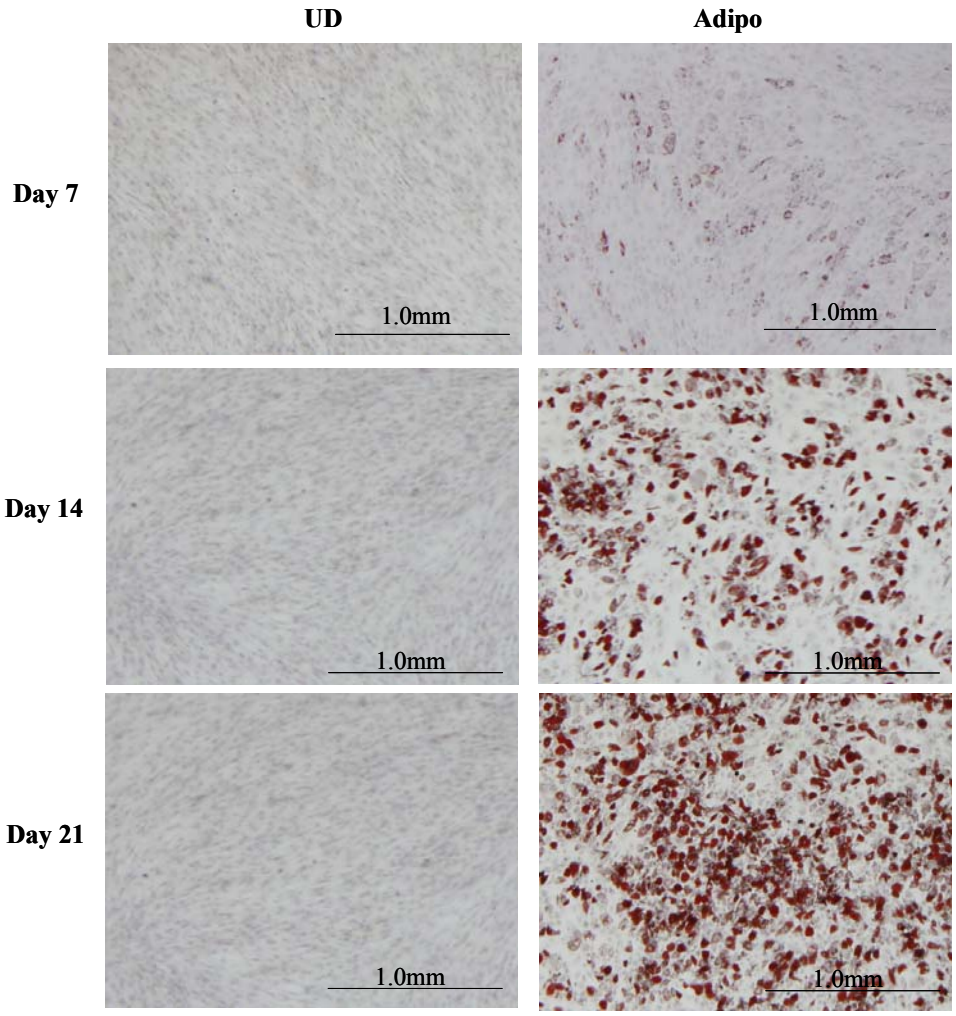


Figure 3-2: Histochemical Oil Red O and hematoxylin staining of UD and Adipo cultures at day 7, day 14 and day 21.

3.1.2 Quantitative aspect of adipogenesis

In the present study, Oil Red O staining is employed to qualitatively estimate the extent of differentiation. In order to quantitate extent of MSC adipogenic differentiation, Nile Red staining and flow cytometry are employed. Cells detected on the FL2 channel are gated and counted by marker 1 (M1). Consistent with our microscopic observations (Figures 3-1 and 3-2), no adipocytes were observed in undifferentiated MSC cultures (the small amount of cells (< 1%) gated in M1

represents auto-fluorescence commonly observed in flow cytometry of UD). For the adipogenic samples, 21.95% of cells contain neutral lipids following 7 days of adipogenic stimulation, which further increase to 35.40% by day 14 (Figure 3-3A) again validating our working hypothesis that extent of adipogenesis increases as a function of time. At day 21, the percentage of cells, 36.03%, remains almost similar to that of day 14 (Figure 3-3A). This observation does not comply with those seen in the histochemical staining using Oil Red O (Figure 3-2). The lower percentage value in day 21 is due to the loss of mature adipocytes during the preparation of sample. Usually, the cell pellet is collected, stained with Nile red and analysed by flow cytometry (Materials and Methods: 2.3). However, unique to day 21 adipogenic samples, floating materials are observed in the supernatant. When the floating materials are subjected to the same protocol, 88.02% of cells are detected on the FL2 channel (Figure 3-3B). As these FL2 positive events were of a similar (or greater) size and granularity (measured as FSC and SSC respectively) than adipocytes, suggests that these were more mature adipocytes which could not be pelleted due to their increased buoyancy (larger LD) (Appendix 4). This implies that there is probable terminal differentiation at day 21. On the whole, when MSC are subjected to adipogenic hormonal inducers for increasing periods of time, there is increasing extent of adipogenesis, thereby validating observations seen above (Figures 3-1 and 3-2).

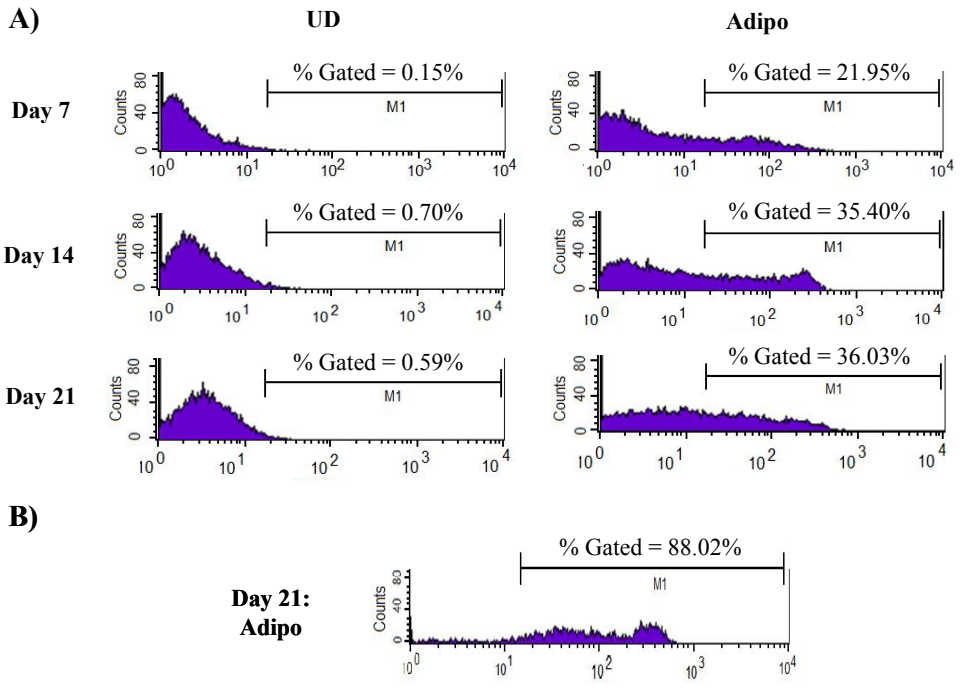


Figure 3-3: Quantitation of cells containing LD.

A) FACS analysis of UD and Adipo samples at day 7, 14 and 21. B) FACS analysis of floating material in day 21 adipo sample.

3.1.3 Expression of genes related to adipogenesis

Formation of LD is a phenotypic determination that adipogenesis occurs. To demonstrate that adipogenesis also occurred transcriptionally, real time-polymerase chain reaction (Real time PCR) is employed to determine changes in mRNA expression of adipogenic gene markers. The results are illustrated as a relative expression of adipocytes to UD.

C/EBPα is 300 folds more in day 7 adipocytes, which is increased to 400 folds by day 14 and finally to 1000 folds at day 21 (Figure 3-4). On the contrary, C/EBPδ expression is modestly upregulated in day 7, at about 2.3 folds more than UD. By day 14 and day 21, C/EBPδ expression is similar to that of UD (Figure 3-4). This

implies that expression of C/EBPδ is probably required during the initial phase of adipogenesis. The expression of C/EBPδ could assist in the expression and activation of C/EBPα, thus the initial upregulation of C/EBPδ and the perpetual upregulation of C/EBPα.

Gene expression of PPARγ1 and PPARγ2 follow a similar trend as C/EBPα. PPARγ1 is upregulated by 30 folds in day 7, followed by 50 folds in day 14 and finally by 160 folds in day 21 (Figure 3-4). PPARγ2 is also upregulated by 1600 folds in day 7, which increased to 2300 folds in day 14 and finally 15000 folds in day 21 (Figure 3-4). PPARγ are transcription factors that regulate adipogenesis. An increase in their expression level suggests that there is the occurrence of adipogenesis in the differentiated MSC. Since both PPARγ and C/EBPα behave in an upward trend manner, this seems to suggest that they may have synergistic relationship in regulating adipogenesis. Furthermore, the relative expression for PPARγ2 is greater than PPARγ1. This indicates that the differentiated MSC could be adipocytes as PPARγ2 is more abundantly expressed in adipocytes.

LPL and aP2 are the downstream adipogenic genes. They are expressed upon activation by transcription factors such as C/EBPα and PPARγ. LPL is expressed at 45000 folds more than UD at day 7. The relative expression decreases to 20000 folds at day 14. Nonetheless, LPL is still upregulated in adipocytes at day 14. At day 21, LPL relative expression is at 34000 folds (Figure 3-4). Similarly, gene expression of aP2 steadily increases from 39000 folds to 125000 folds and finally to 905000 folds at days 7, 14 and 21 respectively (Figure 3-4). Such gene expression profile illustrates that MSC indeed undergoes adipogenesis.

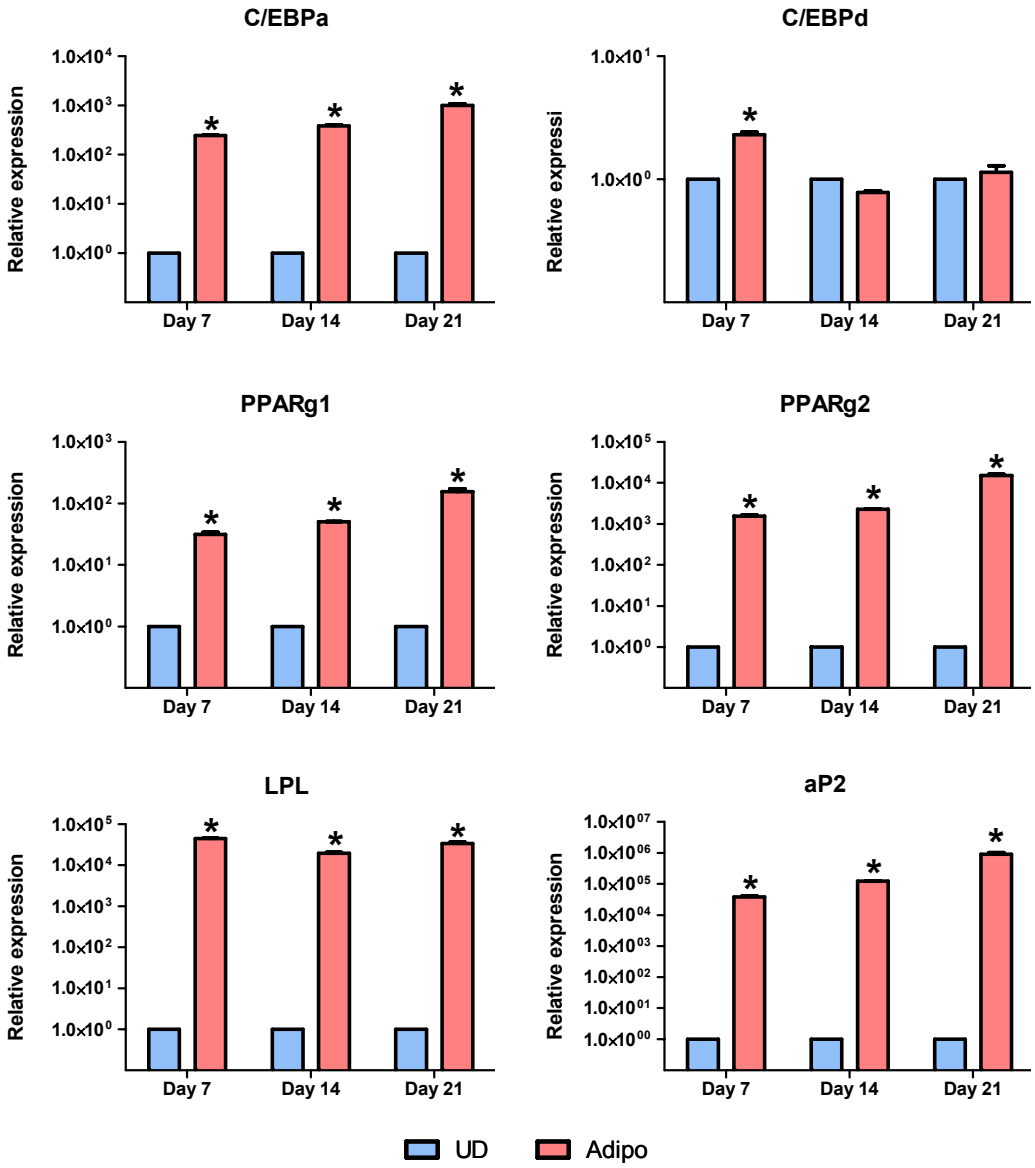


Figure 3-4: Comparison of mRNA transcript levels between UD and adipo overtime using real time PCR analysis.

Each bar represents the mean and standard deviation of n = 3 independent samples. * represents at least 2.5 fold change and $p < 0.05$, significantly different between UD and Adipo.

C/EBPa: CCAAT enhancer binding protein α; C/EBPd: CCAAT enhancer binding protein δ; PPARg1: peroxisome proliferators-activated receptor γ 1; PPARg2: peroxisome proliferators-activated receptor γ 2; LPL: lipoprotein lipase; aP2: fatty acid-binding protein.

3.2 Lipid profiling

3.2.1 Thin Layer Chromatography (TLC)

After validating the differentiative status of MSC (i.e. whether it is maintained at an undifferentiated state or differentiated state), we can now extract lipids using an optimized method to determine the changes in lipid profile between UD and adipocytes. TLC is adopted to acquire a general overview of how lipids change when MSC undergo adipogenesis. Hexane:Diethyl ether:Formic acid (HEFA) and Chloroform:Methanol:Water (C:M:W) systems are used to study the separation of neutral and polar lipids respectively. In this study, only day 0 and day 21 samples are examined. This is to illustrate the distinct differences in lipid types and amounts between UD and adipocytes when equal amounts of total lipids are loaded into each system. By comparing the position of each spot to those of standards, identity of spots is revealed. Using the NIH ImageJ software, densitometric values of each lipid class is derived. After which, these values are normalised to their respective total DNA amounts.

Intuitively, neutral lipids are more abundantly available in adipocytes than in UD (Figure 3-5A, 3-5B). With the same amount of lipids being loaded for each condition, there is a dramatic increase of TAG in day 21 adipocyte samples. In the UD samples, there is negligible amount of TAG. This suggests that accumulation of TAG is characteristic to adipocytes which comply with the commonly known observation. Similarly, there is also more MAG in adipocyte samples than in UD. MAG acts as a precursor to TAG synthesis. The presence of more MAG in adipocytes suggests its role in TAG biosynthetic pathway to satisfy the increased need for TAG. Cholesterol levels remain relatively the same in all three samples.

This implies that cholesterol is tightly regulated during adipogenesis which conforms to literature reports.

Unexpectedly, there are lower amounts of phospholipids in the adipocytes than in the UD when equal amounts of lipids are spotted for each condition (Figure 3-5C, 3-5D). Between day 0 and day 21 UD samples, there is minimal difference in the phospholipids changes. This implies that MSC in maintenance culture over prolonged periods do not affect the phospholipid levels. However, when MSC undergoes adipogenesis, PE decreases by 40% in day 21 samples, while PC decreases by 25%. PS possesses the greatest decrease of 50%. This illustrates that phospholipids decrease when MSC undergoes adipogenesis.

