THE EFFECTS OF *d*-δ-TOCOTRIENOL AND *trans, trans*-FARNESOL ON THE DIFFERENTIATION OF MURINE 3T3-F442A PREADIPOCYTES

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DEDICATION

This dissertation is dedicated to:

My loving children, Mohammad and Fatemeh,

And my supportive mom, Mehrzad Bastani.

Thank you for unconditional love and never-ending patience.

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ABSTRACT

SHEIDA TORABI

THE EFFECTS OF *d*-δ-TOCOTRIENOL AND *trans, trans*-FARNESOL ON THE DIFFERENTIATION OF MURINE 3T3-F442A PREADIPOCYTES

AUGUST 2015

Suppression of adipogenesis is one of the potential approaches in obesity prevention. The statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, suppress the differentiation of adipocytes, suggesting the role of mevalonate in adipocyte differentiation. We investigated the effect of *d*-δ-tocotrienol, a suppressor of HMG CoA reductase, and *trans, trans*-farnesol (*t,t*-farnesol or farnesol), a mevalonate-derived sesquiterpene, on differentiation of murine 3T3-F442A preadipocytes. With AdipoRed assay and Oil Red O staining, we showed that an 8 day incubation with 2.5 - 10 μmol/L *d*-δ-tocotrienol dose-dependently reduced the intracellular triglyceride content of murine 3T3-F442A adipocytes, reminiscent of the lovastatin (1.25 μmol/L) effect. Concomitantly, *d*-δ-tocotrienol dose-dependently inhibited glucose uptake by 3T3-F442A cells and the expression of GLUT4, pAKT, and HMG CoA reductase proteins. Rosiglitazone (1 μmol/L), an agonist of peroxisome proliferator-activated receptor γ (PPARγ), reversed the effects of *d*-δ-tocotrienol on cellular lipid content and glucose uptake. *d*-α-Tocopherol, a vitamin E molecule with no effect on HMG CoA reductase, at 10 μmol/L did not have any impact on 3T3-F442A

differentiation. Trypan blue staining showed no changes in cell viability following 48-h incubation of 3T3-F442A cells with *d*-δ-tocotrienol (0-80 μmol/L), suggesting that the adipogenesis-suppressive activity of *d*-δ-tocotrienol was independent of cytotoxicity.

Farnesol dose-dependently (25 - 75 μmol/L) increased glucose uptake and intracellular triglyceride content of 3T3-F442A cells without affecting cell viability. GW9662 (10 μmol/L), an antagonist of PPARγ, and Ly294002 (10 μmol/L), a phosphatidyl-inositol 3 kinase (PI3K) inhibitor, reversed the effects of farnesol on cellular lipid content. Real-time qPCR and western-blot showed that farnesol increased the mRNA and protein levels of GLUT4 and PPARγ. The mRNA levels for fatty acid binding protein 4, lipoprotein lipase, and adiponectin were also upregulated. Farnesol did not affect the protein or mRNA level of HMG CoA reductase. Farnesol may induce adipocyte differentiation via upregulation of PPARγ and PI3K, which may lead to the increased expression of GLUT4 and adipogenic genes.

d-δ-Tocotrienol, a mevalonate pathway suppressor, and *trans, trans*-farnesol, a mevalonate-derived sesquiterpene, imposed opposite effects on the differentiation of 3T3-F442A preadipocytes. Their effects were mediated by PI3K-PKB/Akt signaling pathway that is responsible for the translocation of GLUT4 protein to plasma membrane and glucose uptake. Future studies may further delineate the role of mevalonate pathway in adipocyte differentiation and the value of the mevalonate pathway as a target for obesity intervention.

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CHAPTER I

INTRODUCTION

Obesity is a prevalent health hazard in industrialized countries and is closely associated with a number of pathological disorders, including type 2 diabetes, hypertension, coronary heart disease, and cancer. With regard to the wide range of health implications, the need to develop new and effective strategies in controlling obesity has become an emergency. Obesity results from an excess of fat tissue in the body. Long term positive energy balance results in the expansion of white adipose tissue (WAT) or obesity. WAT is the major energy reserve in the human body; in periods of energy excess it stores triacylglycerol (TAG) which is mobilized during energy deprivation. Because of its crucial impact for development of obesity, abnormal regulation of adipocyte differentiation and lipogenesis is also linked to important pathological conditions such as obesity, type 2 diabetes, and lipodystrophy [Moller and Flier 1991].

At the cellular level, obesity is characterized by increases in the number and size of adipocytes, named hyperplasia and hypertrophy, respectively. Because terminally differentiated adipocytes cannot divide, hyperplasia is necessary when the existing adipocytes cannot reserve more energy. In humans, the majority of preadipocyte differentiation occurs shortly after birth [Burdi et al. 1985]. This increase in adipose tissue mass enables the newborn to survive during periods of fasting

[MacDougald and Lane 1995, Salvin 1979, Spiegelman et al. 1993]. While the ability of preadipocytes to differentiate continues throughout the life span in response to the fat storage demands, the total number of adipocytes remains constant in adulthood [Spalding et al. 2008]. This supports the idea that increased the number of adipocytes is set during prenatal, childhood, and adolescence [Prins and O'Rahilly 1997]. Measuring adipocyte turnover by analyzing the integration of ^{14}C in genomic DNA [Spalding et al. 2005] revealed that about 10% of adipocytes are renewed annually at adult ages [Spalding et al. 2008]. Even after marked weight loss, the number of adipocytes was constant in both lean and obese individuals in adult ages [Spalding et al. 2008]. Similar results were found in the significant weight gain in adults [Sims et al. 1968], indicating that increased fat storage in already differentiated adipocytes is the most important mechanism responsible for increasing fat tissue or obesity in adults [Bjorntorp 1974, Hirsch and Batchelor 1976].

However, excess accumulation of TAG in adipocytes produces large fat cells or hypertrophic adipocytes which develop to dysfunction and are insulin resistant due to inflammation.This condition results in a metabolic energy imbalance in the whole body system chronically leading to metabolic disorders, such as obesity, metabolic syndrome and diabetes. Accordingly, investigating the regulatory mechanisms of adipocyte differentiation and hypertrophy may help to correct the pathogenesis of metabolic disorders.

The fate of fatty acids (FAs) synthesized in adipocytes depends on the growth activity of organism. In a condition of rapid growth, most of FAs are incorporated into membrane phospholipids, occuring during the proliferation phase of the preadipocyte *in vitro.* However, in the case of high nutrient supply but not active growth, new FAs are used for storage of metabolic energy as TAGs in lipid droplets (LDs) which is observed in culture adipocytes during differentiation period. Accordingly, it is believed that inhibiting lipogenesis is a potential strategy to control adult obesity.

In an isocoloric condition, if lipogenesis is inhibited, the possibility of TAG accumulation in non-adipose tissues increases. In fact, synthesis of TAG in adipocytes is a kind of defense mechanism to clear the extra sources of energy, mainly glucose and fat, from blood. Inhibition of this natural mechanism increases the risk of hyperglycemia and hyperlipidemia leading to glucotoxicity and lipotoxicity. Lipotoxicity is an accumulation of excess lipids in non-adipose tissues leading to cell dysfunction or cell death. Lipotoxicity plays an important role in the pathogenesis of diabetes due to lipid overload in pancreatic β-cells leading to a reduction in β-cells mass [Unger 1995, Unger and Orci 2001, Unger and Zhou 2001]. In animal models, TAG accumulation in pancreatic islets was associated with reductions in β-cells mass and declining insulin production [Unger and Orci 2001].

Accordingly, assessing the effect of dietary ingredients on lipogenesis and glucose uptake in adipocytes may clarify the risk/benefit of recommending to individuals with obesity and insulin resistance. The optimal health of adipose tissue can

be achieved by inducing adipocyte differentiation, while inhibiting the production of dysfunctional or hypertrophic adipocytes.

Most current understanding of the molecular mechanisms of adipocyte differentiation have come from *in vitro* studies on established preadipocyte cell lines [Gregoire et al. 1998, Rosen and Spiegelman 2000]. The 3T3-L1 and 3T3-F442A fibroblasts or preadipocytes are the most common *in vitro* models for studying adipocyte differentiation [Gregoire et al. 1998, Tang and Lane 2012, Green and Kehinde 1975]. Insulin or insulin growth factor-1 (IGF1) are the main hormones required for the differentiation of these established cell lines [Gregoire et al. 1998, Green and Kehinde 1974]. Insulin can induce the translocation of glucose transport protein 4 (GLUT4) vesicles from the cytoplasm to the plasma membrane (PM) by the PI3K-PKB/Akt pathway. This results in increased glucose uptake which provides the main source of energy for TAG synthesis in adipocytes.

Two transcriptional factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) [Gregoire et al. 1998, Tang and Lane 2012], are known as the major regulators of adipogenesis and sit at the core of the adipogenic cascade. PPARγ is highly adipocyte specific with its expression rapidly increasing after hormone induced adipocyte differentiation [Gregoire et al. 1998, Tang and Lane 2012, Green and Kehinde 1975]. Sterol regulatory element binding protein (SREBP) is another key lipogenic transcription factor that is nutritionally regulated by glucose and insulin [Horton et al. 2002, Goldstein and Brown

2008]. SREBP-1c preferentially regulates the lipogenic process by activating genes involved in fatty acid and TAG synthesis. Insulin can function upstream to PPARγ by activation of SREBP-1c [Payne et al. 2009].

Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, have been shown to inhibit adipocyte differentiation through mevalonate deprivation [Brown et al. 1978, Nakanishi et al. 1988, Nishio et al. 1996, Goto et al. 2011, Nakata et al. 2006, Nicholson et al. 2007, Elfakhani et al. 2014]. Isoprenoids, products of the mevalonate pathway in plants, can down-regulate HMG CoA reductase through feedback inhibition [Goldstein and Brown 1990].

Vitamin Es are synthesized in plants from isoprenoids, and includes four tocopherols and four tocotrienols (designated as α-, β-, γ-, and δ-). Unlike other nutrients, these forms of vitamin E cannot be interconverted in the body [Traber 2007]. The tocotrienols have the farnesol side chain with three double bonds. Adminstration of a tocotrienol-rich fraction containing all four tocotrienol homologues plus α-tocopherol to patients reduced serum cholesterol [Qureshi et al. 2001, Tan et al. 1991, Parker et al. 1993]. γ and δ-Tocotrienols reduced triglycerides [Zaiden et al. 2010]. Additive effects were observed when tocotrienol-rich fraction and lovastatin were combined [Qureshi et al. 2001]. Furthermore, γ-tocotrienol inhibits the differentiation of 3T3-L1 preadipocytes *in vitro*, suggested by decreased phosphorylation of PKB-Akt [Uto-kondo et al. 2009]. The effect of other tocotrienols on adipocyte differentiation and the

significance of glucose uptake in the mechanism are important questions in vitamin E research.

Farnesyl pyrophosphate (FPP), a metabolite of mevalonate, can yield farnesol in plants. FPP is the precursor of many isoprenoids and is positioned at branch points leading to the synthesis of long chain isoprenoids. FPP can be produced from farnesol through "salvage pathway" [Crick et al. 1997]. It has been shown that FPP can activate PPARγ as an agonist during adipocyte differentiation [Goto et al. 2011]. FPP activated PPARγ in a dose dependent manner as an endogenous agonist ligand [Goto et al. 2011]. However, the effect of farnesol on the differentiation of preadipocytes and glucose uptake has not been investigated.

Purpose of study

The purpose of this study was to investigate the effect of *d*-δ-tocotrienol and *trans, trans*-farnesol on differentiation of 3T3-F442A preadipocytes and the underlying mechanism. According to the hypothesized model (Figure 1.1), we are trying to answer the following research questions:

1. What is the effect of *d*-δ-tocotrienol and *trans, trans*-farnesol on the differentiation of 3T3-F442A preadipocytes?
2. Is the mevalonate pathway, or the PI3K-PKB/Akt signaling pathway, involved in the underlying mechanism of these effects?

3. Is there a positive correlation between glucose uptake and intracellular TAG accumulation during differentiation of preadipocytes treated by these compounds?

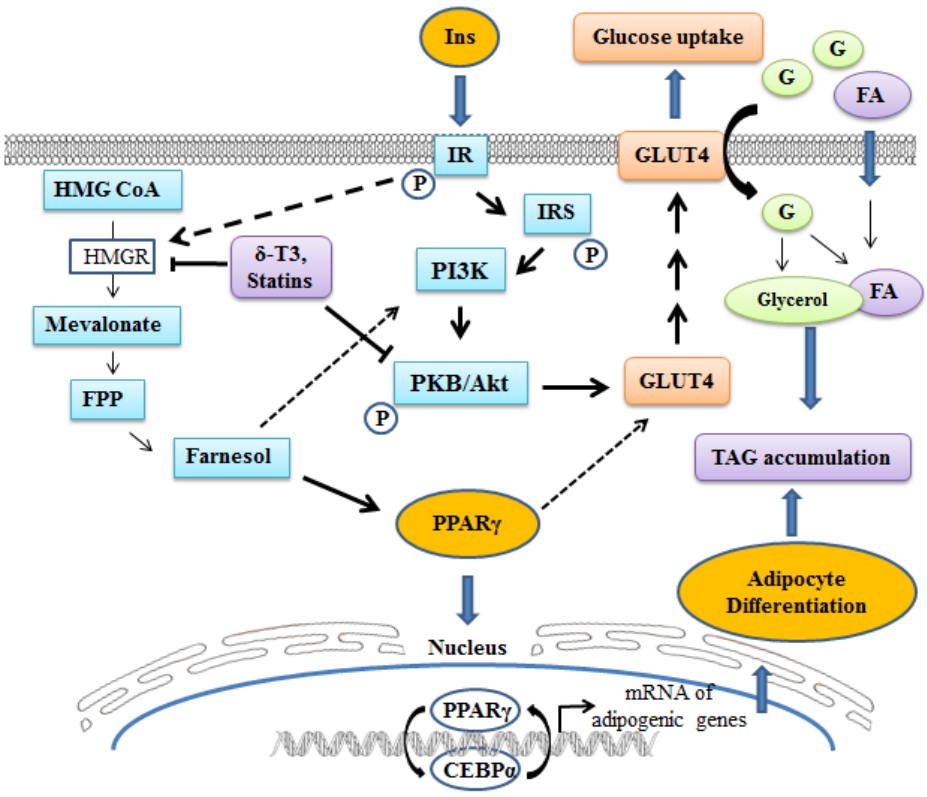

Figure 1.1 Hypothesized model for the effect of *d*-δ-tocotrienol and farnesol on adipocyte differentiation. Insulin (Ins) activates the insulin receptor- insulin receptor substrate-phosphatidylinositol 3-kinase (IR-IRS-PI3K) axis by phosphorylation which results in glucose transporter 4 (GLUT4) translocation from intracellular to plasma membrane and increased glucose uptake. Glucose produces FAs and glycerol, required for TAG synthesis. *d*-δ-Tocotrienol (δ-T3) and statins down-regulate both HMG CoA reductase (HMGR) and PKB/Akt proteins. Farnesol activates peroxisome proliferator-activated receptor γ (PPARγ). PPARγ and CCAAT/enhancer binding protein α (CEBPα) activate each other and induce the expression of adipogenic genes. PKB/Akt, protein kinase B/Akt; G, glucose; FPP, farnesyl pyrophosphate. ⟶ based on cited references, ⇢ hypothesized.

Hypothesis and aims

1. *d*-δ-Tocotrienol, a suppressor of HMG CoA reductase, dose dependently suppresses adipocyte differentiation, and *trans, trans*-farnesol, a metabolite of mevalonate, induces differentiation of murine 3T3-F442A preadipocytes.
2. The effects of *d*-δ-tocotrienol and *trans-trans*-farnesol on the differentiation of 3T3-F442A preadipocyte are mediated by the PI3K-PKB/Akt signaling pathway, which are consequently related to glucose uptake and the expression of GLUT4 protein.

Aims: To evaluate the impact of *d*-δ-tocotrienol and *trans, trans*-farnesol on adipocyte differentiation, intracellular triglyceride content, glucose uptake, and the expression of GLUT4 protein in murine 3T3-F442A preadipocytes.