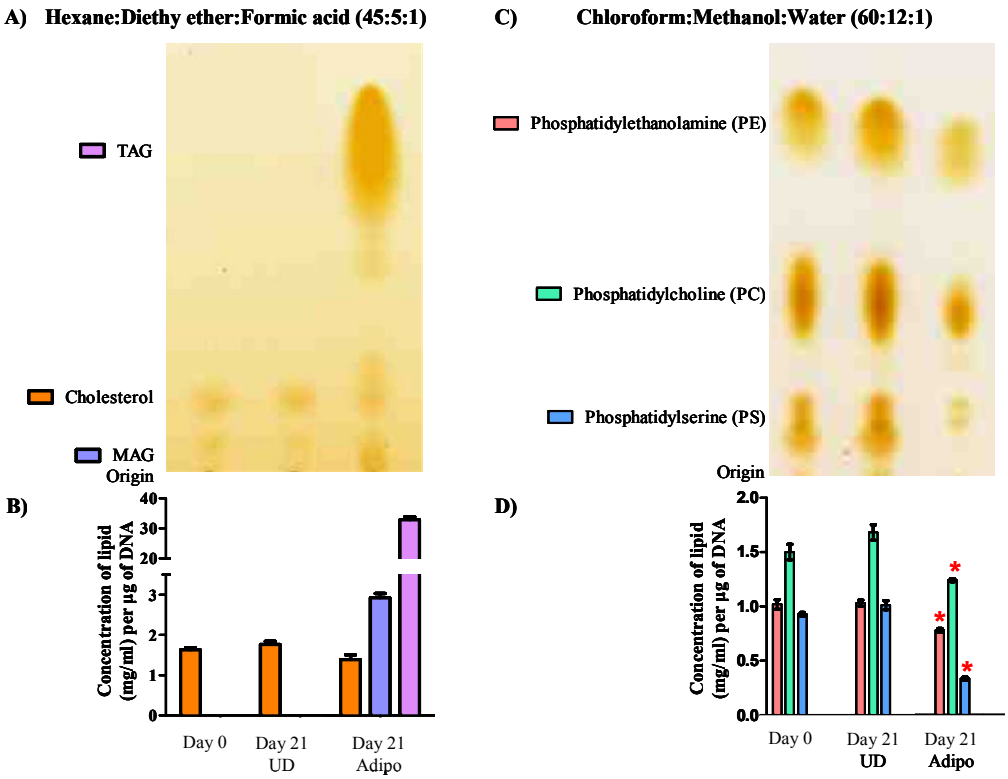


Figure 3-5: General lipid profile.

A) TLC of day 0, day 21 UD and day 21 Adipo samples using HEFA system. B) Densitometry analysis of neutral lipids using NIH ImageJ software. C) TLC of day 0, day 21 UD and day 21 Adipo samples using C:M:W system. D) Densitometry analysis of phospholipids using NIH ImageJ software. Each bar represents the mean and standard deviation of n = 3 independent samples. * represents $p < 0.05$, significantly different between UD and Adipo.

3.2.2 Quantification of triacylglycerols (TAG) species

As exemplified by TLC, when MSC undergoes adipogenesis, there is accumulation of TAG. In order to validate and quantitate the increase in TAG accumulation as MSC differentiates down the adipogenic lineage, selected ion monitoring (SIM), one of the MS methods, is used. This MS method is programmed such that Q3 is set at pre-determined values. Consequently, SIM records intensity of parent ions that possess daughter ions of interest upon collision induced dissociation (CID). For each resulting ion intensity value, it is normalized to the TAG standard and their

corresponding total DNA amounts, followed by the computation of the mean value and finally expressed as relative abundance between adipocytes and UD, which is illustrated by the coloured bars (Figure 3-6).

At each timepoint, there is more TAG in adipocytes than in UD. Taken together, there is increasing abundance of TAG in adipocytes over the three timepoints (Figure 3-6). This demonstrates the buildup of TAG in adipocytes when adipogenesis progresses, which substantiates the results shown in TLC. Amongst the increase, there are some TAG species that exhibit greater extent of enhancement. These TAG species comprise of fatty acyl chains with three or less double bonds, with the majority going to those with three and two double bonds. This suggests that saturated and/or monounsaturated fatty acids are preferentially used for the synthesis of TAG during adipogenesis. In addition, TAG species that increase dramatically over the three timepoints encompass 44 to 58 carbon length. This implies that each of the fatty acyl chain of interest may be made up of 14 to 20 carbons.

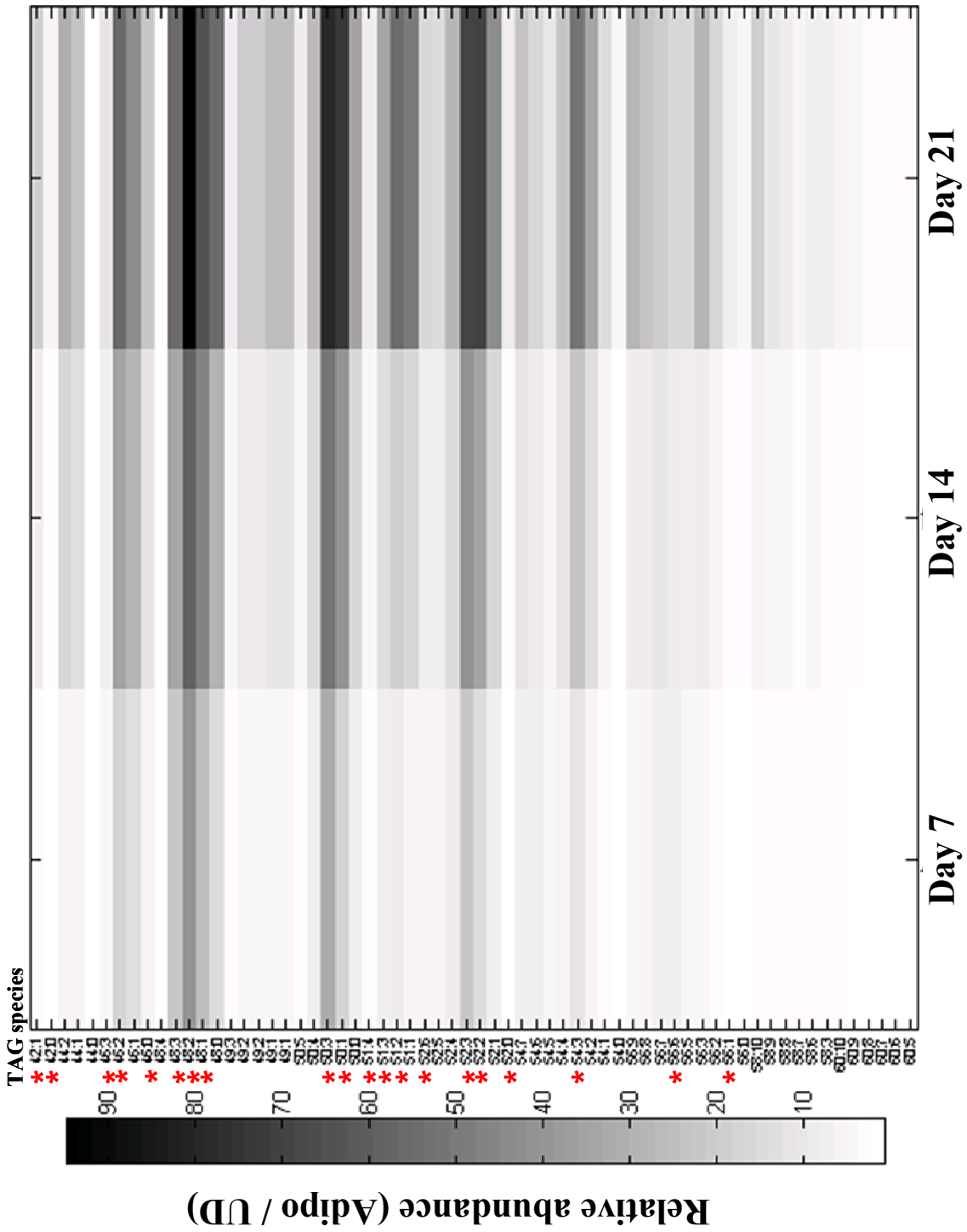


Figure 3-6: Relative abundance of TAG between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single TAG species. * represents $p < 0.05$, significantly different across three timepoints.

3.2.3 Non-targeted profiling of lipids in MSC undergoing adipogenesis

From the TLC analysis, general overview of changes in lipids during adipogenesis is determined. An unexpected decrease of PE, PC and PS are discovered in adipocytes. However, the remaining classes of phospholipids, such as PA, PI and PG, are not resolved distinctly. In order to investigate deeper into the reduced levels of phospholipids during adipogenesis, a more sensitive technique, such as mass spectrometry (MS) needs to be adopted.

There are many MS methods available. The strategy in characterizing phospholipid profile when MSC undergoes adipogenesis is to first adopt a non-targeted approach to obtain an unbiased representation of phospholipids in UD and adipocytes. This is carried out using single scan electrospray ionization MS (ESI-MS). Briefly, single scan ESI-MS comprises of introducing the sample (in this case, the total lipid extract) into the mass spectrometer via ESI method. Individual ionized molecule is then allowed to travel through the electric and magnetic fields within the machine and finally detected based on its nominal mass to charge (m/z) ratio.

For a particular experimental condition (for example the UD), each ion intensity is normalized to the total sum of ion intensities for that condition. Next, the normalized data is aligned to a reference set and the mean normalized intensity for each ion is determined. Subsequently, the mean spectrum from one experimental condition, such as the adipocytes, is warped against the control condition, UD. The relative difference between the two conditions is computed, expressed in the logarithm scale and illustrated in the form of up/down plots (Figure 3-7). Ions that depict positive values indicate that there are more of these ions in the adipocytes than in UD and

vice versa. Noise level spans from -0.1 to 0.1 on the y-axis. Consequently, probable lipid ions are those beyond this range. By simple arithmetic calculations, logarithm ratio of 0.1 refers to a 1.2 folds difference. Hence, ions with at least 1.2 folds differences for all timepoints are taken into considerations for other deeper MS analysis that is to be discussed in the next section. For simplicity, only ions with distinct peaks are illustrated and elaborated in the following up/down plots.

Most of the ions fall between the m/z region of 650 and 900; while some are being detected at the lower range of 400 to 650 (Figure 3-7). Previous knowledge tells us that phospholipids are ionized to give m/z values within the range of 650 to 900 and lysophospholipids at 400 to 650.

At day 7, there are subtle lipid changes between UD and adipocytes (Figure 3-7A). Ions that exhibit logarithm ratio (log ratio) of about 0.2 to 0.4 represent those that are greater in amounts in adipocytes (i.e. increased). Those ions that are lower in amounts in adipocytes (i.e. decreased) also illustrate similar log ratio in the negative sense. At day 14, the lipid ion changes become more definite (Figure 3-7B). Not only are there more lipid ions that display distinct changes, their relative differences are also greater in value, ranging from log ratio of 0.4 to 0.8 and -0.2 to -1. By day 21, there are dramatic lipid differences between UD and adipocytes (Figure 3-7C). Lipid ions that are more abundant in adipocytes are reduced to three, namely 745.9, 773.9 and 801. Despite this, these ions illustrate log ratio of 0.4 to 1. This translates to 2.5 to 10 folds increase. On the other hand, the number of lipid ions that are decreased remained the same. However, their relative differences are greatly decreased with respect to UD, exhibiting folds decrease of 2.5 to 40. In general, over the three

timepoints, there is increasing number of peaks in the "down" plots and their fold changes are also greater, especially within the 650 to 900 m/z range. This suggests that there is decreasing amounts of phospholipids in adipocytes overtime, which validates the TLC results.

Through theoretical calculations, tentative lipid identity can be assigned to each of the m/z values. 773.9, which refers to 36:2 PG, is steadily increasing from day 7 to day 21 (Figure 3-7; Table 3-1). Interestingly, it is the only lipid ion that increases throughout the three timepoints. There are other ions, 745.9 and 801, that start increasing from day 14 onwards. They are also PG in nature, namely 34:2 PG and 38:2 PG respectively (Figure 3-7; Table 3-1). This seems to indicate that PG increases as adipogenesis progresses.

Besides the observed increase of lipid ions in adipocytes, majority of the ions illustrate a downward trend from day 7 to day 21. 559.4 (18:0 LPI) shows a striking decrease from being increased by 2 folds at day 7 to being decreased by 5 folds at day 14. By day 21, it decreases by 14 folds (Figure 3-7; Table 3-1). There are also other PI ions, such as 571.5 (16:0 LPI) and 865.9 (36:0 PI), that display perpetual decrease as adipogenesis progresses. Although 859.8 (36:3 PI) and 885.2 (38:4 PI) are more abundant in the adipocytes at day 7 and day 14, their fold change decreases from 3 folds to 2.5 folds respectively. By day 21, there is relatively similar amount in both UD and adipocytes. This suggests that there is progressive decrease of PI during adipogenesis and that the resultant metabolites may be vital to adipogenic differentiation.

Other than the PIs, there are ions such as 450.4 (16:1a LPE), 690.5 (32:0a PE or 30:0a PC) and 734.8 (32:0 PS) that demonstrate similar downward trend. Their log ratio decreases from about 0.3 to 0.2 during day 7 and day 14 respectively (Figure 3-7). By day 21, their log ratio further decreases to almost zero (Figure 3-7; Table 3-1). This indicates that there is a transient decrease in such phospholipids during adipogenesis. Based on their fatty acyl constituents, they can be described as saturated phospholipids (i.e. phospholipids comprising of saturated fatty acids). This further implies that enzymes, such as desaturases, may be activated at later stage of adipogenesis.

Furthermore, there are ions that are perpetually at lower amounts in adipocytes over the three timepoints. A majority of these ions fall in the PE (684.8, 702.8, 744.9, 790.7) phospholipid class, of which 684.8 (32:3a PE) illustrates the most significant decrease over the three timepoints (Figure 3-7; Table 3-1). This suggests that PE experience tremendous decrease during adipogenesis and that major structural changes may have occurred as PE is the second most common structural phospholipid in cellular membranes.

Lastly, lipid ions from other classes such as PC (744.9, 766.6) and PS (814.8, 837.8) also decrease steadily from day 7 to day 21(Figure 3-7; Table 3-1). This further reiterates that phospholipids decrease during adipogenic differentiation.

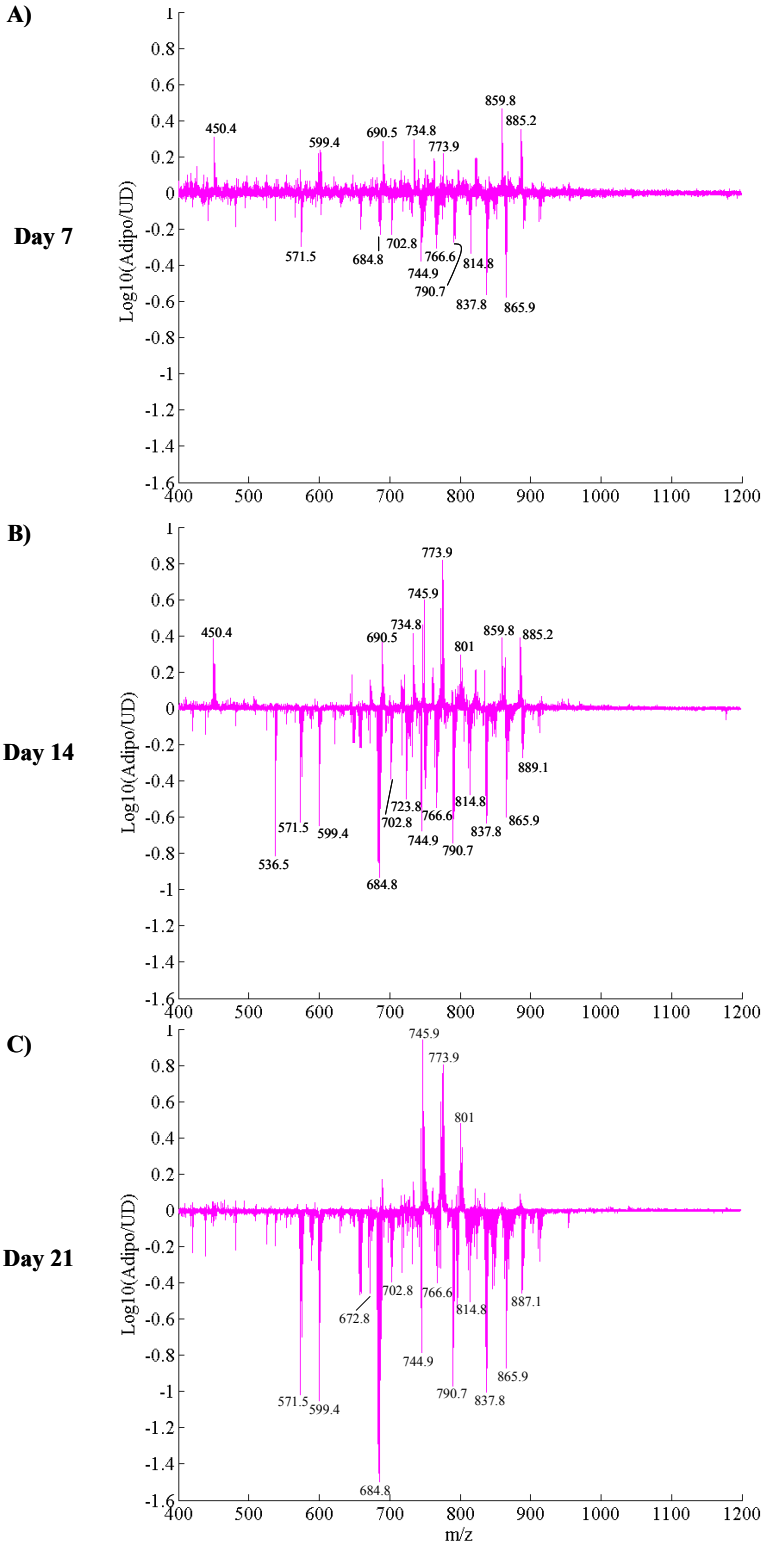


Figure 3-7: Up/Down plots of non-targeted phospholipid profile.

A) day 7, B) day 14 and C) day 21. Data are presented as means from three independent experiments.

UPWARD TREND					
m/z	**PG**	**PE**	**PC**	**PS**	**PI**
Perpetual increase					
773.9	36:2 PG				
Increase from Day 14 to Day 21					
745.9	34:2 PG				
801	38:2 PG				

DOWNWARD TREND					
	PG	**PE**	**PC**	**PS**	**PI**
Greater in amounts at Day 7 and Day 14, but same/lower at Day 21					
450.4		16:1 LPE			
599.4					18:0 LPI
690.5		32:0 PE	30:0 PC		
734.8				32:0 PS	
859.8					36:3 PI
885.2					38:4 PI
Perpetual decrease					
571.5					16:0 LPI
684.8		32:3 PE			
702.8		34:0p / 34:1e PE			
744.9		36:1 PE	34:1 PC		
766.6			38:4 PC		
790.7		40:6 PE			
814.8				38:2 PS	
837.8				40:4 PS	
865.9					36:0 PI

Table 3-1: Summary of phospholipid ion changes.

3.2.4 Tandem MS

Although the single scan MS provides the spectrum of ions that are present in adipocytes and UD, it does not illustrate the identity of these ions, such as the type of fatty acyl chains bound to each phospholipid ion. In order to acquire the identity of phospholipid species present, tandem MS is used. Briefly, tandem MS comprise of fragmenting a single specified ion into its daughter ions via CID. Subsequently, ion intensity of all daughter ions are recorded and illustrated in the form of a spectrum (Figure 3-8). An example using m/z 885 is demonstrated. Tandem MS of m/z 885 illustrates the presence of characteristic daughter ions for phospholipid at m/z 78, 96 and 153, thus verifies that m/z 885 is a phospholipid. In addition, m/z 885 dissociates to yield inositol containing fragment at m/z 241. This indicates that m/z 885 is a PI. Besides this, m/z 283 and 303 are ions with the next highest ion intensity. Since these

two ions translate to FA 18:0 and FA 20:4, they are considered the major fatty acyl groups for m/z 885. Hence, m/z 885 is 38:4 PI. Similar analysis is done for the remaining m/z values illustrated in the single scan.

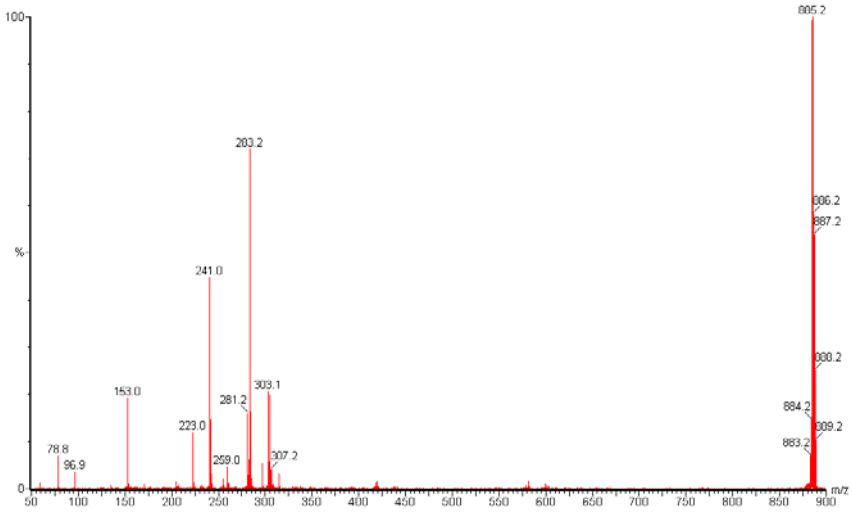


Figure 3-8: Tandem MS of m/z 885.

3.2.5 Precursor Ion Scanning (PREIS)

In addition to tandem MS, PREIS is also carried out to classify ions in their respective phospholipid classes. Different phospholipid classes possess different daughter fragment ions (Appendix 2). There are six phospholipid classes that are of interest. PI and PE each have their own unique fragment structure with m/z value of 241 and 196 respectively in the negative mode. Ions that fall into the PS category loose its amide group, which makes up the m/z value of 87, upon CID. Thus, a neutral loss of 87 in the negative mode implies the PS nature in these ions. PG and PA possess a fragment structure with m/z value of 153 in the negative mode. For PC, it comprises of a product ion of m/z value of 184 in the positive mode. Ions that yield a specific daughter ion upon CID are illustrated in the form of a spectrum.

Using precursor of 196 as an example, the spectrum of ions illustrates ions that generate daughter ion with m/z value of 196 upon CID (Figure 3-9). This shows that these ions are of PE in nature since m/z value of 196 reflects the ethanolamine headgroup of PE. After identifying all ions in the spectrum, those that correspond to the m/z values in the single scan MS and possess at least 1.5 folds difference are used to build the MRM transitions list. PREIS is carried out for all samples and similar analysis are done.

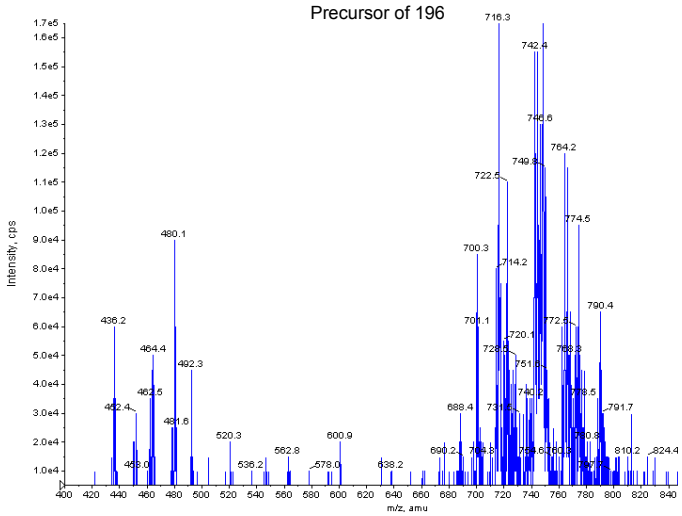


Figure 3-9: PREIS spectrum for PE.

3.2.6 Quantification of phospholipid species

Single scan MS provides a spectrum of ions that are possibly found in UD and adipocytes. Results illustrate prominent reduction of phospholipid amounts in adipocytes. In order to quantify the extent of phospholipids changes, another MS method needs to be adopted. Multiple reaction monitoring (MRM) is a method that selects for parent ions of interest that dissociates to form characteristic daughter ions. For instance, a particular PI has a parent ion of 885. Upon optimized CID, 885 yields daughter ion of 241. Consequently, the programme is designed such that only ions that fit into this criterion (also known as the MRM transition) is recorded, which in

turn provides the ion intensity for 885 PI. Information retrieved from the single scan MS and precursor ion scan is used to build the MRM transition lists (Appendix 3). The final ion intensity of each phospholipid species is normalised to their respective phospholipid standard and total DNA amounts; followed by the computation of mean normalised ion intensity for each phospholipid species. Finally, relative abundance between adipocytes and UD, illustrated by coloured bars, is used to demonstrate the lipid changes during differentiation. Coloured bars denote values indicated on the colour chart. The six phospholipid classes are represented by individual heat plots.

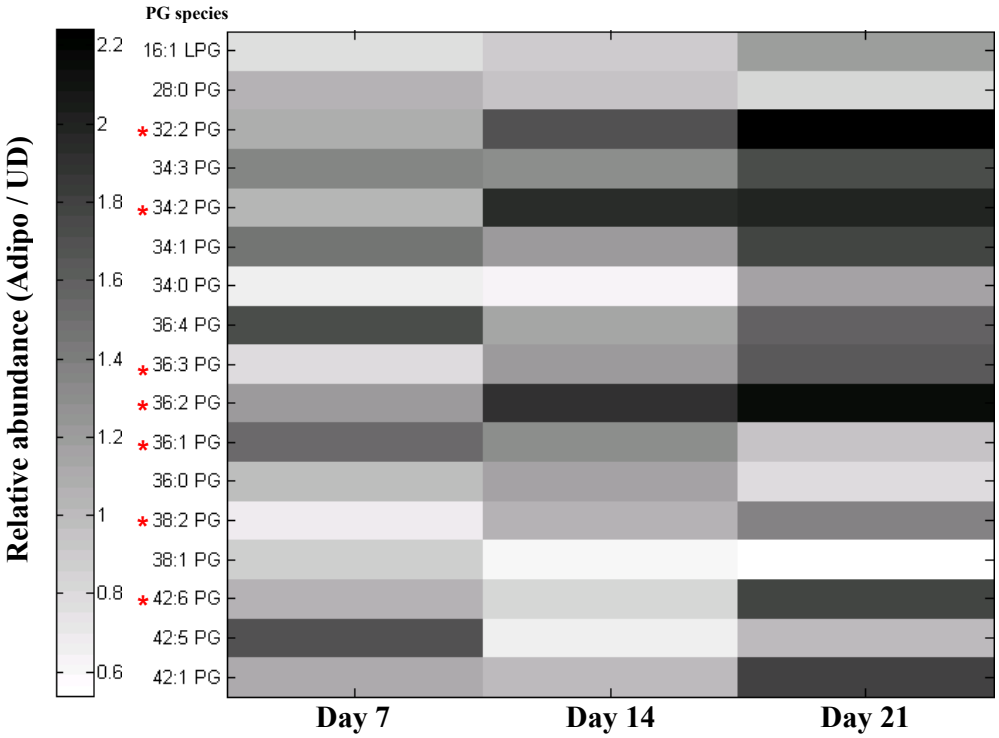


Figure 3-10: Relative abundance of PG between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PG species. * represents $p < 0.05$, significantly different across three timepoints.

In the single scan MS, although a majority of the ions illustrate reduced amounts in the adipocytes, there are some that demonstrate otherwise and they are hypothesized

to be PG in nature. In the MRMs, PG species illustrate an upward trend (Figure 3-10). Consistent with the single scan MS data, 36:2 PG (773.9), 34:2 PG (745.9) and 38:2 PG (801) prominently display increasing amounts in adipocytes over the three timepoints, thereby validating the phenomenon observed in the single scan MS. In addition, there are other species of PG evident in the MRM. They too illustrate an upward trend. The feature in PG lipids that are increased in adipocytes, is that they are comprised of two to three double bonds in their fatty acyl chains. This suggests that there are specific types of PG found in adipocytes.

PG are found in mitochondria membranes. Since there are more PG in adipocytes over the three timepoints, a likelihood is that there are more mitochondria in adipocytes as they differentiate and mature. In order to investigate this hypothesis, the expression of a mitochondria-specific protein, voltage-dependent anion channel (VDAC) protein is determined by western immunoblotting. Densitometric comparison between VDAC value and its respective β-actin is performed to yield the relative abundance of VDAC between adipocytes and UD samples. There is a transient increase of VDAC in adipocytes at day 7, 14 and 21 (Appendix 5).

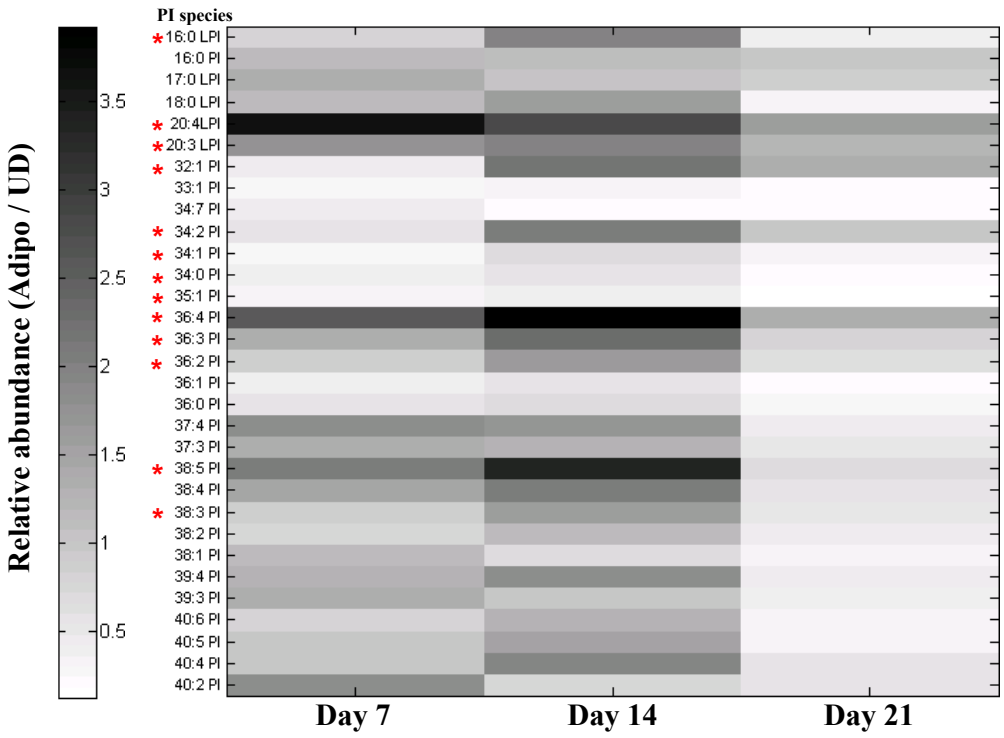


Figure 3-11: Relative abundance of PI between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PI species. * represents $p < 0.05$, significantly different across three timepoints.

At day 7, most PI are more abundant in the adipocytes than in the UD. They continue to behave in this manner at day 14. However, by day 21, there are significantly lower amounts of PI in adipocytes (Figure 3-11). This is also consistent with the single scan MS results, where 36:3 PI (859.8) and 38:5 PI (885:2) are increased at day 7 and day 14. By day 21, no peaks are observed for these ions. Besides this unusual trend, there are ions that illustrate perpetual reduction in adipocytes, such as 36:0 PI (865.9) which is also evident in single scan MS thereby validating our single scan MS data. Although there are some lyso-PI (LPI) and PI that remain slightly more abundant in the adipocytes, such as the 20:4 LPI, 20:3 LPI, 32:1 PI, 34:2 PI and 36:4 PI, these PI experience tremendous reduction in amounts by day 21. This suggests that there is increased metabolism of PI during the later phase of adipogenesis. The resulting

metabolites may aid in the maintenance of the adipogenic phenotype and/or the assist in the progression into terminal differentiation.

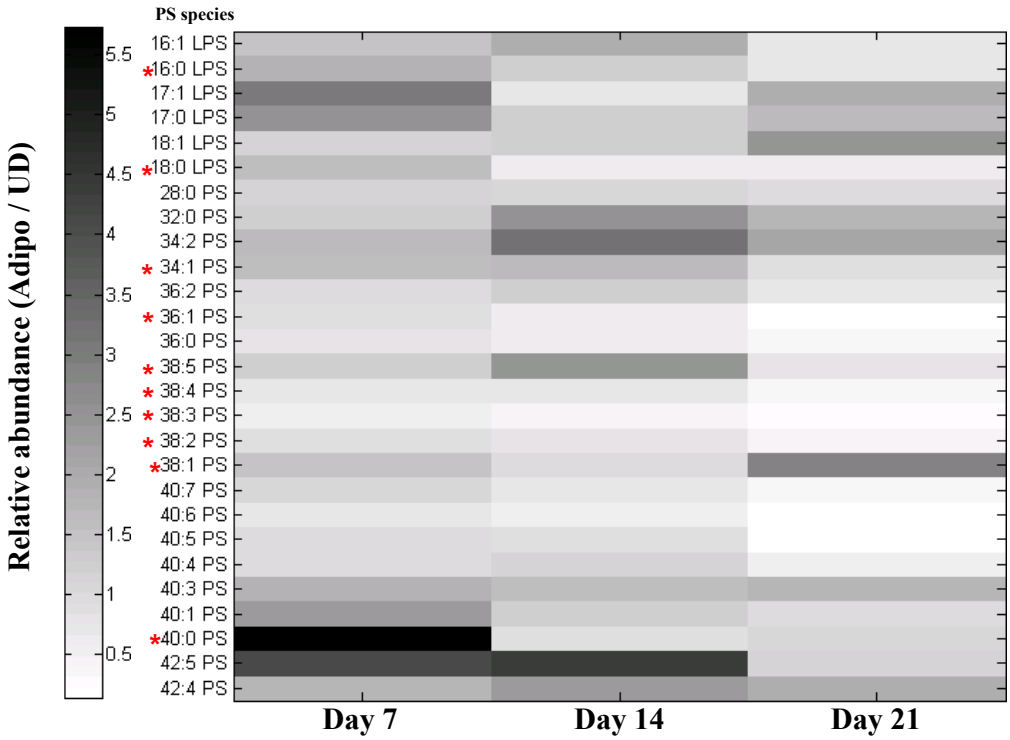


Figure 3-12: Relative abundance of PS between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PS species. * represents $p < 0.05$, significantly different across three timepoints.

As exemplified in the TLC, PS are of lower amounts in the adipocyte. The relative abundance between adipocytes and UD decreases over time (Figure 3-12). This illustrates that PS decrease when MSC differentiate into adipocytes. On the other hand, there are some PS that display an upward trend, namely 18:1 LPS, 32:0 PS, 34:2 PS and 38:1 PS. These PS that increase possess fatty acyl chains that are 16, 18 and /or 20 carbon length, thereby implying the preferential increase for PS with such FA configuration.