Significance of study

The significance of this study is to investigate the effect of *d*-δ-tocotrienol and *trans, trans*-farnesol on intracellular TAG accumulation in adipocytes and the underlying mechanism. The results of this study may help to elucidate the role of the mevalonate pathway in adipocyte differentiation and the potential application of mevalonate-targeting compounds in clinical conditions such as insulin resistance and obesity.

CHAPTER II

REVIEW OF LITERATURE

Obesity is characterized by, among many other physiological changes, increased number of hypertrophic adipocytes and is a risk factor for chronic diseases such as type 2 diabetes, hypertension, coronary heart disease, and cancer. At the cellular level, obesity is characterized by hyperplasia and hypertrophy of adipocytes differentiated from fibroblastic preadipocytes which are regulated by genetic, endocrine, metabolic, neurological, and nutritional factors. Although adipocyte differentiation starts as a physiological response to energy imbalance, dysregulation of adipogenesis leads to pathological conditions. Suppressing adipocyte hypertrophy is one potential approache in the treatment and prevention of obesity and obesity related metabolic disorders.

Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, have been shown to inhibit adipocyte differentiation [Brown et al. 1978, Nakanishi et al. 1988, Nishio et al. 1996, Goto et al. 2011, Nakata et al. 2006, Nicholson et al. 2007, Elfakhani et al. 2014]. In a previous study, we showed that lovastatin inhibits the differentiation of 3T3-F442A preadipocytes and the expression of adipogenic genes, and the inhibition was reversed by supplemental mevalonate [Elfakhani et al. 2014].The effect of *d*-δ-tocotrienol, a suppressor of HMG CoA reductase, on adipocyte differentiation and the significance of glucose uptake in the underlying mechanism of adipogenesis have not been investigated.

Farnesyl pyrophosphate (FPP), a metabolite of the mevalonate pathway can yield farnesol in plants. FPP is the precursor of almost all isoprenoids and is positioned at branch points leading to the synthesis of long chain isoprenoids. FPP can be produced from farnesol through the "salvage pathway" [Crick et al. 1997]. It has been shown that FPP can activate PPARγ as an agonist during adipocyte differentiation [Goto et al. 2011].The effect of farnesol on the differentiation of preadipocytes and glucose uptake has not been investigated.

In the following, the process of adipocyte differentiation and the regulatory mechanisms and hypothesized underlying mechanism of *d*-δ-tocotrienol and *trans, trans*-farnesol on the differentiation of murine preadipocytes are reviewed.

Origin of adipocytes

The molecular events leading to the commitment of embryonic stem cells to the adipocyte lineage are still poorly understood. However, the pluripotent fibroblasts (stem cells) are known to have mesodermal origins [Cornelius et al. 1994] and can differentiate into committed preadipocytes, cartilage, bone or muscle tissue (Figure 2.1). Studies on the embryos of most species, including human, pig, mouse, and rat, have shown that the formation of WAT begins before birth [Piossonnet et al. 1983, Piossonnet et al. 1988]. The chronology of WAT appearance, however, is strictly dependent on the species [Robelin et al. 1985]. In humans, differentiation of preadipocytes begins during the embryonic development while the majority of adipose tissue formation occurs shortly after birth [Brudi et al. 1985]. This enables the newborn to cope efficiently with the

intervals between nutrient intake [MacDougald and Lane 1995]. Rat and mouse preadipocytes do not begin differentiation and conversion into adipose tissue until after birth [Aihaud et al. 1992]. In spite of difference in chronology of WAT formation, all species have the ability to differentiate preadipocytes throughout their life spans.

The potential to generate new fat cells persists even at the adult stage in response to the body's fat storage demands and the kind of diet. For example, fat cell number can increase when rats are fed a high-carbohydrate or high fat diet [Faust et al. 1984] or when humans are getting obese. Even in adipose tissue of very old mice, there are the early differentiation markers of adipocyte differentiation [Kirkland et al. 1990]. Moreover, isolated fat cell precursors from adult WAT of various species can be differentiated *in vitro* into mature adipocytes [Gregoire et al. 1995, Litthauer and Serrero 1992, Suryawan et al. 1997]. However, the efficiency of preadipocyte differentiation varies greatly when preadipose cells are derived from young versus old animals [Gregoire et al. 1990, Serrero and Mills 1987], with the capacity of preadipocyte differentiation decreasing significantly with age [Gregoire et al. 1995, Kirkland et al. 1990]. Recently, it has been shown that the total number of adipocytes remains constant in adults, relatively, with about 10% of adipocytes are renewed annually at adult ages [Spalding et al. 2008].

Pluripotent mesenchymal stem cells (MSCs), a subpopulation of MSCs, have the capacity to differentiate into adipocytes and have been used to produce adipocyte lineage. Established preadipocyte cell lines, such as 3T3L1 or 3T3-F442A cells, are committed to differentiate only into adipocyte lineage (Figure 2.1). The pluripotent MSCs are residents

in the vascular stroma of adipose tissue and have the potential to undergo commitment to differentiate into adipocytes, chondrocytes, myocytes, and osteocytes (Figure 2.1). Pluripotent C3H10T1/2 cells are used as an MSC model for *in vitro* studies of adipocyte genetics. These stem cells can be induced to commit and differentiate into different cell lineages by the effect of several factors in the culture medium which are different from *in vivo* studies. Recruitment to the adipocyte lineage *in vivo* is stimulated by prolonged excessive energy intake and elevated glucose uptake [Shepherd et al. 1993]. This prolonged positive energy balance may generate the signals required for inducing MSCs to generate new preadipocytes which are committed to differentiate into mature adipocytes. Identified factors that affect the conversion of pluripotent stem cells to adipocyte lineage include BMP family members, BMP4 and BMP2 [Bowers et al. 2006], Wnt [Ross et al. 2000], and Hh (hedgehog) [Zehentner et al. 2000, Spinella-Jaegle et al. 2001, van der Horst et al. 2003]. BMP4 and BMP2 have an activating effect, but Hh and Wnt signaling pathway appears to have an inhibitory effect in adipocyte differentiation [Bowers and Lane 2008, Bennett et al. 2005]. For example, Wnt10b promotes osteogenesis but inhibits adipogenesis [Bennett et al. 2005]; BMP4 promotes adipogenesis while inhibiting myogenesis [Kang et al. 2007], and PPARγ inhibits chondrogenesis but stimulate adipogenesis. Thus, lineage determination is regulated by extracellular signaling factors that lead to the coordinated activation or inhibition of several lineage-specific transcription factors.

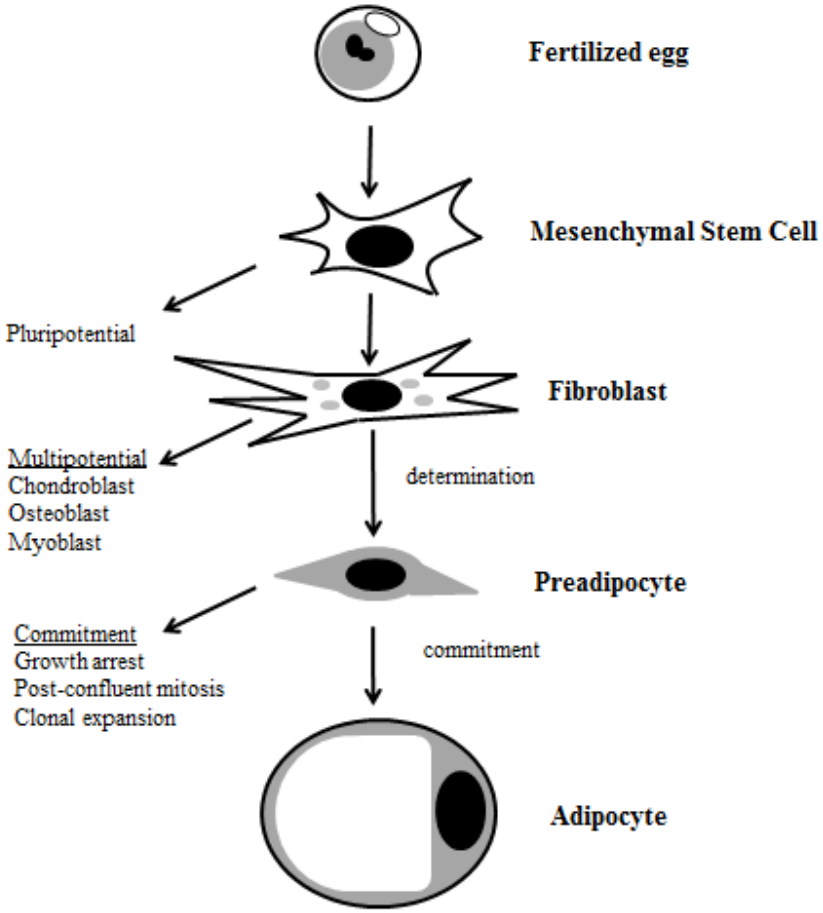

Figure 2.1 The development of adipocyte from fertilized egg.