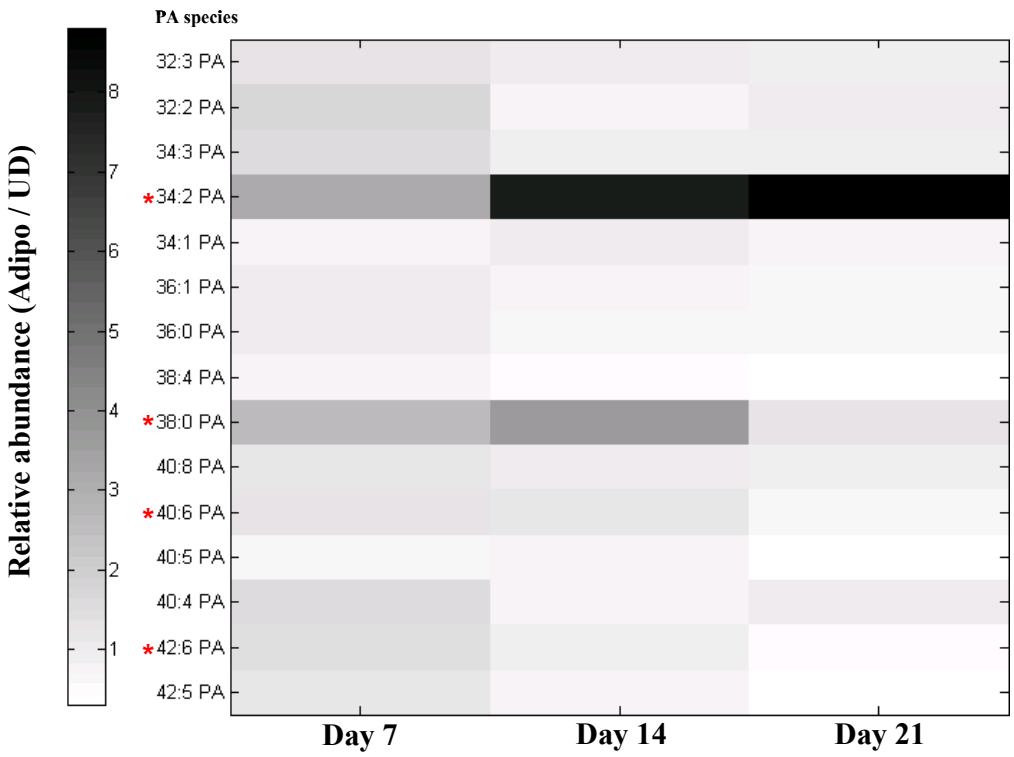


Figure 3-13: Relative abundance of PA between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PA species. * represents $p < 0.05$, significantly different across three timepoints.

Species of PA clearly illustrate a downward trend over the three timepoints (Figure 3-13). Most of the species start off with being more abundant in the adipocytes at day 7. As adipogenesis progresses, the levels of PA in adipocytes decline gradually. This suggests the decreasing amounts of PA in adipocytes as adipogenic differentiation proceeds. Despite the majority of PA species experiencing decrease, 34:2 PA increases steadily throughout the three timepointes. Interestingly, it is the only PA lipid that exhibits such a phenomenon. This implies the importance of this PA species to adipogenesis.

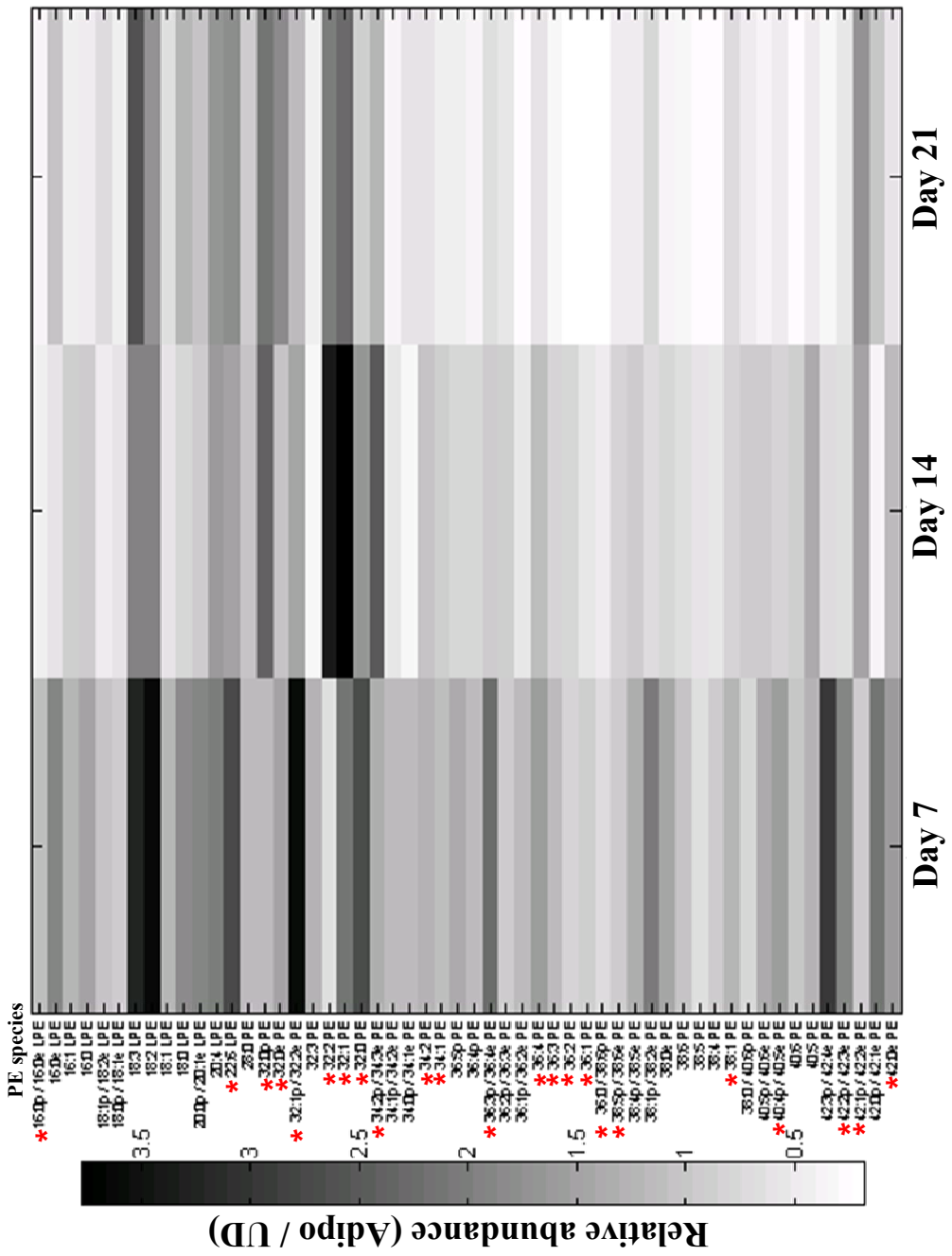


Figure 3-14: Relative abundance of PE between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PE species. * represents $p < 0.05$, significantly different across three timepoints.

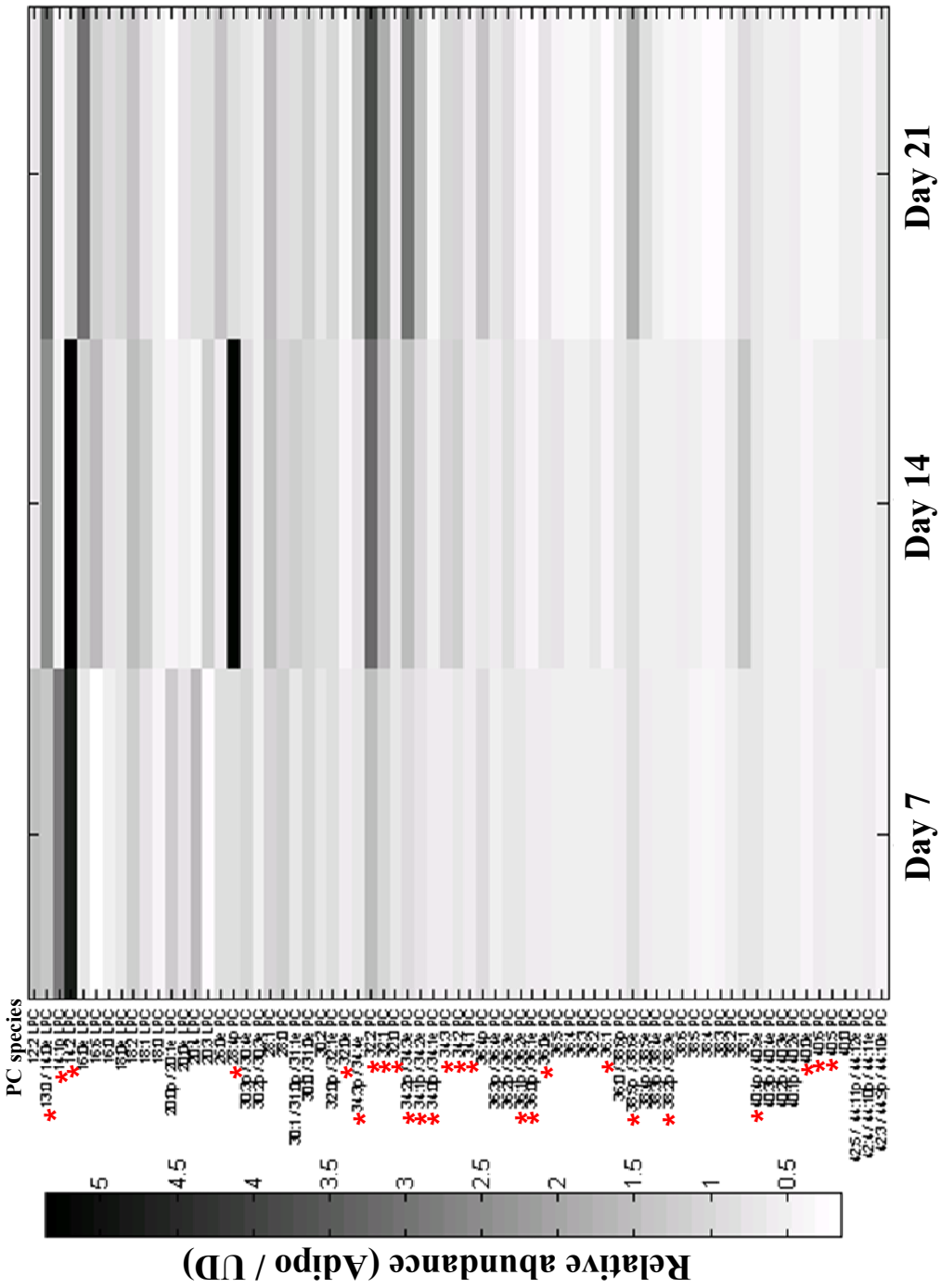


Figure 3-15: Relative abundance of PC between Adipo and UD at day 7, day 14 and day 21.

Each of the colour bars within a row represents mean values from three independent samples. Each row across the heat map illustrates a single PC species. * represents $p < 0.05$, significantly different across three timepoints.

PE exhibit progressive decrease in adipocytes overtime (Figure 3-14). Similar to PA, there are more PE in adipocytes at day 7. By day 14 and day 21, PE decrease in adipocytes, leaving only some of the PE to remain higher in the adipocyte. These exceptional PE are lyso-PE (LPE) (18:3 LPE, 18:2 LPE, 20:4 LPE), PE (32:2 PE and 32:1 PE) and plasmalogen PE (34:2p/34:3e PE, 42:1p/42:2e PE). This suggests that the increase in some of these PE may be catered for the increase need for membrane lipids. As MSC differentiates to adipocytes, MSC undergoes change in cell shape, probable increase in cell size and formation of LD.

PC are of lower amounts in adipocyte at all timepoints (Figure 3-15). Similarly, there are some PC that display transient increase. 32:2 PC and 32:1 PC increase steadily through out the three timepoints. 18:2 LPC also illustrates increase progressively. Since PC are also known for their structural function, the observed increase indicates the role of these PC to satisfy the structural changes involved during differentiation. In addition, there are also plasmalogen PC, such as 34:2p/34:3e PC and 38:5p/38:6e PC, that increase slightly overtime, thereby implying that plasmalogen lipids increase upon adipogenesis. This increase in plasmalogen lipids is also evident in PE, thus presenting a unique lipid signature for adipocyte.

In summary, there are lower amount of phospholipids in adipocytes over the three timepoints, which in turn verifies the results illustrated in the TLC and single scan MS. In spite of the phenomenal decrease, MRM also demonstrate that there are some intriguing lipid species that behaved in the reverse manner. These phospholipids tend to be comprised of 32 to 38 carbon chain length with zero to four double bonds in a single fatty acyl chain (Table 3-2). This suggests that there is preferential inclusion

of such fatty acid into phospholipids when MSC undergoes adipogenesis. Besides the unexpected increase of specific phospholipid species, there is a phospholipid class, PG, which exhibits a surprising overall increase too. This indicates that PG increases upon adipogenic differentiation and can serve as a unique lipid profile to represent adipogenesis.

Carbon Length \ Double Bonds	0	1	2	3	4
32	32:0p PE, 32:0e PE, 32:0 PS	32:1 PC, 32:1 PE, 32:1 PI	32:2 PC, 32:2 PE, 32:2 PG		
34		34:1p PC	34:2 PC, 34:2p PC, 34:2e PC, 34:2p PE, 34:2 PI, 34:2 PS, 34:2 PA, 34:2 PG	34:3 PC, 34:3e PC, 34:3p PC, 34:3e PE	34:4e PC
36	36:0e PC		36:2 PI, 36:2 PG	36:3 PI, 36:3 PG	36:4 PI
38		38:1 PS	38:2 PG		38:4 PI

Table 3-2: Summary of phospholipids species that demonstrate an upward trend over the three timepoints, day 7, day 14 and day 21.

3.3 *Gene expression of Lipins, Lipid Phosphate Phosphatase (LPP) and Phospholipases*

In order to understand the cause for the unexpected decrease in phospholipid levels during adipogenesis, expression of genes related to the biosynthetic and metabolic pathways of phospholipids are investigated. Biosynthesis of phospholipids and TAG are closely related and both have a common precursor, PA. Since there is predictable increase of TAG and surprising decrease of phospholipids during adipogenesis, it will be interesting to study the gene expression level of the enzyme involved in determining the fate of PA, lipin. Mammals possess three lipin forms, namely lipin 1, lipin 2 and lipin 3 (Peterfy *et al.*, 2001). All three possess phosphatidic acid phosphatase (PAP) enzyme activity. In addition to lipin, there is another enzyme that also has PAP activity. That is lipid phosphate phosphatase (LPP) and it comprises of two isoforms LPPa and LPPb. Gene expressions of these genes are examined. Housekeeping genes used and the method of analysis are as described in sections **2.4** and **3.1.3**. The results are expressed in the form of relative expression of adipocytes to UD. Only gene expression with at least 2.5 fold change is considered significant.

Lipin 1 is strongly upregulated and its relative expression increases steadily from day 7 to day 21 (Figure 3-16). At day 7, gene expression of lipin 1 in adipocytes is 2.6 folds higher than UD. This increases further to 6 folds on day 14. By day 21, the relative expression in adipocytes is 12 folds higher than UD. Conversely, there is no significant change in expression level for lipin 2 and lipin 3 as adipogenesis progresses (Figure 3-16). Similarly, there is also no major change in expression level for LPPa and LPPb (Figure 3-16). This implies that lipin 1 exhibits high expression level in adipocytes as compared to UD and increasing presence of lipin 1 may be

responsible for the shift away from the phospholipids and towards the TAG biosynthetic pathway. Hence, the possible cause of decreased phospholipid levels during adipogenesis prevails.

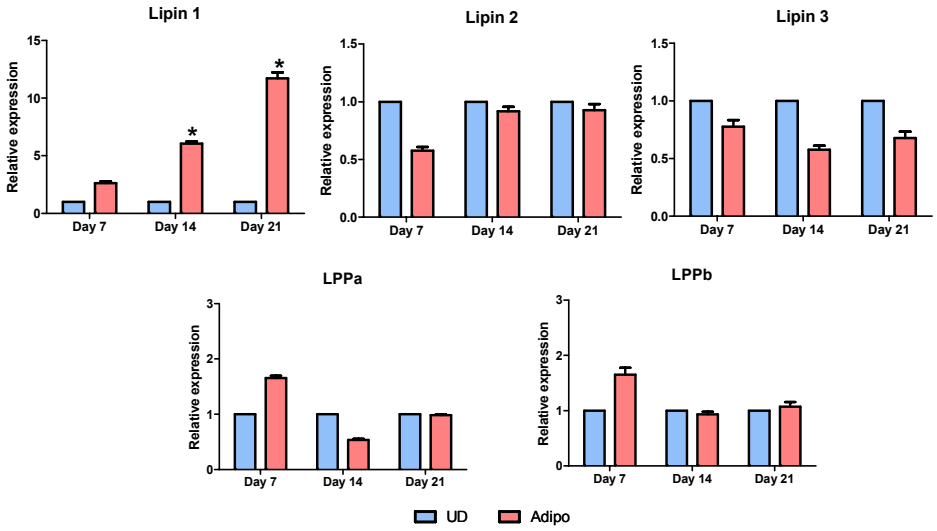


Figure 3-16: Gene expression levels of lipin 1, lipin 2, lipin 3 LPPa and LPPb over three timepoints, day 7, day 14 and day 21 using real time-PCR analysis.

Each bar represents the mean and standard deviation of n = 3 independent samples. * represents at least 2.5 folds change and $p < 0.05$, significantly different between UD and Adipo.

Besides lipins, catabolism of phospholipids can also be used to explain the observed decrease in phospholipids during adipogenesis. The enzyme of interest is the phospholipase. Amongst the many phospholipases, only those that target the *sn*-1 and *sn*-2 positions of the phospholipids, such as phospholipase A_1 A (PLA_1A), phospholipase A_2 (PLA_2) and phospholipase B (PLB) are investigated. Under the PLA_2 category, there is a variety of PLA_2, those that are cytosolic in nature are of interest, namely PLA_2 group 4a (PLA_2 G4a) and PLA_2 group 6 (PLA_2 G6).

PLA_1A is downregulated at day 7 and day 14 (Figure 3-17), where its expression level is 10 and 3 folds lower than UD respectively. By day 21, expression level of PLA_1A is on par with UD. This illustrates that the expression level of PLA_1A is silenced early in adipogenesis and returns to baseline as adipogenesis progresses.

Both PLA_2 isoforms (PLA_2 G4a and PLA_2 G6) diplay similar expression pattern during adipogenesis. There is no significant change in expression of PLA_2s during the first 14 days of differentiation, however 3 folds higher levels of PLA_2 G4a and 4 folds higher levels of PLA_2 G6 are found in day 21 adipocytes compared to UD (Figure 3-17).

PLB does not exhibit any changes in its expression level throughout the three timepoints (Figure 3-17). This suggests that PLB may not play a vital role in adipogenesis. On the whole, PLA_2s possess an upward trend expression level. This implies that proteins that act on the *sn*-2 position of phospholipids are more abundantly available, which suggests that the release of fatty acyl at the *sn*-2 position occurs more prevalently during adipogenesis.

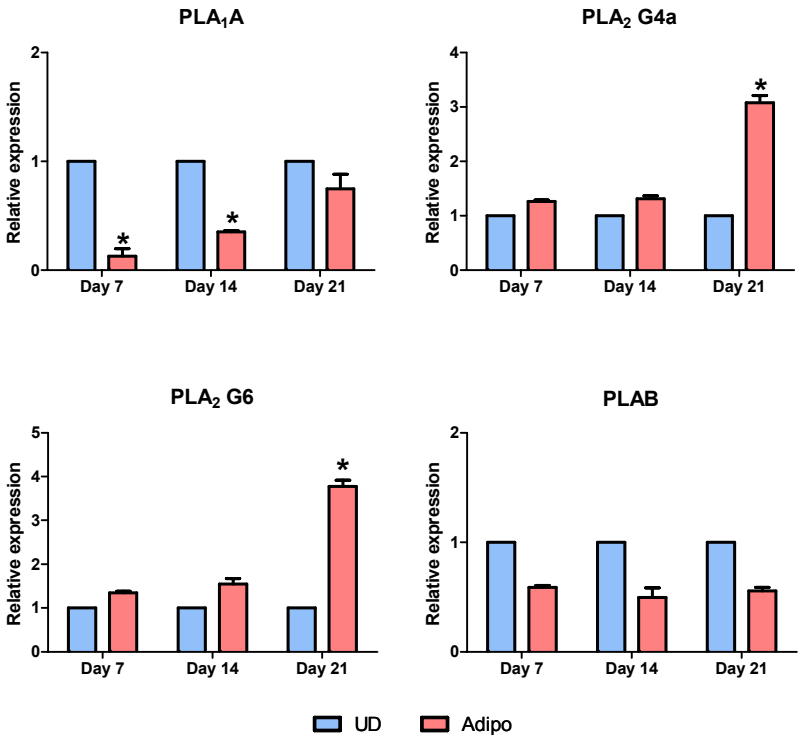


Figure 3-17: Gene expression levels of PLA1A, PLA2 G4a, PLA2 G6 and PLB over three timepoints, day 7, day 14 and day 21 using real time-PCR analysis.

Each bar represents the mean and standard deviation of n = 3 independent samples. * represents at least 2.5 fold change and $p < 0.05$, significantly different between UD and Adipo.

PLA_1A: Phospholipase A_1 A; PLA_2 G4a: Phospholipase A_2 group 4a; PLA_2 G6: Phospholipase A_2 group 6; PLB: Phospholipase B

DISCUSSIONS AND FUTURE DIRECTIONS

4 Discussions and Future Directions

Obesity has been viewed as the top ten health problem by the WHO. With the increasing prevalence not just in affluent societies, but also in developing countries, there is a need to combat this emerging global epidemic. Although dietary habits and lifestyle patterns are the major contributing factors of obesity, intrinsic mechanisms that lead to obesity should also be taken into consideration, so as to achieve a wholesome approach to fighting this problem. Other than hypertrophy of adipocyte, hyperplasia of adipocyte is also an etiology of obesity especially in morbid obesity of humans and rodents (Hirsch *et al.*, 1989). Recently, progenitors from the bone marrow can be recruited to the adipose tissue and differentiate into adipocytes (Crossno *et al.*, 2006). This poses as an alternative source of adipocytes contributing to obesity, thus it is essential to understand the adipogenic pathway of MSC.

Lipids are more than just energy storage and structural entities. They also function as signaling molecules and modulators of inflammatory responses. Importantly, changes in cellular lipid composition modify cellular function. For instance, supplementation of EPA to T-cells leads to modification of fatty acyl composition of phospholipids within their lipid rafts, resulting in suppression of proliferation (Li *et al.*, 2006). In addition, changes to membrane lipid composition lead to changes in the endocytic organization of lipids intracellularly (Mukherjee & Maxfield, 2004). Besides this, alteration to major phospholipid composition in erythrocyte membrane is associated to hyperinsulinemia (Candiloros *et al.*, 1996). Identification of lipidome changes during MSC adipogenesis provides a fresh perspective, bringing us one step closer to understanding the adipose cell development and physiology and battling the globally increasing prevalence of obesity and its related metabolic diseases.

LD formation is characteristic of adipocytes. Through light microscopy, histochemical Oil Red O staining and FACS using Nile red staining, we are able to illustrate and validate the differentiation of MSC to adipocytes. As adipogenesis progresses, some of the cells exhibit few large LD; while others display many small LD. This observation is consistent with those seen in rat stromal vascular cells undergoing adipogenesis, where at later stages of adipogenic differentiation, many of the small LD fused together to from larger ones (Nagayama *et al.*, 2007). In addition, the fusion of LD to form larger LD is also exemplified in 3T3-L1 where observations are made using three-dimensional constructions (Böstrom *et al.*, 2005). This, rules out the possibility that LD "disappear" due to vertical movement resulting in one LD on top of another. Other than the fusion of small LD to from larger ones, LD may also have grown overtime. With each media change in an *in vitro* setting, there is fresh supply of nutrients, this enable more TAG to be synthesized, thus the excess TAG are stored in LD leading to the increase in size of LD.

Furthermore, as an additional measure to ensure definitive adipogenesis, expression levels of adipogenic genes were also investigated. Consistent with phenotypic observations that extent of adipogenesis increases proportionally to time, so too does the expression of adipogenic gene markers. When there is upregulation of transcription factors, C/EBPα and PPARγ, there is upregulation of adipogenic gene markers, LPL and aP2. Coherent with literature findings, the synergistic effects of C/EBPα and PPARγ result in the expression of genes necessary for adipocyte differentiation (Lefterova *et al.*, 2008). Furthermore, the greater expression levels of PPARγ2 as compared to PPARγ1 illustrates that the differentiated MSC are

adipocytes, as PPARγ2 is highly and exclusively expressed in adipocytes (Braissant *et al.*, 1996; Tontonoz *et al.*, 1994).

Conversely, the expression of C/EBPβ is only increased at day 7. At later timepoints, its expression level returns to baseline. This agrees with literature findings that C/EBPβ is expressed early during differentiation (Gregoire *et al.*, 1998) and its expression level subsequently decreases as adipogenesis progresses (Lane *et al.*, 1999). Mitotic expansion occurs prior to differentiation (Tang *et al.*, 2003; Reichert & Eick, 1999; Yeh *et al.*, 1995). Since C/EBPβ is endogenously expressed during clonal expansion (Lane *et al.*, 1999), there is likelihood that C/EBPβ plays a role in mitotic expansion. Furthermore, there is evidence demonstrating that C/EBPβ (-/-) mouse embryonic fibroblasts cannot undergo mitosis (Tang *et al.*, 2003). Phosphorylation of C/EBPβ activates its DNA binding function, which is vital in mitotic clonal expansion (Tang *et al.*, 2005). Following this, cells undergo a second growth arrest, termed G_D (Scott *et al.*, 1982). This marks the point of no return where cells are committed and determined to undergo adipogenesis (Otto & Lane, 2005). This suggests that MSC subjected to the hormonal inducers are committed to the adipogenic lineage after day 7. In addition, reports have illustrated that C/EBPβ speeds up the expression of C/EBPα, which in turn expresses adipogenic genes (Darlington *et al.*, 1998; Yeh *et al.*, 1995). Once C/EBPα is expressed, C/EBPα can regulate its own expression and maintains the adipocyte phenotype (Lin *et al.*, 1993; Tang & Lane, 1999).

After verifying the maintenance of undifferentiated state for MSC (UD) and the adipogenic state for differentiated MSC (Adipo), characterization of their lipids

begins with TLC. TLC has long been adopted to analyse lipids in biological samples. Its ease of use and rapid retrieval of data makes TLC the preferred technique in providing a general overview of lipid changes between different experimental conditions (Wenk, 2004). Under the HEFA system, there is a tremendous increase of TAG in day 21 Adipo sample. This is expected of adipocytes due to the formation of LD which contain mainly TAG (Martin & Parton, 2005).

Amongst the variety of TAG types that are detected using MS, TAG comprising of fatty acyl chains with three or two double bonds and encompassing 44 to 58 carbon lengths possess significant upward trend overtime. This suggests that saturated and/or mostly monounsaturated FA (MUFA) with 14 to 20 carbons are preferentially used for the synthesis of TAG during adipogenesis. AcylCo-A:DAG acyltransferase (DGAT) is the enzyme involved in the biosynthesis of TAG (Weiss *et al.*, 1960). A lack of DGAT2, one of DGAT isoforms, leads to severe reduction of TAG deposition in tissues (Stone *et al.*, 2004), thereby illustrating the significance of DGAT in TAG biosynthesis. Stearoyl-CoA desaturase (SCD) is an enzyme responsible for the synthesis of MUFA (Ntambi & Miyazaki, 2004). SCD-/- mice exhibit considerable decrease in TAG in white adipose tissue and liver (Ntambi *et al.*, 2002; Miyazaki *et al.*, 2000). When they are fed with high fat diet, these mice are resistant to diet-induce obesity and liver steatosis (Ntambi *et al.*, 2002). In spite of these observations, DGAT expression and activity in SCD-/- mice remain similar to that of wild type (WT) (Dobrzyn *et al.*, 2005). This indicates the importance of SCD in TAG production. Besides this, the close proximity of SCD and DGAT in the ER allows enhanced access of MUFA to DGAT for the synthesis of TAG (Man *et al.*, 2006). Consequently, TAG containing MUFA are more abundantly found in

adipocytes. The above described processes can be used to explain the observed increase in specific types of TAG.

In addition, there is more MAG in day 21 Adipo sample from the TLC analysis. In the MAG pathway, it begins with the acylation of MAG with fatty acyl-CoA catalyzed by acylCo-A:MAG acyltransferase (MGAT) to form DAG. Subsequently, the same process occurs with DAG in the presence of DGAT to form TAG (Figure 4-1). Although MAG pathway is commonly demonstrated in the small intestines, it has also been shown to occur in adipose tissue (Polheim *et al.*, 1973). There are three MGAT isoforms, MGAT1, MGAT2 and MGAT3. All three forms are expressed in human adipose tissue (Turkish *et al.*, 2005). Cao *et al.* have illustrated that the activity of MGAT3 is greater than DGAT and proposed that MGAT3 can act as a putative TAG synthase (Cao *et al.*, 2007). This suggests that the abundance of MAG in adipo samples can be used to satisfy the increased need for TAG.

Besides this, based on TLC results, cholesterol remains relatively the same in all day 0, day 21 UD and day 21 adipo samples. This is consistent with literature that cholesterol is tightly regulated by an elaborate network of systems to bring about cellular cholesterol homeostasis (Tabas, 2002; Tall *et al.*, 2002; Simons & Ikonen, 2000). In membranes, cholesterol has higher affinity to phospholipids and sphingolipids with saturated fatty acyl chains (Simons & Vaz, 2004; Simons & Ehehalt, 2002). Lipid rafts are regions in the membrane where there are more tightly packed due to the presence of saturated hydrocarbon chains in raft sphingolipids and phospholipids (Simon & Vaz, 2004). As such, cholesterol has the ability to distinguish between raft and non-raft domains (Simons & Ehehalt, 2002).

Furthermore, cholesterol plays an integral component in membranes as it helps to modulate membrane fluidity and construct semipermeable barrier between cellular compartments (Ikonon, 2008). The lack of cholesterol in membranes can lead to dissociation of membrane proteins and render these proteins inadequate (Simons & Ehehalt, 2002), thereby affecting cellular functions. Besides this, cholesterol itself is a toxic compound and is tightly regulated by an array of mechanisms to maintain any variation within a narrow range (Goldstein & Brown, 2001). Inability to control the levels of cholesterol can lead to pathological diseases, such as atherosclerosis (Maxfield & Tabas, 2005). Since adipogenesis is a normal process, one expects minimal changes in cholesterol, which is exemplified in the TLC.

On the other hand, TLC under the C:M:W system identifies an overall decrease in phospholipids as MSC differentiate into adipocytes. Through the use of MS, more detailed examination of phospholipid classes is achieved. Despite a similar overall decrease in phospholipids, certain specific phospholipids and phospholipid class (e.g. PG) demonstrated to have increased.

Most of the phospholipids that demonstrate transient increase overtime possess a "2 double bond" configuration (i.e. 32:2a PC, 32:2a PE, 34:2 PG, 34:2 PI, etc.). In addition, TAG that exhibit a steady increase throughout time also consists of MUFA within them. This suggests that there is preferential inclusion of MUFA to both phospholipids and TAG. MUFA can be synthesized endogenously or exogenously acquired. In the exogenous context, FA analysis on FBS was carried out using gas chromatography-MS (GC-MS). The results show that MUFA is not abundant in FBS (Appendix 6). In fact, a large percentage of FA in FBS are saturated FA, such as

FA16:0 and FA18:0. This suggests that the source of MUFA derives mainly from *de novo* synthesis. In the ER, SCD acts on fatty acyl-CoA substrates to yield MUFA (Ntambi & Miyazaki, 2004). Reports have displayed that SCD is responsible for the incorporation of MUFA into TAG of 3T3-L1 (Gomez *et al.*, 2002) and that SCD activity increases by 20-100 folds during adipogenic differentiation of 3T3-L1 (Katsuri & Joshi, 1982). Hence, we have shown that the embodiment of MUFA is not only to TAG, but also to phospholipids.

Increased amounts of phospholipids containing MUFA can affect membrane fluidity, in turn influence biological functions. Plasma membrane fluidity is maintained by the ratio of cholesterol to phospholipids and the ratio of saturated to unsaturated FA integrated into phospholipids (Thewke *et al.*, 2000). Since there is an observed increase of MUFA-containing phospholipids, membrane fluidity of adipocytes is likely to be different from that of MSC. A recent report has illustrated that lipid structures regulate function of G protein-coupled receptor (GPCR) (Yang *et al.*, 2005). GPCR43 is found to be expressed in differentiated 3T3-L1 adipocytes and that exogenously added propionate increases the extent of lipid accumulation and also elevates the expression of GPCR43 in 3T3-L1 (Hong *et al.*, 2005). Furthermore, silencing GPCR43 gene expression through small-interfering RNA (siRNA) treatment inhibits adipogenesis (Hong *et al.*, 2005). Similarly, GPR120, another member of the GPCR family, is expressed in differentiated 3T3-L1 and the down-regulation of GPR120 via siRNA also prevents adipogenesis (Gotoh *et al.*, 2007). These evidences illustrate that members of the GPCR play a role in modulating adipogenesis and their functions can be modified by membrane structures. Incorporation of more MUFA containing phospholipids may have modified

membrane structure to better suit the localization of respective GPCRs, e.g. GPCR43 and GPR120, to the membrane, thus allowing more efficient adipogenesis to take place.

Other than the increase in phospholipids containing MUFA, some plasmalogen lipids are found to be more abundant in adipocytes. Recent report documents the presence of plasmalogen PC and PE in adiposomes (Bartz *et al.*, 2007). This suggests that the increased plasmalogen lipids observed in MSC-derived adipocytes is due to the increased and/or larger LD within them. Plasmalogens have been shown to be involved in membrane biogenesis and fusion. Rapid vesicular events are evident at membranes high in plasmalogen composition (Gremo *et al.*, 1985). *In vitro* experiments have also shown that membrane fusion occurs more swiftly with vesicles containing PE plasmalogens (Glaser & Gross, 1994). This suggests that the increased abundance of plasmalogens in adipocytes may be used for the formation of LD. In addition, vinyl-ether linkage in plasmalogen has been reported to be more susceptible to ROS attack (Hahnel *et al.*, 1999; Zoeller *et al.*, 1999; Hagar *et al.*, 1996). As a result, presence of plasmalogens can serve as antioxidants, thereby shielding neighbouring molecules against ROS attack. With report verifying that there is enhanced oxidative phosphorylation in adipocytes (Luo *et al.*, 2008), there will thus be increased release of ROS (Gutterman, 2005). The greater abundance of plasmalogens in adipocytes can serve as a form of protection against ROS.