In vitro models of adipocyte differentiation

In vivo study of preadipocyte differentiation is difficult because of several problems. Adipocytes compose about one third of fat tissue in animals with the remaining are a combination of small blood vessels, nerve tissue, fibroblasts and preadipocytes in various stages of development [Ge'loen et al. 1989]. A large amount of fat tissue is required to isolate preadipocytes from other fibroblasts. The distinction between

preadipocytes and fibroblasts is difficult to make, and it is hard to align preadipocytes at similar developmental stages. Primary cultures have a limited life span. Preadipocyte differentiation has, therefore, been studied primarily by using *in vitro* models of adipogenesis; and much of the knowledge of adipocyte differentiation is based on using the cell line models.

There are advantages and disadvantages to using a cell line. A cell line derived from cloning gives a homogenous population of cells which are all at the same stage of differentiation. In addition, these cells can be passaged indefinitely and provide a consistent source of preadipocytes for study. Injection of these preadipocyte cell lines into mice produces mature fat pads similar to the normal adipose tissue [Green and Kehinde 1979]. However, primary cultures are useful for validating results obtained by preadipose cell lines. Primary preadipocytes have been successfully cultured from a number of species including humans [Gregoire et al. 1990, Gregoire et al. 1995, Hauner et al. 1989, Kirkland et al. 1990, Litthauer and Serrero 1992, Serrero and Mills 1987]. Primary cells are diploid and may reflect the *in vivo* context better than aneuploidy cell lines, and can be derived from adipose tissue of various adipose depots [Deslex et al. 1987], reflecting the molecular and biochemical differences in fat pads [Gregoire et al. 1991, Masuzaki et al. 1995]. These differences may have important clinical applications. For example, these differences can explain the observation that excessive centralized fat accumulation is associated with more increased morbidity in humans.

Adipocyte precursor cell lines can be classified into two groups, pluripotent fibroblasts and unipotent preadipocytes. The pluripotent fibroblasts (e.g., 10T1/2, Balb/c 3T3, and CHEF/18 fibroblasts) have the ability to generate several cell types. For example, 10T1/2 fibroblasts can convert to preadipose, premuscle and precartilage tissue when treated with 5-azacytidine, a DNA methylation inhibitor [Taylor and Jones 1979]. These pluripotent fibroblasts have been used as good models for understanding the underlying mechanisms responsible for cellular determination of the separate cell fates. For example, it has been shown that MyoD is a critical regulator of muscle cell determination [Edmondson and Olson 1993]. Unipotent preadipocytes (e.g., 3T3-L1, 3T3-F422A, Ob1771 and 30A5), have undergone determination and commitment for differentiation to mature adipocytes. The 3T3-L1 and 3T3-F442A preadipocytes are the most widely used cell lines for such studies. These preadipocytes were clonally isolated from Swiss 3T3 cells derived from disaggregated 17-19-day mouse embryos [Green and Kehinde 1975, Green and Kehinde 1976, Green and Meuth 1974]. The 3T3-L1 cell line is one of the most well-characterized and reliable models for studying the conversion of preadipocytes into adipocytes.

There is some variation in the differentiation requirements of each cell line and it is believed that these differences are due to the developmental stage at which cells were arrested [Cornelius et al. 1994, Smas and Sul 1995] or the age and source of tissue from which primary cells are isolated. Although they may represent different stages of adipocyte development, the preadipocyte cell lines, as well as primary preadipocytes, are

already commited solely to the adipocyte lineage. In most cases, primary preadipocytes derived from young animals require only either low concentrations (1-10 nM) of insulin in the presence of fetal bovine serum (FBS) or high concentrations (1-10 mM) of insulin in serum-free medium [Deslex et al. 1987, Hauner et al. 1989]. Other enhancing agents such as glucocorticoids and methylisobutylxanthine (MIX) may be necessary to accelerate the differentiation process depending on the species, the age of the donor, and/or the adipose depot source [Deslex et al. 1987, Gregoire et al. 1998, Hauner et al. 1989, Syryawan et al. 1997]. Depending on the culture system, the insulin may either be critical for differentiation or necessary only to achieve the maximal rate of triglyceride synthesis and fat accumulation. Table 2.1 summerizes the characteristics of the most frequently employed preadipocyte cell line models [modified from Grégoire et al. 1998].

Table 2.1 The common *in vitro* cell line models of adipocyte differentiation.

Cell lines	**Origin**	**Inducing agents used for differentiation**
ES cells	Mouse blastocyst	Retinoic acid
CH3 10T1/2	Mouse embryo	Demethylating agent 5′-azacytidine
3T3-L1	Mouse embryo	10% FBS, DEX, MIX, and Insulin (high concentration)
3T3-F442A	Same as 3T3-L1	10% FBS, Insulin
Ob17	Epididymal fat pads of adult *ob/ob* mouse	8% FBS, insulin, and 3,3',5-triiodothyronine

FBS, fetal bovine serum; MIX, methylisobutylxanthine; DEX, dexamethasone.

Process of adipocyte differentiation

During the proliferation phase, preadipocytes are morphologically similar to fibroblasts. At confluence, induction of differentiation leads to drastic cell shape changes. In the differentiation phase, the preadipocytes convert to a spherical shape due to TAG synthesis, accumulated in lipid droplets. Progressively, the adipocytes acquire the morphological and biochemical characteristics of the mature white adipocyte.

A variety of differentiation protocols have been used for preadipocyte cell lines. Embryonic stem cells can be differentiated into adipocytes if exposed to retinoic acid, followed by treatment with adipogenic hormones. Maximal differentiation is achieved upon treatment with the combination of insulin, dexamethasone (DEX), methylisobutylxanthine (MIX) and fetal bovine serum (FBS) (Student et al. 1980). Insulin is known to act through the insulin-like growth factor 1 (IGF-1) receptor, and IGF-1 can be substituted for insulin in the adipogenic cocktail [Smith et al. 1988]. The adipogenic cocktail, containing MIX, DEX and insulin, is commonly abbreviated MDI. MIX, a cAMP-phosphodiesterase inhibitor, is traditionally used to stimulate the cAMP-dependent protein kinase pathway.

Adipocyte differentiation is characterized by chronological changes in the expression of numerous genes which can lead to the appearance of early, intermediate, and late mRNA/protein markers and TAG accumulation. In addition to the activation of genes required for adipogenesis, those genes that are unnecessary for adipocyte function are repressed. Growth arrest is required for subsequent differentiation. Growth-arrested

cells begin to express late markers of differentiation at day 3 of differentiation. These late markers consist of lipogenic and lipolytic enzymes, as well as other proteins responsible for modulating the mature adipocyte phenotype.

The molecular events of adipocyte differentiation are classified and summarized below as: (1) Early events, including growth arrest, mitotic clonal expansion, and gene expression; and (2) Late events, including the changes in gene expression and the accumulation of TAG in fat droplets.

Early events

Growth arrest. In general, when preadipocytes are seeded in culture plates with appropriate density and incubated in high glucose medium, Dulbecco's modified Eagle's medium (DMEM) including 10% calf serum, they start growing by proliferation. However, growth arrest and not cell confluence or cell-cell contact per se appears to be necessary for the following adipocyte differentiation. It has been shown that confluent 3T3-442A cells still undergo differentiation when shifted to methylcellulose-stabilized suspension culture differentiation [Pairault and Green 1979]. Primary rat preadipocytes can also differentiate in the absence of cell-cell contact [Gregoire 1998].