Surprisingly, PG steadily increases in adipocytes over the three timepoints despite a general decrease in all other classes of phospholipid. Since PG are synthesized and found in mitochondrial membranes (Dowan, 1997), increase in PG maybe related to

an increase in mitochondria density (number of mitochondria per cell). Western immunoblotting of voltage dependent anion channel protein (VDAC), a channel protein found at the outer mitochondria membrane (Colombini, 2004), illustrates a transient increase in adipocytes overtime (Appendix 5). This seems to imply that there is escalating mitochondria as adipogenesis progresses. Literature has demonstrated that there is increase of cytochrome c and mitochondrial heat shock protein 70 by about 20-30 folds during adipogenesis of 3T3-L1 (Wilson-Fritch *et al.,* 2003). Similarly, proteomic analysis of human adipose derived stem cells undergoing adipogenic differentiation also reveals an increase in similar mitochondrial proteins from 8% to more than 18% (DeLany *et al.*, 2005). Light and electron microscopy of the adipocyte progeny from human adipose derived stem cells further validate the increase in mitochondria numbers in adipocytes (Wilson-Fritch *et al.,* 2003).

Mitochondria biogenesis is usually associated with adaptive thermogenesis in brown adipose tissue and skeletal muscles (Butow & Bahassi, 1999). The discovery of mitochondria biogenesis in white adipose tissue is unanticipated. PPARγ coactivator-1α (PGC-1α) is a vital transcription coactivator that is involved in the interaction with an array of transcription factors regulating various types of biological functions, such as mitochondrial biogenesis and adaptive thermogenesis (Liang & Ward, 2006). PGC-1α can also regulate intracellular FA transport and FA β-oxidation (Vega *et al.*, 2000). There may be factors required for adipogenesis modulating PGC-1α, thereby enhancing mitochondrial biogenesis. For instance, there is evidence that pioglitazone (TZD and PPARγ agonist) treatment increases the expression of PGC-1α (Bogacka *et al.*, 2005). Besides this, dexamethsone and a cAMP elevating agent, affects PGC-1α expression level. Dexamethasone alone increases expression of PGC-1α slightly,

but when coupled with 8-bromo-cAMP, there is a synergistic effect on the expression level of PGC-1α (Yoon *et al.*, 2001). As such, there is likelihood that factors modulating adipogenesis can also promote mitochondrial biogenesis. As a result, increased number of mitochondria prevails. With mitochondria being part of the citric acid cycle, they can provide glycerol-3-phosphate (G3P) for the maintenance of the rate at which TAG is synthesized (Olswang *et al.*, 2002). The presence of PGC-1α and increased number of mitochondria allow for β-oxidation of fatty acid in mitochondria to occur, thus contributing an alternative source of energy for biological functions in adipocytes.

In order to further investigate the relationship between PG, mitochondria and adipogenesis, more work needs to be done. Firstly, the observation that mitochondrial biogenesis occurs during adipogenesis needs to be verified. This can be carried out by determining the mitochondrial copy number through real time PCR of genomic and mitochondrial DNA. Furthermore, gene expression level of major proteins in mitochondria, such as citrate synthase, can also be monitored to give an indication that there may be mitochondrial biogenesis when adipogenic pathway is induced. Lastly, the use of Mito Tracker dyes can easily provide a visual effect to prove the hypothesis. After the phenomenon of increased mitochondria during adipogenesis has been substantiated, thwarting PG synthesis by inhibiting PG-phosphoric acid (PGP) synthase in MSC undergoing adipogenesis can be one of the ways to examine the importance of PG in mitochondria and the consequences in adipogenesis.

Apart from the increase in some phospholipid species and PG, the general trend observed is a surprising decrease of phospholipids and an expected increase in TAG. The decrease in phospholipids observed during adipogenic differentiation appears counterintuitive at first. During adipogenic differentiation, the cells hypertrophy (grow in size). Thus, one will expect to see increased phospholipids, so as to form the larger plasma membrane required to envelope the cellular contents. However, this in turn implies that lipids perform only structural functions. Hence, our data also support a more dynamic role of lipids during differentiation.

It is classically known that phospholipids and TAG synthesis occurs at the ER and sometimes at the mitochondrial membranes. However, when this synthesis is measured on microsomal membranes, little amounts of DAG and TAG are detected. When the cytosolic fraction is added exogenously into the above *in vitro* system, increased lipids are detected and thus suggest the presence of stimulating factors in the cytosol (Hubscher *et al.*, 1967; Stein & Shapiro, 1957). This factor is identified as soluble phosphatidic acid phosphatase (PAP) (Johnston *et al.*, 1967; Smith *et al.*, 1967). Based on the phospholipids and TAG biosynthetic pathway (Figure 4-1), a common denominator between the two is PA. Since PA serve as a branch point between subsequent phospholipid and TAG synthesis and PAP enzymatic reaction operates as a committed step in the production of TAG (Brindley, 1984), regulation of PAP activity may determine the fate of PA. There are two types of PAP found in yeast and mammalian systems, namely PAP1 and PAP2. PAP1 exists as a cytosolic protein that can translocate to the ER (Han *et al.*, 2006). PAP2, later renamed to lipid phosphate phosphatases (LPP), is an integral membrane protein (Toke *et al.*, 1999;

Toke *et al.*, 1998) that normally does not participate in phospholipid and TAG synthesis (Brindley, 2004).

Recent studies have shown that lipin proteins exhibit PAP1 enzyme activity (Donkor *et al.*, 2007; Harris *et al.*, 2007; Han *et al.*, 2006). In mammals, there are three lipin proteins found in the cytosol, lipin1, 2 and 3 (Peterfy *et al.*, 2001). All three are specific for PA and dependent on Mg^{2+} (Donkor *et al.*, 2007). Lipin 1 is highly expressed in white and brown adipose tissue (Nadra *et al.*, 2008; Verheijen *et al.*, 2003) and regulates lipid metabolism in mammalian cells (Phan *et al.*, 2005). However, little information is known about lipin 2 and 3, except that lipin 2 can be found in 3T3-L1 preadipocytes (Grimsey *et al.*, 2008).

In our hands, lipin1 gene expression increases steadily as adipogenesis progresses, but there do not seem to have any change in gene expression for lipin 2 and lipin 3. Studies have shown that lipin 1 is not only found in the cytosol, but also in the nucleus (Peterfy *et al.*, 2005) and that lipin 1 can regulate the expression of adipogenic transcription factors, PPARγ and C/EBPα (Phan *et al.*, 2004). As a result, maintenance of the adipocyte phenotype reigns. Deficiency of lipin 1 in mice led to lipodystrophy and insulin resistance, while an excess resulted in obesity and insulin sensitivity (Phan & Reue, 2005). In the work of Grimsey and colleagues, they have demonstrated that Lipin 1-depleted 3T3-L1 results in enhanced expression of lipin 2 but these cells are unable to express aP2 and accumulate lipids within lipid droplets, thus implying their inability to differentiate into mature adipocytes. Conversely, Lipin 2-depeletd 3T3-L1 allow adipogenesis to persist and in fact there is greater expression of aP2 (Grimsey *et al.*, 2008). This suggests the greater importance of

lipin 1 than lipin 2 in adipogenesis, thus explains the unchanged expression level of lipin 2 when MSC undergoes adipogenesis. Similarly, the unchanged expression level of lipin 3 may imply its minor role in adipogenesis in MSC.

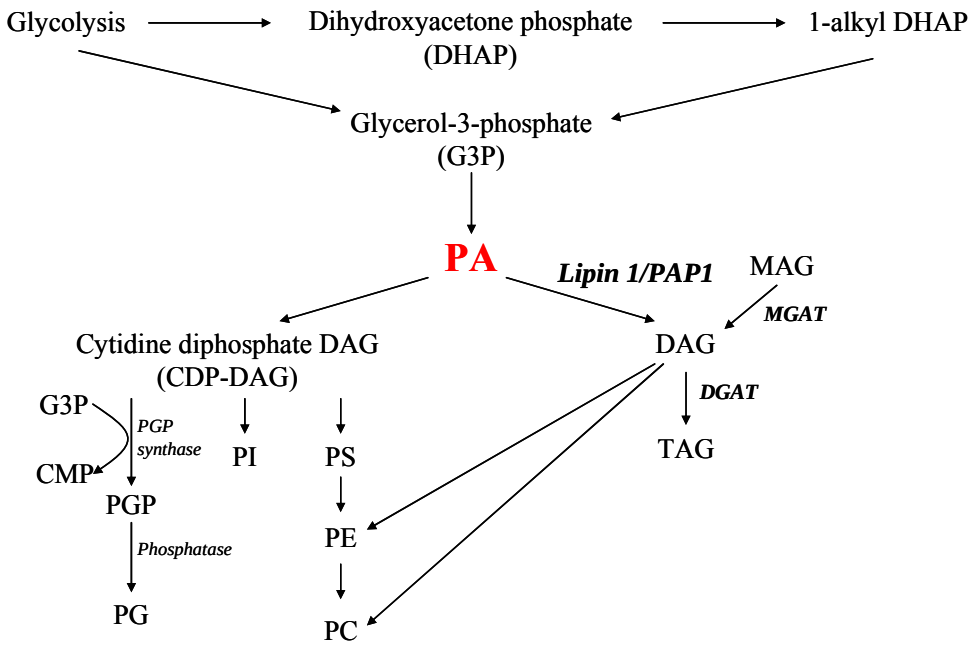
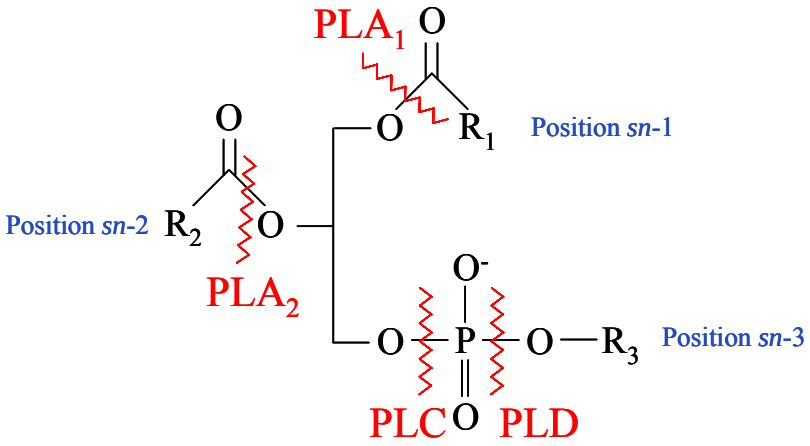


Figure 4-1: An overview of phospholipids and TAG biosynthesis.

CDP-DAG: Cytidine diphosphate-DAG; **CMP**: Cytidine monophosphate; **DHAP**: Dihydroxyacetone phosphate; **DGAT**: acyl-CoA:DAG acyltransferase; **G3P**: Glycerol-3-phosphate; **MGAT**: acyl-CoA:MAG acyltransferase; **PAP1**: phosphatidic acid phosphatase 1; **PGP**: PG-Phosphoric acid.

In addition, LPP also do possess PAP activity and has a large substrate preference for lipid phosphate species (Brindley, 2004). From the gene expression profile of LPPa and LPPb, there is no change over the three timepoints. This implies that they may not be responsible for the changes seen in phospholipids and TAG. With the importance of lipin 1 in adipogenesis and its ability to synthesis TAG, the observed phenomenon that there is a general decrease in PI, PS, PE, PC and PA but an increase in TAG may be due to lipin 1 and thus result in a shift towards TAG biosynthesis.

In addition to lipin and LPP, phospholipases may play a role in the decreased phospholipids observed in adipocytes. Mammalian systems possess many phospholipases that hydrolyze phospholipids and yield a variety of unique phospholipid breakdown products that influence cell function through extracellular and/or intracellular receptors (Feige *et al*, 2006; Hla, 2005; Marrache *et al.*, 2005). There are four types of phospholipases that hydrolyze different *sn*-positions in phospholipids (Figure 4-2). Of which, phospholipase A (PLA) and phospholipase B (PLB) are of interest. PLB has the ability to act on both *sn*-1 and *sn*-2 positions. PLA and PLB release fatty acyl chains, which are also active lipid mediators (Zimmerman *et al.*, 2002; Funk, 2001). The resulting FA can be used for the synthesis of TAG. In addition, there are reports illustrating the ability of FA to modulate adipogenesis. Exogenously added medium chain fatty acids, octanoic acid (FA 8:0) and decanoic acids (FA 10:0), has been shown to increase lipid accumulation when 3T3-L1 underwent adipogenesis (Yang *et al.*, 2008; Takenouchi *et al.*, 2004). Similarly, long chain fatty acids, linoleic acid (FA 18:2) and oleic acid (FA 18:1), exhibit similar phenomenon in human and mouse preadipocytes respectively (Hutley *et al.*, 2003; Guo *et al.*, 2000).



R_1 and R_2 = alkyl groups

R_3 = Phospholipid headgroups

Figure 4-2: Sites of action by phospholipases on phospholipids.

PLA_1: phospholipase A_1; PLA_2: phospholipase A_2; PLC: phospholipase C; PLD: phospholipase D

Within PLA group of enzymes, there is PLA_1 and PLA_2. PLA_1 hydrolyze phospholipids at the *sn*-1 position to form 2-acyl-lysophospholipids, while PLA_2 that hydrolyze phospholipids at the *sn*-2 position to yield 1-acyl-lysophopsholipids. Besides this, PLA_2 comprise of many different proteins grouped into five classes. They are namely secreted PLA_2s, cytosolic PLA_2s, cytosolic Ca^{2+}-independent PLA_2s, platelet activating factor (PAF) acetylhydrolases and lysosomal PLA_2s (Schaloske & Dennis, 2006). Of all the PLA_2s, the cytosolic (Group IV) and cytosolic Ca^{2+}-independent (Group VI) PLA_2s are the most relevant in this study because the decreased levels of phospholipids in adipocytes is determined through the use of whole cell lipid extract, thus any changes will denotes changes within the intracellular environment.

Both PLA_2 G4a and PLA_2 G6 exhibit upregulation throughout the three timepoints, thus imply their possible roles in adipogenesis. A recent report has shown that

smaller adipocytes and reduced level of hepatic TAG content are evident in PLA_2 G4a deficient mice (Gruben *et al.*, 2008). In addition, silencing of PLA_2 G6a and PLA_2 G6b in 3T3-L1 results in inhibition of hormone induced adipogenesis (Su *et al.*, 2004). These finding imply that these phospholipases are essential for adipogenesis which complements the upregulation of PLA_2 G4a and PLA_2 G6 seen in adipogenesis of MSC. In addition, knockdown of PLA_2 G6 has been shown to block the expression of PPARγ and C/EBPα in 3T3-L1 and the authors suggest that it may be the result of lipids produced by PLA_2 G6 that elicit such an effect (Su *et al.*, 2004).

Besides, FA and some metabolites of FA, such as 15dPGJ2 and nitrolinoleic acid, serve as PPARγ ligands and in turn promote adipogenesis (Madsen *et al.*, 2005; Schopfer *et al.*, 2005; Forman *et al.*, 1995). These evidences show the functional aspects of lipids in adipogenesis.

Both PLA_2 G4a and PLA_2 G6 cleave off FA at the *sn*-2 position of phospholipids. FA in that position are usually unsaturated FA (Yamashita *et al.*, 1997). PLA_2 G4a preferentially hydrolyses arachidonic acid (AA) at the *sn*-2 position of phospholipids (Krammer *et al.*, 1991; Takayama *et al.*, 1991; Wijkander & Sundler, 1991; Clark *et al.*, 1990; Gronich *et al.*, 1990; Leslie *et al.*, 1988). This implies that phospholipids with AA within them are greatly decreased. Coincidentally, our results have shown that phospholipids with AA within them (i.e. at least 4 double bonds configuration) are also decreased overtime. *In vivio*, the release of AA allows for the synthesis of prostaglandins, such as 15dPGJ2, and brings about adipogenesis (Forman *et al.*, 1995). However, there are other types of phospholipids that are also reduced. PLA_2

G6 has been reviewed to be able to hydrolyze a wide variety of phospholipids (Winstead *et al.*, 2000). The reduction in other forms of lipids may due to PLA_2 G6. In turn, the release of other types of FA modulating adipogenesis prevails.

Many tissues exhibit PLA_1 activity, but physiological functions still remain unknown. So far, what is known is that some PLA_1 exhibit broad substrate specificity such that they are able to hydrolyse phospholipids, TAG and even galactolipids (Aoki *et al.*, 2007). On the other hand, there are some, such as PS-specific PLA_1 and PA-specific PLA_1, which elucidate strict substrate specificities (Sonoda *et al.*, 2002; Higgs *et al.*, 1998). In the database, only one PLA_1 hit is returned which is the first mammalian PLA_1 cloned, PS-specific PLA_1 (denoted as PLA_1A). PLA_1A hydrolyses PS specifically at the *sn*-1 position to yield 2-acyl lysoPS (Higgs *et al.*, 1997). Induction of endotoxin shock in rats through injection with lipopolysaccharide results in immense upregulation of PLA_1A gene expression, thus suggesting the role of PLA_1A in pathological states (Deaciuc *et al.*, 2004). In addition, the released lysophosphatidylserine (LPS) has been shown to be involved in T-cell growth suppression and mast cell activation (Lourenssen & Blennerhassett, 1998; Bellini & Bruni, 1993). These evidences point towards the notion that PLA_1A and its breakdown products are related to immunological response and function.