Two families of transcription factors, C/EBP and PPAR, are known as early markers of gene expression during adipocyte differentiation. PPARγ is highly adipocyte specific and its expression rapidly increases after hormonally induced differentiation. PPARγ expression is dectectable two days after induction of 3T3- L1 adipocyte differentiation with the maximal expression level in mature adipocytes. C/EBPα and

PPARγ may act cooperatively to bring about growth arrest [Altiok et al. 1997]. Although C/EBPα and PPARγ expression increase dramatically during adipocyte differentiation, the low level of these factors expressed in preadipocytes may be sufficient to mediate growth arrest preceding differentiation.The antimitotic activity of C/EBP prevents postconfluent mitosis when added to preadipocytes [Umek et al. 1991]. This prevents the cells from progressing through intermediate differentiation. The antimitotic activity of C/EBPα has been demonstrated by using the C/EBPα-estrogen fusion protein, resulting in cessation of cell growth as assessed by cell number and DNA synthesis [Umek et al. 1991].

During growth arrest, cyclic AMP response element-binding protein (CREB) becomes phosphorylated and activates the expression of C/EBPβ immediately (≤5 min) after induction of differentiation [Tang 2012]. G1- to S-phase transition occurs at 14-16 h after induction. C/EBPβ acquires DNA-binding activity as the preadipocytes reenter the cell cycle. Between 16-20 h after induction, the cell-cycle markers of S-phase entry are synchronously expressed (or inactivated) concomitant with the initiation of DNA replication [Tang 2012]. Acquisition of DNA-binding activity is delayed for ∼16 h when C/EBPβ undergoes phosphorylation by GSK3β coincident with reentry into the cell cycle at the G1/S boundary. These events are closely correlated with the expression of histone H4 [Zhang et al. 2011], the coordinated expression of cell-cycle proteins and DNA replication [Tang et al. 2003].

After the growth arrest, cells are committed to becoming adipocytes. The committed preadipocyte has the capacity for growth and conversion to an adipocyte.

Mitotic clonal expansion. After the hormonal stimulation, post-confluent growth-arrested preadipocytes reenter the cell cycle. Approximately 24 h after induction by MDI, differentiating preadipocytes undergo a postconfluent mitosis, named mitotic clonal expansion, followed by secondary growth arrest [Bernlohr et al. 1985]. The growth-arrested preadipocytes undergo at least one round of DNA replication and cell division and progress to clonal amplification of committed cells [Pairault and Green 1979]. This mitosis is believed to be necessary to unwind DNA, allowing transcription factors to access the regulatory response elements in genes responsible for the adipocyte phenotype [Cornelius et al. 1994]. For 3T3-442A and Ob17 cells, an increase in DNA synthesis precedes expression of adipogenic mRNA markers [Amri et al. 1986]. Blocking DNA replication by inhibitors of the cell cycle progression prevents differentiation, indicating that mitotic clonal expansion is required for adipocyte differentiation [Tang 2012]. For example, the constitutive overexpression of p27, the cell-cycle inhibitor, prevents cells from entering the S-phase of the cycle and stops all subsequent steps of differentiation [Patel and Lane 2000].

The molecular events during adipocyte differentiation. During adipocyte differentiation, the cells convert from a fibroblastic spindle shape to spherical mature adipocytes. This dramatic change in cell morphology accompanies changes in cytoskeletal components and the level and type of extracellular matrix (ECM) components [Gregoire 1998]. It is

likely that these changes are related to the expression of genes during adipocyte differentiation. A decrease in actin and tubulin expression precedes overt changes in morphology and the expression of adipocyte-specific genes [Spiegelman and Farmer 1982]. The morphological differentiation in 3T3-L1 preadipocytes can occur even in conditions when TAG synthesis is blocked by deprivation of biotin, a cofactor for fatty acid synthesis [Kuri-Harcuch et al. 1978], or by addition of lipolytic agents [Spiegelman and Green 1981], suggesting that morphological changes reflect a distinct process and are not the result of fat accumulation [Gregoire 1998].

There is also a decline in collagen gene expression in the early stage of differentiation. The fibroblast-expressed type I and III procollagen mRNA are decreased by 80-90%, while secretion of type IV collagen and entactin/nidogen increase during differentiation of 3T3-L1 cells [Aratani and Kitagawa 1988, Weiner et al. 1989]. The production of soluble chondroitin sulfate proteoglycan-I (versican) is also increased during 3T3-L1 differentiation and may account for increased viscosity of the culture medium [Calvo et al. 1991]. The synthesis of fibronectin and its pericellular amount decrease by 4-5 fold during differentiation of 3T3-F442A cells [Antras et al. 1989].

Changes in the pattern of gene expression can differ with the cell culture models and the differentiation protocols used. Expression of lipoprotein lipase (LPL) is known as the early sign of adipocyte differentiation [Cornelius et al. 1994, Gregoire et al. 1995, Ibrahimi et al. 1993, MacDougald and Lane 1995]. LPL is also synthesized by other cells derived from MSC including cardiac muscle cells and macrophages [Tengku-Muhammad

et al. 1996], indicating that LPL is not adipocyte specific and its expression may be independent of agents required for adipocyte differentiation.

At confluence, 3T3-L1 preadipocytes express early markers of adipocyte differentiation. Within 1 h after the addition of MDI, the expression of c-fos, c-jun, junB, c-myc and CCAAT/enhancer binding proteins (C/EBP) β and δ is observed [Cornelius et al. 1994]. Fos and jun proteins have not been implicated directly in any differentiation-specific events. The expression of c-fos, c-myc, and c-jun is transient and dissipated 2-6 h after induction of MDI, but they are believed to have mitogenic properties. It has been shown that C-myc increases at the time of initiating mitogenesis in differentiating preadipocytes [Cornelius et al. 1994].

Two transcriptional factors, PPARγ and C/EBPα, are known as the major regulator of adipogenesis and sit at the core of the adipogenic cascade [Gregoire et al. 1998, Tang and Lane 2012]. PPARγ expression rapidly increases after hormonally- induced adipocyte differentiation [Gregoire et al. 1998, Tang and Lane 2012, Green and Kehinde 1975]. Once activated, PPARγ and C/EBPα trans-activate each other in a feedback loop to maintain their gene expression [Shao and Lazar 1997, Lane et al. 1999]. C/EBPα and PPARγ function together as pleiotropic transcriptional activators of the large group of genes that produce the adipocyte phenotype [Vertino et al. 2005, Hwang et al. 1997, MacDougald and Lane 1995]. Furthermore, unlike coexpression of these two factors, ectopic expression of either transcription factor does not promote differentiation to the

same extent as the full differentiation initiated by the hormonal MDI cocktail [Brun et al. 1996, Hu et al. 1995, Tontonoz et al. 1994].

Another transcription factor induced early during adipocyte differentiation is sterol regulatory element binding protein-1c (SREBP1c)/adipocyte determination and differentiation factor 1(ADD1) [Kim and Spiegelman 1996, Kim et al. 1995], which is also involved in cholesterol metabolism [Brown and Goldstein 1997].

Preadipocyte factor-1 (pref-1) has been considered as a marker of preadipocyte phenotype [Smas et al. 1997]. Pref-1 is abundant in preadipocytes and is not detectable in mature adipocytes, and is the only known gene whose expression is completely downregulated during adipocyte differentiation [Gregoire 1998].

Now we explain the characteristics of the main genes involved in adipocyte differentiation:

The CCAAT/enhancer-binding family: The expression of C/EBP increases from undetectable levels in preadipocytes to detectable levels 2 d after MDI stimulation and to full expression 5 d after initiation of the differentiation program [Christy et al. 1991, Lin and Lane 1994]. Although C/EBPα was the first C/EBP family member shown to function in adipogenesis [Landschulz et al. 1988], the other isoforms, including C/EBPβ [Akira et al. 1990, Chang et al. 1990] and C/EBPδ [Cao et al. 1991, Williams et al. 1991], are also involved. The C/EBP isoforms (C/EBPα, C/EBPβ, and C/EBPδ) contain a DNA-binding domain and an adjacent C-terminal leucine zipper dimerization domain that allows formation of homo or heterodimers with other C/EBP family members,

required for DNA binding [Landschulz et al. 1989]. Within their proximal promoters, both the C/EBPα and PPARγ genes possess C/EBP regulatory elements at which C/EBPβ binds to coordinately activate transcription [Lane et al. 1999].

The expression of C/EBPβ and C/EBPδ are transiently increased before the expression of PPARγ and C/EBPα mRNA [Mandrup and Lane 1997, Wu et al. 1996, Lane et al. 1996]. Expression of C/EBPβ and C/EBPδ under the control of exogenous promoters of differentiation induces and accelerates adipogenesis in response to hormonal inducers. C/EBPβ is responsive primarily to DEX, whereas C/EBPδ is responsive primarily to MIX. The activity of C/EBPβ and C/EBPδ is thought to mediate the expression of PPARγ [Wu et al. 1995], which is transcriptionally induced during the 2 days after induction of differentiation with maximum level by 3-4 days [Tang 2012]. C/EBPα can then bind to the C/EBP site in the PPARγ promoter, providing for a stable, self-reinforcing regulatory loop [Tontonoz and Spiegelman 2008].

Blocking expression of C/EBPα with antisense RNA suppressed adipogenesis [Lin and Lane 1992]. Furthermore, ectopic expression of C/EBPα and PPARγ in 3T3-L1 preadipocytes enhances adipogenesis without hormonal induction [Lin and Lane 1994]. These and other findings indicate that C/EBPα may be sufficient to initiate the 3T3-L1 adipocyte differentiation program. Once C/EBPα is expressed, its expression is maintained through autoactivation [Christy et al. 1991].

Peroxisome proliferator-activated receptor-γ: The three isoforms of PPARγ (PPARγ1, PPARγ2, and PPARγ3) are transcribed from the same gene through alternative

splicing [Tanaka et al. 1997]; PPARγ2 is the primary adipocyte –specific isoform [Tontonoz and Spiegelman 2008]. All isoforms possess transactivation, DNA-binding, and dimerization domains [Tontonoz and Spiegelman 2008]. To bind at peroxisome proliferator response elements in target genes, PPARs must first form heterodimers with the retinoid X receptor [Tontonoz and Spiegelman 2008]. Although naturally occurring ligands for PPARγ have not yet been identified, several potent synthetic thiazolidinediones and prostanoids, e.g., 15-deoxy-Δ 12, 14 prostaglandin J2 [Green and Meuth 1974], bind it with high affinity.

Although PPARγ2 appears to act as a "master" regulator of the adipogenesis program, like C/EBPα, it also participates in other diverse systems, including hepatic lipogenesis [Tontonoz and Spiegelman 2008]. The ectopic expression of PPARγ2 in native preadipocytes and nonadipogenic fibroblasts activates expression of adipocyte genes and differentiation [Tontonoz and Spiegelman 2008, Egan et al. 1992]. Then, PPARγ2 and C/EBPα transactivate a large group of adipogenic genes.

Sterol regulatory element-binding protein: SREBPs are transcription factors that bind to the sterol regulatory element (SRE) of DNA sequence. The SREBPs are basic helix-loop-helix-leucine zipper proteins which regulate the synthesis of cholesterol and TAG. SREBP2 regulates transcription of the genes of cholesterol metabolism, while SREBP1c and its mouse homolog, adipocyte determination and differentiation factor-1 (ADD1), regulate lipogenesis [Horton 2002, Tontonoz et al. 1993]. SREBP1c/ADD1 presents as a membrane-bound precursor bound to SREBP-cleavage-activating protein

(SCAP) and is tethered in the endoplasmic reticulum (ER). SCAP is a regulatory protein required for the proteolytic cleavage of the SREBP, which is located in the ER as an integral membrane protein. Insulin-induced gene 1 (Insig-1) binds SCAP in the ER and blocks proteolytic processing required for SREBP activation. Upon its release from the ER (stimulated by insulin), SCAP-SREBP1c/ADD1 moves to the Golgi apparatus, where proteolytic cleavage frees its basic helix-loop-helix component for translocation to the nucleus. Because SCAP escorts SREBP from the ER to Golgi for proteolytic processing into an active transcription factor, the binding of SCAP by Insig-1 effectively prevents SREBP activation and thus blocks its action on gene transcription [Yang et al. 2002]. Recent evidence has been also shown that insulin regulates the release of SCAP-SREBP1c/ADD1 from the ER by downregulating the level of Insig-2a through increased turnover of its mRNA [Yellaturu et al. 2009]. Reducing the level of Insig-2a also facilitates export of SCAP-SREBP1c/ADD1 from the ER to the Golgi for proteolytic cleavage. Once in the nucleus, SREBP1c induces transcription of genes encoding lipogenic enzymes [Horton 2002] and adipocyte differentiation [Tontonoz et al. 1993].

In a metabolic sense, these findings are consistent with the recent finding that SREBP1c/ADD1 is phosphorylated and probably disactivated by AMPK [Aagaard et al. 2011], which promotes energy mobilization. Insulin, on the other hand, promotes energy storage by activating lipogenic enzyme expression through inducing SREBP1c/ADD1.The expression of SREBP1c/ADD1 mRNA is activated after the expression of C/EBPα and PPARγ, i.e., at ~20 h after induction of differentiation in

adipocytes [Kim and Spiegelman 1996]. Current evidence implicates SREBP1c/ADD1 in the late events of adipocyte differentiation.

Late events

The changes in gene expression. Adipocytes start adipogenesis and markedly accumulate lipid in the form of fat droplets during the late stages of differentiation. This de novo adipogenesis is concomitant with increased sensitivity to insulin. The activity, protein, and mRNA levels for enzymes involved in triacylglycerol metabolism including ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase (SCD1), glycerol-3 phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, and fatty acid synthase, insulin receptor number, and insulin sensitivity increase [Gregoire 1998]. During adipocyte differentiation, the total adrenergic receptor number increases mainly via increase in the b2- and the b3-subtypes, while there is a loss of b1-adrenergic receptors [Feve et al. 1990, Lai et al. 1982]. Adipocytes also synthesize other adipose tissue-specific markers including αP2, an adipocyte-specific fatty acid binding protein, also named FABP4 [Spiegelman et al. 1983], FAT/CD36 (a putative fatty acid transporter) [Ibrahimi et al. 1996] and perilipin (a lipid droplet-associated protein) [Greenberg et al. 1993]. In addition, mature adipocytes secrete a number of compounds including monobutyrin (an angiogenic agent), adipsin (a homolog of the serine protease complement factor D), Acrp30/AdipoQ, PAI-1, and angiotensinogen II [Hu et al 1995].

Formation of lipid droplets. Lipid droplets (LDs) are the organelles for lipid storage in cells of all organisms [Murphy 2001]. They provide energy by mobilization of fatty acids

when food supplies are limited or cannot fulfill the body's needs. They are important for survival and serve as a source of fuel for egg development. LDs have a protective role by scavenging free fatty acids and fat soluble toxins. Free fatty acids can form reactive lipids through non-oxidative pathways that promote cellular dysfunction and eventually cell death due to lipotoxicity [Kusminski et al. 2009]. The acute increase of fat soluble chemical toxins in blood can lead to acute toxicity syndrome. By storing the chemical toxins, LDs play a role in protecting the body organs from acute harmful effects of toxins. Furthermore, the inability to store lipids leads to lipodystrophy that is also related to multiple systemic problems [Herranz et al. 2008]. However, excessive storage of lipids can be detrimental to the organism by promoting the development of several metabolic disorders, such as obesity, MS, DM-II, CVD, and cancer. Thus, while lipid storage is an elementary feature of life, it must be tightly controlled. Therefore, optimal metabolic heath requires a finely tuned regulation of lipid storage and mobilization from LDs.