Recent report has demonstrated that LPS stimulate glucose uptake in 3T3-L1, which suggests the involvement of LPS in adipogenesis (Yea *et al.*, 2009). Coincidentally, 18:1 LPS exhibits an upward trend. The other species of PS display a general decrease overtime. This suggests that there is likely hydroxylation of PS to LPS and thus the presence of PLA_1A activity. Although there is downregulation of PLA_1A

gene expression, gene expression level does not always translate to protein function and activity (Gygi *et al.*, 1999). There is likelihood that due to the high PLA_1A activity upon adipogenic induction that there is no need for constant expression of PLA_1A gene, thus the observed downregulation prevails. However, protein activity cannot be sustained for perpetual length of time. Overtime, gene of PLA_1A will still need to be expressed to bring about the PLA_1A activity. Hence, gene expression level for PLA_1A returns to baseline at day 21. More research needs to be done to investigate this phenomenon.

Since adipose tissue is regarded as an endocrine organ and PLA_2 illustrates upregulation during adipogenesis, there is likelihood that the resultant products may be secreted out of cells and modulate adipogenesis in neighbouring cells. Evidence has demonstrated that fatty acid and its metabolites, such as eicosanoids, behave as bioactive lipid mediators (Zimmerman *et al.*, 2002; Funk, 2001). Hence, it will be interesting to investigate the profile of FA and eicosanoids in the extracellular domain. Modification of adipogenic cocktail to exclude dexamethasone is required as dexamethasone acts as both a cyclooxygenase 1 and cyclooxygenase 2 inhibitor, thereby preventing the production of eicosanoids. Besides this, exogenous addition of FA can be carried out to determine the effects of FA in adipogenesis of MSC.

Identification of lipid characteristics during adipogenesis allows for deeper understanding of how adipogenesis takes place in a lipid-related manner. The presence of unique trends, such as decreased amounts of phospholipids in adipocytes, increasing trend of PG and selective incorporation of fatty acids to phospholipids in adipocytes, enable the targeting of pathways associated to these trends.

Consequently, combating obesity becomes more efficient. Besides this, current literatures have suggested that a balance between osteogenesis and adipogenesis within bone marrow is essential for the healthy development of bone (Nuttall & Gimble, 2004; Nuttall & Gimble, 2000). Adopting the same methods for the lipid profiling of MSC-derived adipocytes to MSC-derived osteoblasts, followed by comparison between the profiles can help discover lipid species that exhibited significant differences. Further probing of this effect will allow for better understanding of the relationship between the two progenies. As a result, development of improved treatment for bone dysfunction-related diseases, such as osteoporosis and osteopenia, can be achieved.

In conclusion, the results demonstrate generally lower amounts of phospholipids in adipocytes with a surprising increase in PG and selective inclusion of fatty acids into phospholipids. This unique lipid fingerprint for adipocytes provides the first step to understanding adipogenesis further.

CONCLUSIONS

5 Conclusions

The lipid profiling of MSC undergoing adipogenesis has revealed that in spite of the expected increase in TAG, there is also a surprising decrease in phospholipids during adipogenesis. This decrease appears to be counterintuitive at first. During adipogenic differentiation, the cells hypertrophy (grow in size). Thus, one will expect to see increased phospholipids, so as to form the larger plasma membrane required to envelope the cellular contents. However, this in turn implies that lipids perform only structural functions. Hence, our data also support a more dynamic role of lipids during cellular function.

The gene expression levels of lipin 1 and phospholipases demonstrated that these proteins may be responsible for the observed decrease in phospholipids. Literatures have also illustrated the importance of these enzymes during adipogenesis. It will be interesting to investigate further how these enzymes act during adipogenesis so as to understand the intrinsic mechanisms involved and thus add more insight to the adipogenic cascade. Besides this, adipose tissue has been regarded as an endocrine organ. Research on the type of factors released during adipogenesis of MSC can provide more information on the interactions between MSC, preadipocytes and adipocytes. As a result, identification of factors that commit MSC to the adipogenic lineage may be discovered.

Contrary to the general decrease in phospholipids, there is a class of phospholipids that exhibited an overall increase, PG. The increase in PG may indicate an increase in mitochondria, which is exemplified through the transient increase in VDAC protein as adipogenesis progresses. This finding is rather unexpected as mitochondria

biogenesis has usually been associated to brown adipose tissues. It is only in recent reports that scientists too discover this exceptional phenomenon in white adipose tissues. More works are underway to explore this observation.

In addition, there are some species of phospholipids that increased overtime. Similarly, amongst the more abundantly available TAG in adipocytes, those TAG species that display progressive increase encompass similar characteristics to particular phospholipids types that increase overtime. Most of them are made up of MUFA. This finding suggests that there is preferential incorporation of MUFA to TAG and phospholipids and that this process is occurring via the *de novo* pathway. Further development in this area may reveal exciting revelations on the biosynthetic pathways of phospholipids and TAG.

Current literatures have suggested that a balance between osteogenesis and adipogenesis within bone marrow is essential for the healthy development of bone (Nuttall & Gimble, 2004; Nuttall & Gimble, 2000). Adopting the same methods for the lipid profiling of adipocyte-derived MSC to osteoblast-derived MSC, followed by comparison between the profiles can help discover lipid species that exhibited significant differences. Further probing of this effect will allow for better understanding of the relationship between the two progenies. As a result, development of improved treatment for bone dysfunction-related diseases, such as osteoporosis and osteopenia, can be achieved.

Taken together, lipid profiling of MSC undergoing adipogenesis presents the unique lipid fingerprints of cells at distinct differentiative stages. In-depth analysis of the

abundant information acquired can reveal interesting and novel observations, thus enable one to venture into unchartered boundaries of the adipogenic process.

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APPENDICES

Appendix 1

Gene name	Primer Name	Primer (5' → 3')	Primer length
18S ribosomal RNA	18SrRNA F	GACTCAACACGGGAAACCTC	20
	18SrRNA R	AGCATGCCAGAGTCTCGTTC	20
β-Actin	β-Actin F	CACACTGTGCCCATCTACGA	20
	β-Actin R	GTGGTGGTGAAGCTGTAGCC	20
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH F	CCCTTCATTGACCTCAACTACAT	23
	GAPDH R	TCCTGGAAGATGGTGATGG	19
Hydroxymethylbilane synthase	HMBS F	AGGATGGGCAACTGTACCTG	20
	HMBS R	TCGTGGAATGTTACGAGCAG	20
Hypoxanthine phosphoribosyltransferase 1	HPRT1 F	TGAGGATTTGGAAAGGGTGT	20
	HPRT1 R	AATCCAGCAGGTCAGCAAAG	20
Proliferator peroxisome activated receptor γ 1	PPARG1-2 F	CTTCCATTACGGAGAGATCC	20
	PPARG1 R	AAAGAAGCCGACACTAAACC	20
Proliferator peroxisome activated receptor γ 2	PPARG2 R	GCGATTCCTtCACTGATAC	19
CCAAT Enhancer binding protein α	C/EBPa F	GAGGAGGGGAGAATTCTTGG	20
	C/EBPa R	TCTCATGGGGGTCTGCTGTA	20
CCAAT Enhancer binding protein δ	C/EBPd F	CTGTCGGCTGAGAACGAG	18
	C/EBPd R	GAGGTATGGGTCGTTGCTG	19
Lipoprotein lipase	LPL F	CAGCCAGGATGTAACATTGG	20
	LPL R	AGGCTTCCTTGGAACTGCAC	20
Adipocyte fatty acid binding protein	aP2 F	TACTGGGCCAGGAATTTGAC	20
	aP2 R	GTGGAAGTGACGCCTTTCAT	20
Phospholipase A 1	PLA1 F	AGTTCTGCACTGCCCTTTTG	20
	PLA1 R	AATGCAGGGAGATGTGTCCT	20
Phospholipase A 2 Group 4A	PLA2-G4A F	AGCCATATTGGGTTCAGGTG	20
	PLA2-G4A R	GGCCCTTTCTCTGGAAAATC	20
Phospholipase B	PLB F	TCAGGAGAAGACCCACCAAC	20
	PLB R	TCGGGAGTGAGACTTGCTG	19
Lipin 1	LPIN1F	AGTGACCAATCGCCAACTCT	20
	LPIN1R	TCCGTCTTGTTTGCTGTCTG	20
Lipin 2	LPIN2F	ACCTTTTCACGTTCGGTTTG	20
	LPIN2R	CCAAAGGGGTGTCAATATCTTT	22
Lipin 3	LPIN3F	TCAGTGAAGGGTGACAGCAG	20
	LPIN3R	GTGTGTCATGTGCTGAGATGC	21
Lipid Phosphate Phosphatase a	LPPa F	GACTGCGGCCTCACTTCTT	19
	LPPa R	AAACAGCATGCAGTACATGGAG	22
Lipid Phosphate Phosphatase b	LPPb F	AAATGACGCTGTGCTCTGTG	20
	LPP2b R	CTGTGAAAGACTGGCTGATGG	21

Table A1-1: Primer pair sequences.

Appendix 2

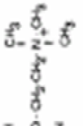
Specificity	Polarity	Fragment structure	Scan mode
All glycerophospholipids / Phosphatidic acid / Phosphatidylglycerol	Negative		Precursor of 153
Phosphatidylinositol	Negative		Precursor of 241
Phosphatidylethanolamine	Negative		Precursor of 196
Phosphatidylserine	Negative		Neutral loss of 87
Phosphatidylcholine	Positive		Precursor of 184

Table A2-1: Structure specific daughter product.

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylcholine				
PC	12:2 LPC	436.6	184.1	90	50
PC	14:1p LPC	450.6	184.1	90	50
PC	14:2 LPC	464.6	184.1	90	50
PC	16:0e LPC	482.6	184.1	90	50
PC	16:6 LPC	484.6	184.1	90	50
PC	16:0 LPC	496.6	184.1	90	55
PC	18:0e LPC	510.6	184.1	90	50
PC	18:2 LPC	520.6	184.1	90	50
PC	18:1 LPC	522.6	184.1	90	50
PC	18:0 LPC	524.6	184.1	90	50
PC	20:0p / 20:1e LPC	536.6	184.1	90	50
PC	20:0e LPC	538.6	184.1	90	50
PC	20:4 LPC	544.6	184.1	90	54
PC	20:3 LPC	546.6	184.1	90	54
PC	26:0e PC	636.7	184.1	90	60
PC	28:4p PC	638.7	184.1	90	60
PC	30:4e PC / 30:3p PC	668.8	184.1	90	60
PC	30:3e / 30:2p PC	670.8	184.1	90	60
PC	28:1 PC	676.7	184.1	90	60
PC	32:0p / 32:1e PC	703.9	184.1	90	60
PC	32:0e PC	705.9	184.1	90	60
PC	32:2 PC	717.9	184.1	90	60
PC	32:1 PC	719.9	184.1	90	60
PC	32:0 PC	718.9	184.1	90	60
PC	34:2p / 34:3e PC	720.9	184.1	90	60
PC	34:1p / 34:2e PC	729.9	184.1	90	60
PC	34:0p / 34:1e PC	730.9	184.1	90	60
PC	34:3 PC	732.9	184.1	90	60
PC	34:2 PC	734.9	184.1	90	60
PC	34:1 PC	742.9	184.1	90	60
PC	36:4p PC	744.9	184.1	90	60
PC	36:3p / 36:4e PC	746.9	184.1	90	60
PC	36:2p / 36:3e PC	756.9	184.1	90	60
PC	36:1p / 36:2e PC	758.9	184.1	90	60
PC	36:0p / 36:1e PC	760.9	184.1	90	60
PC	36:5 PC	766.9	184.1	90	60
PC	36:4 PC	768.9	184.1	90	60

Table A3-1: MRM transition list in the positive mode.

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylcholine				
PC	36:3 PC	770.9	184.1	90	60
PC	36:2 PC	772.9	184.1	90	60
PC	36:1 PC	774.9	184.1	90	60
PC	38:4p / 38:5e PC	780.9	184.1	90	60
PC	38:3p / 38:4e PC	782.9	184.1	90	60
PC	38:2p / 38:3e PC	784.9	184.1	90	60
PC	38:6 PC	786.9	184.1	90	60
PC	38:5 PC	788.9	184.1	90	54
PC	38:4 PC	794.9	184.1	90	54
PC	38:3 PC	796.9	184.1	90	54
PC	40:4p / 40:5e PC	798.9	184.1	90	54
PC	40:3p / 40:4e PC	806.9	184.1	90	54
PC	40:2p / 40:3e PC	808.9	184.1	90	54
PC	40:1p / 40:2e PC	810.9	184.1	90	54
PC	40:0e PC	812.9	184.1	90	54
PC	40:6 PC	813.9	184.1	90	54
PC	40:5 PC	815.9	184.1	90	54
PC	13:0 PC / 14:0e PC	822.9	184.1	90	54
PC	30:1 / 31:1e / 31:0p PC	824.9	184.1	90	54
PC	30:0 / 31:0e PC	826.9	184.1	90	54
PC	30:2 PC	827.9	184.1	90	54
PC	34:4e / 34:3p PC	828.9	184.1	90	54
PC	36:0e PC	832.9	184.1	90	65
PC	36:0 / 38:6p PC	834.9	184.1	90	54
PC	38:5p / 38:6e PC	836.9	184.1	90	54
PC	38:2 PC	814.8	184.1	90	60
PC	38:1 PC	816	184.1	90	60
PC	40:0 PC	846.1	184.1	90	60
PC	42:5 / 44:12e / 44:11p PC	848.2	184.1	90	60
PC	42:4 / 44:11e / 44:10p PC	850.1	184.1	90	60
PC	42:3 / 44:10e / 44:9p PC	852.1	184.1	90	60

Table A3-1: MRM transition list in the positive mode (cont'd).

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidic acid				
PA	32:2 PA	643.7	153	-80	-40
PA	34:2 PA	671.7	153	-80	-30
PA	34:1 PA	673.7	153	-80	-50
PA	34:3 PA	669.8	153	-80	-50
PA	36:1 PA	701.8	153	-80	-50
PA	36:0 PA	703.8	153	-80	-50
PA	38:4 PA	723.8	153	-80	-50
PA	38:0 PA	731.9	153	-80	-50
PA	40:6 PA	747.9	153	-80	-50
PA	40:5 PA	749.9	153	-80	-50
PA	40:4 PA	751.9	153	-80	-50
PA	42:6 PA	775	153	-80	-50
PA	42:5 PA	777	153	-80	-50
PA	32:3 PA	796.9	153	-80	-40
	Phosphatidylglycerol				
LPG	16:1 LPG	481.2	153	-80	-40
PG	32:2 PG	717.8	153	-80	-50
PG	34:3 PG	743.9	153	-80	-50
PG	34:2 PG	745.9	153	-80	-50
PG	34:1 PG	747.9	153	-80	-50
PG	34:0 PG	749.9	153	-80	-50
PG	36:4 PG	769.9	153	-80	-50
PG	36:2 PG	773.9	153	-80	-50
PG	36:1 PG	775	153	-80	-50
PG	36:0 PG	777	153	-80	-50
PG	38:2 PG	801	153	-80	-50
PG	38:1 PG	803	153	-80	-50
PG	42:6 PG	849.1	153	-80	-50
PG	42:5 PG	851.1	153	-80	-50
PG	42:1 PG	859.1	153	-80	-50
PG	36:3 PG	771.8	153	-90	-55

Table A3-2: MRM transition lists in the negative mode.

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylinositol				
PI	17:0 LPI	585.7	241	-90	-55
PI	16:0 LPI	571.7	241	-90	-55
PI	18:0 LPI	599.7	241	-90	-55
PI	20:4LPI	619.7	241	-90	-55
PI	32:1 PI	807.8	241	-115	-55
PI	33:1 PI	821.8	241	-115	-55
PI	34:7 PI	823.8	241	-115	-55
PI	34:2 PI	833.8	241	-115	-55
PI	34:1 PI	835.8	241	-115	-55
PI	35:1 PI	849.8	241	-115	-55
PI	36:4 PI	857.8	241	-115	-55
PI	36:3 PI	859.8	241	-115	-55
PI	36:2 PI	861.9	241	-115	-55
PI	36:1 PI	863.9	241	-115	-55
PI	37:4 PI	871.9	241	-115	-55
PI	37:3 PI	873.9	241	-115	-55
PI	38:5 PI	883.9	241	-115	-55
PI	38:4 PI	885.9	241	-115	-60
PI	38:3 PI	887.9	241	-115	-60
PI	39:4 PI	899.9	241	-115	-60
PI	39:3 PI	901.9	241	-115	-60
PI	40:6 PI	909.9	241	-115	-55
PI	40:5 PI	911.9	241	-115	-55
PI	40:4 PI	913.9	241	-115	-55
PI	40:2 PI	917.9	241	-115	-55
PI	20:3 LPI	621.7	241	-90	-55
PI	34:0 PI	837.8	241	-115	-55
PI	36:0 PI	865.9	241	-115	-55
PI	38:2 PI	889.9	241	-115	-60
PI	38:1 PI	891.2	241	-115	-60

Table A3-2: MRM transition lists in the negative mode (cont'd).

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylserine				
PS	16:1 LPS	494.7	407.7	-80	-25
PS	16:0 LPS	496.7	409.7	-80	-25
PS	17:1 LPS	508.7	421.7	-80	-25
PS	17:0 LPS	510.7	423.7	-80	-25
PS	18:1 LPS	522.7	435.7	-80	-25
PS	18:0 LPS	524.7	437.7	-80	-25
PS	34:2 PS	758.6	671.6	-80	-25
PS	34:1 PS	760.6	673.6	-80	-25
PS	36:2 PS	786.6	699.6	-80	-25
PS	36:1 PS	788.6	701.6	-80	-25
PS	38:4 PS	810.6	723.6	-80	-25
PS	38:3 PS	812.6	725.6	-80	-25
PS	40:7 PS	832.6	745.6	-80	-25
PS	40:6 PS	834.6	747.6	-80	-25
PS	40:5 PS	836.6	749.6	-80	-25
PS	32:0 PS	734.8	647.8	-80	-25
PS	36:0 PS	790.7	703.7	-80	-25
PS	38:5 PS	808	721	-80	-25
PS	38:2 PS	814.8	727.8	-80	-25
PS	38:1 PS	816	729	-80	-25
PS	40:4 PS	838.8	751.8	-80	-25
PS	40:3 PS	840.1	753.1	-80	-25
PS	40:1 PS	844.1	757.1	-80	-25
PS	40:0 PS	846.1	759.1	-80	-25
PS	42:5 PS	864.9	777.9	-80	-25
PS	42:4 PS	866.9	779.9	-80	-25

Table A3-2: MRM transition lists in the negative mode (cont'd).

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylethanolamine				
PE	16:0p / 16:0e LPE	436.5	196.1	-80	-30
PE	16:1 LPE	450.5	196.1	-80	-30
PE	16:0 LPE	452.5	196.1	-80	-30
PE	18:1p / 18:2e LPE	462.5	196.1	-80	-40
PE	18:0p / 18:1e LPE	464.5	196.1	-80	-30
PE	18:2 LPE	476.5	196.1	-80	-50
PE	18:1 LPE	478.5	196.1	-80	-50
PE	18:0 LPE	480.5	196.1	-80	-55
PE	20:0p / 20:1e LPE	492.5	196.1	-90	-55
PE	20:4 LPE	500.5	196.1	-90	-55
PE	22:6 LPE	524.6	196.1	-90	-55
PE	32:0p PE	660.8	196.1	-90	-55
PE	32:0e PE	662.8	196.1	-90	-55
PE	32:2 PE	686.8	196.1	-90	-55
PE	32:1 PE	688.8	196.1	-90	-55
PE	32:0 PE	690.8	196.1	-90	-55
PE	34:2p / 34:3e PE	698.8	196.1	-90	-55
PE	34:1p / 34:2e PE	700.8	196.1	-100	-55
PE	34:2a PE	714.8	196.1	-100	-55
PE	34:1a PE	716.8	196.1	-110	-55
PE	36:5p PE	720.8	196.1	-110	-55
PE	36:4p PE	722.8	196.1	-115	-55
PE	36:2p / 36:3e PE	726.8	196.1	-115	-55
PE	36:1p / 36:2e PE	728.8	196.1	-115	-55
PE	36:4 PE	738.8	196.1	-115	-55
PE	36:3 PE	740.8	196.1	-115	-55
PE	36:2 PE	742.8	196.1	-115	-55
PE	36:1 PE	744.8	196.1	-115	-55
PE	36:0 / 38:6p PE	746.8	196.1	-115	-55
PE	38:5p / 38:6e PE	748.8	196.1	-115	-55
PE	38:4p / 38:5e PE	750.8	196.1	-115	-55
PE	38:1p / 38:2e PE	756.8	196.1	-115	-55
PE	38:0e PE	760.8	196.1	-115	-55

Table A3-2: MRM transition lists in the negative mode (cont'd).

Appendix 3

Phospholipid species	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
	Phosphatidylethanolamine				
PE	38:6 PE	762.8	196.1	-115	-55
PE	38:5 PE	764.8	196.1	-115	-55
PE	38:4 PE	766.8	196.1	-115	-55
PE	38:0 / 40:6p PE	774.8	196.1	-115	-55
PE	40:5p / 40:6e PE	776.8	196.1	-115	-55
PE	40:4p / 40:5e PE	778.8	196.1	-115	-55
PE	40:6 PE	790.8	196.1	-115	-55
PE	40:5 PE	792.8	196.1	-80	-40
PE	16:0e PE	438.1	196.1	-80	-30
PE	18:3 PE	474.6	196.1	-80	-50
PE	32:1p / 32:1e PE	672.8	196.1	-80	-30
PE	32:3 PE	684.8	196.1	-90	-55
PE	34:0p / 34:1e PE	702.8	196.1	-100	-55
PE	36:3p / 36:4e PE	724.8	196.1	-115	-55
PE	38:1 PE	772.9	196.1	-115	-55
PE	42:3p / 42:3e PE	808	196.1	-115	-55
PE	42:2p / 42:3e PE	810	196.1	-115	-55
PE	42:1p / 42:2e PE	812	196.1	-115	-55
PE	42:0p / 42:1e PE	814.8	196.1	-115	-55
PE	42:0e PE	816	196.1	-115	-55

Table A3-2: MRM transition lists in the negative mode (cont'd).

Standards	Phospholipid Identity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (eV)	CE (eV)
DAPA std	40:8 PA	743.8	153	-80	-40
DMPG std	28:0 PG	665.6	153	-90	-50
Di-C8 PI std	16:0 PI	585.5	241	-90	-55
DMPS std	28:0 PS	678.6	591.6	-80	-25
DMPE std	28:0 PE	634.6	196.1	-90	-50
DMPC std	28:0 PC	678.8	184.1	90	50

Table A3-3: Internal standards.

Appendix 3

Triacylglycerol species	Selected ion (*m/z*)	Triacylglycerol species	Selected ion (*m/z*)
49:2	834.8	54:4	900.9
49:1	836.8	54:3	902.9
42:1	738.8	54:2	904.9
42:0	740.8	54:1	906.9
44:2	764.8	54:0	908.9
44:1	766.8	56:9	918.9
44:0	768.8	56:8	920.9
46:3	790.8	56:7	922.9
46:2	792.8	56:6	924.9
46:1	794.8	56:5	926.9
46:0	796.8	56:3	930.9
48:4	816.8	56:2	932.9
48:3	818.8	56:1	934.9
48:2	820.8	56:0	936.9
48:1	822.8	58:10	944.9
48:0	824.8	58:9	946.9
49:3	832.8	58:8	948.9
49:2	834.8	58:7	950.9
49:1	836.8	58:6	952.9
50:5	842.8	58:3	956.9
50:4	844.8	60:10	972.9
50:3	846.8	60:9	974.9
50:1	850.8	60;8	976.9
50:0	852.8	60:7	978.9
51:4	858.9	60:6	980.9
51:3	860.9	60:5	982.9
51:2	862.9		
51:1	864.9		
52:6	868.9		
52:5	870.9		
52:4	872.9		
52:3	874.9		
52:2	876.9		
52:1	878.9		
52:0	880.9		
54:7	894.9		
54:6	896.9		
54:5	898.9		

Table A3-4: SIM transition lists for TAG.

Appendix 4

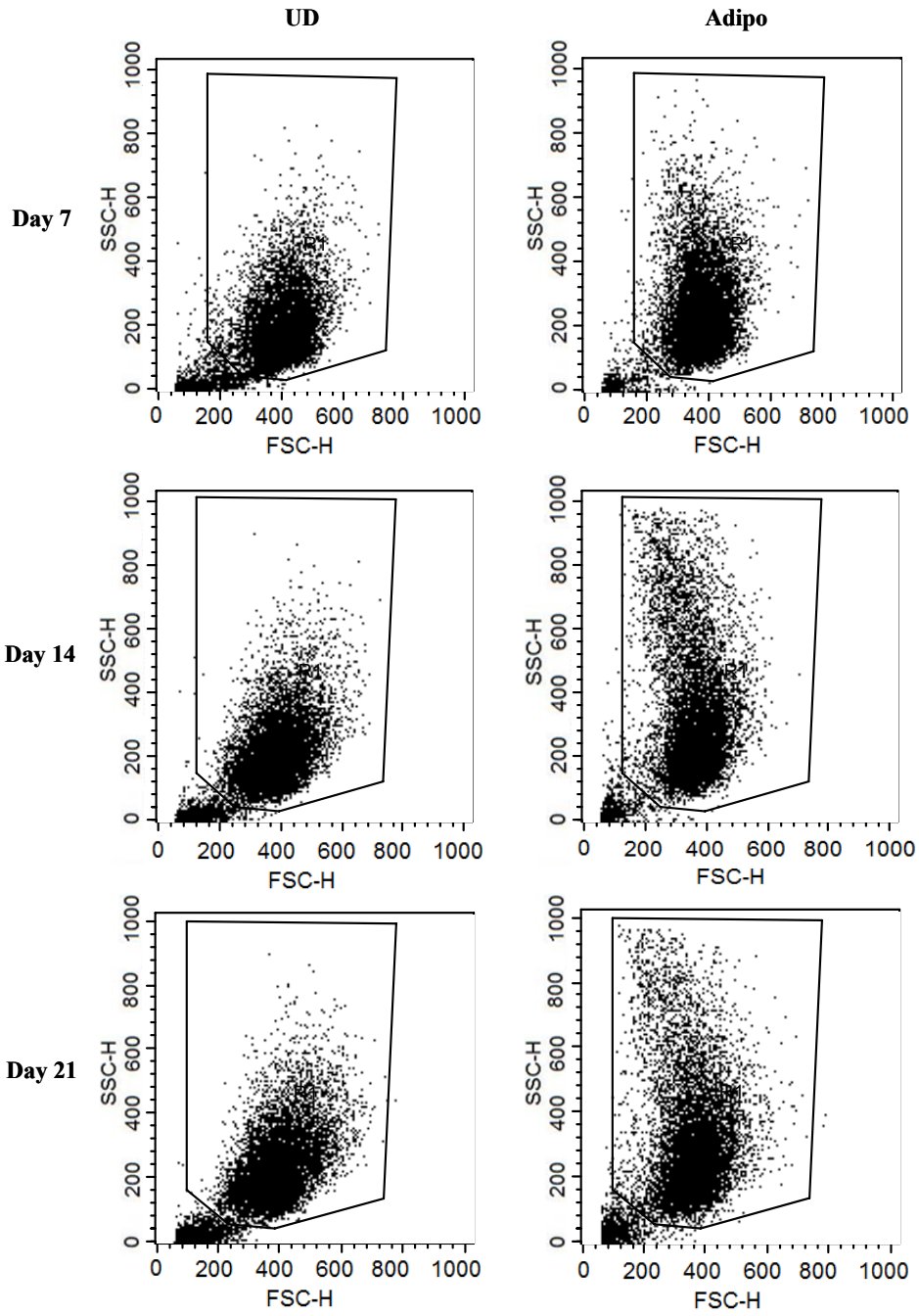


Figure A4-1: Forward scatter (FSC) and side scatter (SSC) of UD and Adipo at day 7, 14 and 21.

Appendix 5

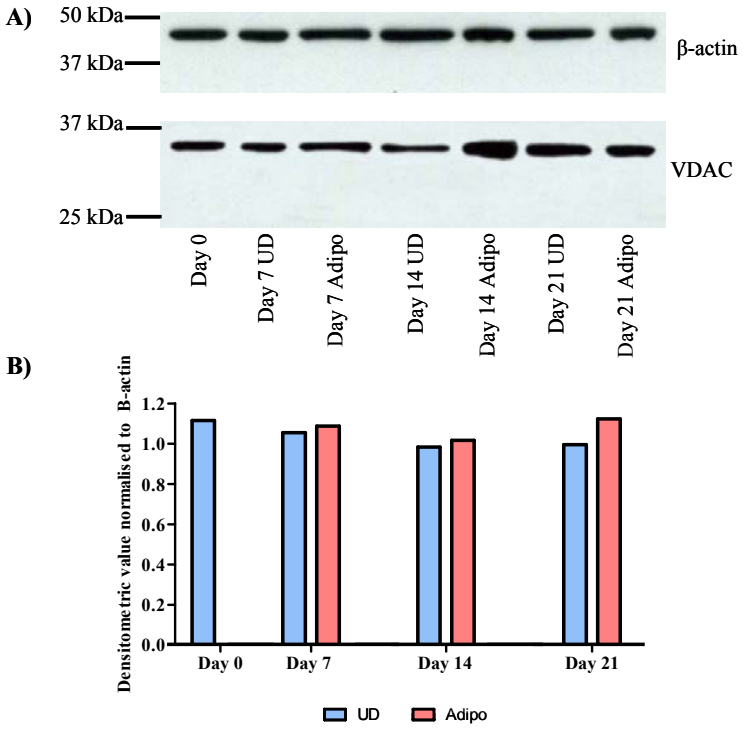


Figure A5-1: VDAC protein in UD and Adipo samples overtime.
A) Western blot of VDAC and β-actin. B) Densitometic analysis of bands. (Representative of 1 data set)

Appendix 6

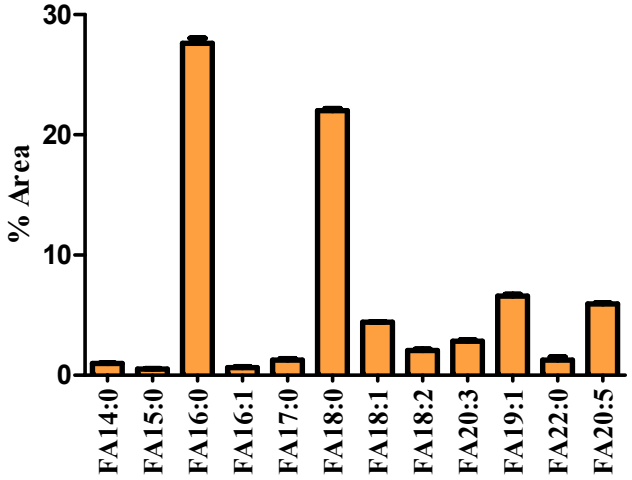


Figure A6-1: FA analysis of FBS.
Each bar represents the mean and standard deviation of n=3 independent samples.

Appendix 7

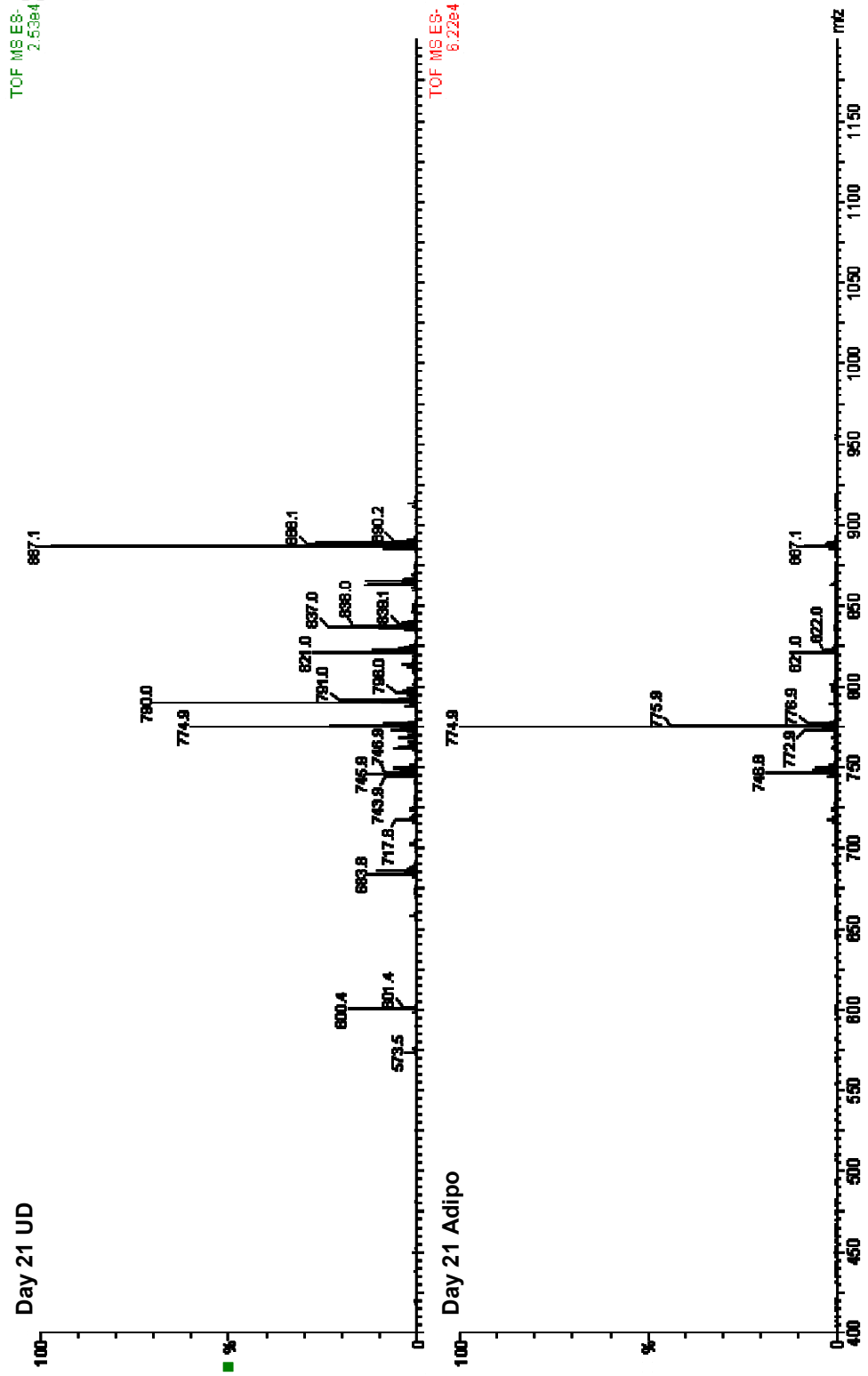


Figure A7-1: Raw TOF spectra for UD and Adipo samples
Representative raw data profiles of 1 data set for Day 21 samples. Day 7 and Day 14 profiles are not presented here.