Although all cell types have ability to store fat in LDs, these organelles are highly enriched in mammalian adipocytes. Despite variations in size and appearance, the structure and organization of LDs are highly conserved. They consist of a hydrophobic core of neutral lipids, such as triacylglycerols and sterol esters, which is surrounded by hydrophilic phospholipid membrane. Among all cytoplasmic organelles, LDs are exceptional in that they possess only a monolayer membrane. LD biogenesis involves the incorporation of neutral lipids in the interspace between the bilayer leaflets of the ER membrane, followed by a budding-out of the cytoplasmic hemimembrane to form the fat

–bearing LD. In this model, the cytoplasmic leaflet of the ER membrane provides the phospholipid monolayer surface of the nascent LD. Once formed and released from ER, cytoplasmic LDs are likely to increase their volume either by localized lipid synthesis [Kuerschner et al. 2008] or by fusion of LDs [Boström et al. 2007]. Upon cellular demand, i.e. when energy is needed by the cell, stored lipids are remobilized by the tightly controlled activity of specific lipases. The fatty acids liberated by LD breakdown are subsequently utilized by the cell as an energy source and/or for metabolite production, or they are shuttled outside the cell to be used in other cells or target tissues. While the LD life cycle as a whole seems to be clear, many steps in the various processes are still unknown or controversial. It is possible that the LDs within a cell differ distinctly with respect to function, maturation and/or metabolic status, and that these characteristics are reflected in LD-specific protein coat compositions [Martinez-Botas et al. 2000].

Regulation

Excess accumulation of fat causes adipocytes to become dysfunctional and insulin resistant, which produces a major problem in regulation of metabolic energy balance. In long term, this condition can lead to chronic metabolic disorders such as obesity and obesity related MS and DM-II. Investigating the effect of different food ingredients on adipocyte differentiation helps to add a new strategy for control of obesity. It seems that the current strategies for control of obesity are not efficient. Most of diet therapies fail in the first year or there is a rebound phenomenon following cessation of diet and exercise therapies. On the other hand, some obese people demonstrate a resistance to these

strategies, explainable by individual variation due to genetic and biological factors or the functional status of adipocytes.

In general, approach to every disease is primarily based on histopathology and pathophysiology. Obesity, as the mother of many other disorders, is not excluded. To resolve the problem of obesity, we need to correct the problem at a cellular level. Regardless of the different etiologies, obesity is characterized by an increase in the number and size of adipocytes that represent the histopathology of obesity. Accordingly, evaluating the effect of various food ingredients on adipocyte differentiation and its functional status may reveal critical points in diet therapy.

While some specific biomarkers of adipocyte function, such as leptin, can be detected in blood at levels proportional to that in the fat tissue, others such as tumor necrosis factor-alpha (TNFα), are released mainly in adipose tissue. TNFα is a multi-functional cytokine capable of regulating cellular and biological processes such as immune function, cell differentiation, proliferation, apoptosis and energy metabolism. A high level of TNFα is related to adipose tissue dysfunction and insulin resistance. TNFα is secreted from very large adipocytes; its level in blood represents the inflammation in the whole body system but not within the adipocytes. In obesity related type 2 diabetes, TNFα levels increase in adipose tissue, while the circulating TNFα is lower, relatively [Hotamisligil et al. 1993, Xu et al. 2002]. Therefore, whether adipose-derived TNFα exerts endocrine effects in systemic insulin resistance has been debated. With improved sensitivity and specificity of detection methods, circulating TNFα seems well correlated

with BMI [Zahorska-Markiewicz et al. 2000] and impairment in TNFα processing can improve systemic insulin sensitivity [Togashi et al. 2002, Serino et al. 2007]. *In vitro* studies initially indicated that TNFα affects glucose homeostasis in adipocytes [Stephens and Pekala 1991], promotes lipolysis in cultured adipocytes [Kawakami et al. 1987] and strongly inhibits adipocyte differentiation and lipogenesis [Beutler et al. 1985, Torti et al. 1985]. However, a human study from Spiegleman's group has shown no correlation between the TNF-α level in blood and adipose tissue of obese people [Hotamisligil et al. 1995]. TNF-α protein concentrations in plasma and in conditioned medium of explanted adipose tissue were measured in obese individuals. Obese individuals expressed 2.5-fold more TNF-α mRNA and protein in fat tissue, but the plasma level of TNF-α were undetectable [Hotamisligil et al. 1995]. Furthermore, they found a strong positive correlation between TNF-α level in fat tissue and the level of hyperinsulinemia, an indirect measure of insulin resistance. The body weight reduction in obese subjects was also associated with a decrease in TNF-α level in fat tissue [Hotamisligil et al. 1995]. TNF-α treatment of mature TA1 or 3T3-L1 adipocytes or newly differentiated primary human adipocytes down regulated the expression of adipocyte markers [Petruschke and Hauner 1993, Torti et al. 1985, Torti et al. 1989]. This anti-adipogenic effect of TNF-α can be due to insulin resistance. Accordingly, adipose tissue biopsy of obese people would help in evaluating the size and functional status of adipocytes. Then, based on the information from *in vitro* studies about the effect of different ingredients on adipocyte function, appropriate food ingredients can be selected to correct adipocyte sickness

specifically in individuals. This strategy can help to control obesity beside the other strategies such as diet, exercise, and behavioral therapies.

Now we explain the regulation of adipocyte differentiation by different factors:

Regulation of adipocyte differentiation by transcriptional factors

CEBP family. Following the induction of differentiation, the expression of C/EBPβ and C/EBPδ is rapidly (<4 h) increased before other transcriptional factors [Tang and Lane 1999]. However, C/EBPβ is inactive and unable to bind DNA until later (~ 16 h after induction) when the cells enter into S phase and mitotic clonal expansion [Tang 2012]. Beginning 17-24 h after the induction of differentiation, the C/EBPα and PPARγ genes are transcriptionally activated by C/EBPβ through C/EBP regulatory elements in their proximal promoters [Tang 2012]. A knockout of the C/EBPβ gene in mice had little effect on adipose tissue accumulation [Tanaka et al. 1997] while in the double knockout $[(\mathrm{C/EBP\beta})^{-/-}/(\mathrm{C/EBP\delta})^{-/-}]$, adipose tissue mass was markedly reduced [Tanaka et al. 1997]. Phosphorylation is an important posttranslational modification of C/EBPβ and leads to the acquisition of DNA-binding function as preadipocytes traverse to the G1-S checkpoint at the onset of mitotic clonal expansion [Tang et al. 2005]. C/EBPβ is phosphorylated sequentially, first by MAP kinase and then much later by GSK-3β [Tang et al. 2005]. Phosphorylation on Thr188 by MAP kinase occurs ~4 h after induction of differentiation. However, Thr188 phosphorylation is insufficient on its own to produce DNA-binding activity by C/EBPβ [Tang et al. 2005]. Phosphorylation on Thr179 or

Ser184 by GSK3β occurs between 12 and 16 h after induction. Dual phosphorylation of C/EBPβ at two of these sites leads to acquisition of DNA-binding activity.

PPARγ. PPARγ is induced during the differentiation of preadipocytes into adipocytes and is highly expressed in both white and brown adipose tissue (WAT and BAT) [Tontonoz et al. 1994, Sears et al. 1996]. The expression of adipose-specific genes is induced by exogenous PPARγ agonists and results in morphologic differentiation in fibroblasts, including the accumulation of triglyceride droplets [Tontonoz et al. 1994]. It has been shown that PPARγ mutants with dominant-negative activity inhibit adipogenesis in cultured preadipocytes [Barroso et al. 1999, Berger et al. 2000].

PPARγ, like all members of PPAR family, functions as an obligate heterodimer with RXRs [Kliewer et al. 1992], and its high-affinity binding to DNA requires this dimerization with RXR. The PPARγ protein has domains that are found in nearly all nuclear hormone receptors. The C-terminal region is responsible for dimerization with RXR and contains the major transcriptional activation domain. The C-terminal region also forms the ligand-binding pocket, including many hydrophobic residues occurring inside the pocket. Like most members of the nuclear receptor group, the N-terminal 120 amino acids have transcriptional activity when linked to a heterologous DNA-binding domain. The PPARγ protein has two N-terminal variants formed by an alternative promoter usage and an alternative first exon. PPARγ2 has an additional 30 amino acids at its extreme N terminus and is expressed in a more adipose-selective manner [Torontoz et

al. 1994]. It has been suggested that only PPARγ2 can induce adipose differentiation [Ren et al. 2002].

When the N-terminal region of PPARγ is deleted, this factor has greater transcriptional activity and more powerful adipogenic action [Tontonoz et al. 1994], suggesting that the N terminus might also have some inhibitory function in the context of the holoreceptor. Much of this inhibitory action was later shown to be linked to a phosphorylation of PPARγ by Erk 1 or 2, members of the MAP kinase family. Murine PPARγ2 can be phosphorylated by MAP kinase at serine 112, severely decreasing the biological action of the receptor [Adams et al. 1997, Hu et al. 1996].

PPARγ activity can be regulated by many of the common cofactors for the nuclear receptor family of proteins. One of the first highly regulated coactivators was discovered as a regulator of PPARγ is PPARγ coactivator 1 (now PGC-1α). This protein is highly enriched in brown fat cells, relative to white fat cells, and binds to PPARγ in a ligand-independent manner [Puigserver et al. 1998].

The major regulatory mechanism for switching on PPARγ activity is presumably the binding of an agonist ligand, natural or synthetic. The identity of biological ligands for PPARγ remains unresolved and is an area of active investigation. It has been suggested that an endogenous PPARγ activator is produced during adipogenesis [Tzameli et al. 2004], though it has been difficult to purify a ligand. Numerous studies have shown that polyunsaturated fatty acids and related molecules can activate PPARγ as well as other PPARs [Kliewer et al. 1997, Keller et al. 1993]. Certain prostanoids, including 15-

deoxy-δ 12,14 prostaglandin J2 (15-dpGJ2), are excellent activators of PPARγ [Kliewer et al. 1995].

Forced expression of PPAR in preadipocytes does not cause differentiation without the addition of an exogenous ligand [Tontonoz et al. 1994]. This indicates that an endogenous PPAR ligand is not present in 3T3-L1 preadipocytes. It is suggested that the upregulation of ADD1/SREBP-1c gene expression during preadipocyte differentiation leads to the production of endogenous PPAR ligands required for transcriptional activity [Kim et al. 1999].

The nuclear hormone receptor PPARγ is activated by thiazolidinediones (TZDs) which are widely used as insulin-sensitizers in the treatment of diabetes.The TZDs were initially discovered because of their ability to lower glucose levels in rodents. Subsequently, they were confirmed to improve insulin sensitivity in human subjects [Nolan et al. 1994]. Two members of this drug class, rosiglitazone and pioglitazone, are currently in widespread use for the treatment of type 2 diabetes. Several lines of evidence support the relation between PPARγ activation and insulin sensitivity. TZDs, direct agonist ligands of PPARγ [Lehmann et al. 1995], exert their biological effects on insulin sensitivity through binding to PPARγ. The synthetic non-TZD agonists for PPARγ also improve insulin sensitivity [Henke et al. 1998]. Furthermore, mutations in PPARγ in both rodents and humans are associated with insulin resistance [Barroso et al. 1999, Agostini et al. 2006, He et al. 2003].

The production of bioactive molecules, named adipokines, derived from adipose tissue, may play a role in cross talk between PPARγ pathway and insulin sensitivity. Plasma levels of adiponectin, an adipokine selectively released by adipocytes [Bouskila et al. 2005], are correlated with adipose tissue mass and insulin sensitivity [Hu et al. 1996]. The adiponectin gene is a direct target for regulation by PPARγ [Berg et al. 2002]. Adiponectin mRNA and plasma protein levels are induced in rodents and humans following TZD administration [Yu et al. 2002, Pajvani et al. 2004]. Administration of adiponectin in mice results in the suppression of hepatic glucose output and improvement in glucose uptake, like the effects of TZDs [Yamauchi et al. 2002]. Furthermore, mice lacking adiponectin show impaired responses to TZDs [Nawrocki et al. 2006]. These evidences indicate the relation between PPARγ pathway and insulin sensitivity that results in increased glucose uptake.

Ligand activation of PPARγ in adipocytes is also associated with decreased production of tumor necrosis factor TNFα and resistin (Steppan et al. 2001), proteins postulated to cause insulin resistance. It has been shown that the knockouts of TNF, TNF receptors, and resistin improve insulin sensitivity, consistent with a physiological and/or pathophysiological role for these proteins in modulating insulin responses and systemic metabolism [Uysal et al. 1997].

The mechanistic studies of the effects of PPARγ agonists on insulin resistance led to the conclusion that adipose tissue is the primary tissue responsible for the therapeutic effects of TZDs. At the core of this conclusion is the “lipid steal” hypothesis [Rangwala

and Lazar 2004, Ye et al. 2004]. Type 2 diabetes is associated with increased plasma levels of free fatty acids (FFA) and the inappropriate deposition of lipids in tissues other than fat, including liver and skeletal muscle. The use of TZD drugs in diabetes cause incorporation of FFAs into fat by activation of PPARγ. Thereby, FFAs are sequesterated away from tissues where their accumulation could have deleterious effects on insulin action. Upregulation of PPARγ target gene expression in WAT is believed to enhance its capacity to store dietary fatty acids [Tontonoz et al. 1994, Tontonoz et al. 1995, Schoonjans et al. 1996, Martin et al. 1997]. Consistent with this hypothesis, TZDs effectively lower FFA levels [Boden et al. 2005]. Genetic studies in mice strongly support the contention that adipose tissue is the primary site of TZD action. First, studies in lipodystrophic mice have established that the presence of WAT is critical for the antidiabetic effects of TZDs [Chao et al. 2000]. Second, insulin-resistant mice lacking expression of PPARγ specifically in adipose tissue are markedly deficient in their response to TZD treatment [He et al. 2003].

Adipose tissue exhibits the highest levels of PPARγ expression in the human body, and it is also the primary site of TZD action in human diabetic subjects [Semple et al. 2006]. The ability to lower FFA levels by PPARγ agonists in humans is consistent with the hypothesis that they act at least in part by promoting partitioning of lipids into adipose tissue and away from liver and muscle [Maggs et al. 1998]. In particular, TZD treatment causes a preferential increase in subcutaneous adipose tissue compared to visceral adipose tissue [Mori et al. 1999, Kelly et al. 1999], indicating that PPARγ

agonists also have depot-selective effects on adipose tissue structure in humans. The ability of TZDs to regulate expression of adipokines, at least in part, explains the hypothesis that PPARγ agonists improve human insulin resistance.

SREBP1c. Sterol-regulatory-element-binding protein 1c (SREBP1c) is another important adipogenic transcription factor that can directly regulate the expression of several key genes of lipid metabolism [Eberle et al. 2004]. Moreover, SREBP1c appears to contribute to the expression of PPARγ and the production of an endogenous PPARγ ligand in adipocyte differentiation [Kim and Spiegelman 1996, Kim et al. 1998]. SREBP1c expression and activity, via cleavage and nuclear translocation, are acutely responsive to insulin [Eberle et al. 2004, Brown and Goldstein 1997]. In addition to controlling genes involved in lipid metabolism, the regulation of the expression of the adipokines, leptin and adiponectin, by insulin is also mediated by SREBP1c [Kim et al. 1998, Seo et al. 2004]. Thus, in both developing and mature adipocytes, SREBP1c can potentially integrate information of nutritional and metabolic status to control new adipocyte formation, lipid metabolism, insulin sensitivity and, via adipokines, whole-body energy homoeostasis and appetite [Payne 2010].

SREBP1c plays a central role in lipid metabolism of the liver and adipose tissue. Despite the importance of SREBP1c and its established role in adipocyte development, relatively little is known about the factors controlling its expression during adipogenesis [Seo et al. 2004]. The C/EBP family of transcription factors play a critical role in both the early induction of SREBP-1c and the maintenance of its expression in maturing

adipocytes [Payne 2009]. AMPK also interacts with and directly phosphorylates sterol regulatory element binding proteins (SREBP-1c and SREBP-2). Ser372 phosphorylation of SREBP-1c by AMPK is necessary for inhibition of proteolytic processing and transcriptional activity of SREBP-1c in response to polyphenols and metformin. AMPK stimulates Ser372 phosphorylation, suppresses SREBP-1c cleavage and nuclear translocation, and releases SREBP-1c target gene expression in hepatocytes exposed to high glucose, leading to reduced lipogenesis and lipid accumulation [Li et al 2011].

Regulation of adipocyte differentiation by leptin

Adipose tissue is an endocrine organ. Leptin and adiponectin are the most important hormones produced by adipose tissue which have a significant role in energy homeostasis. Leptin is a cytokine produced in proportion to fat stores and acts on hypothalamic nuclei to reduce food intake and promote energy expenditure in rodents [Friedman 1998, Elmquist et al. 1998]. Conversly, lack of leptin signaling because of a mutation in leptin or leptin receptor gene results in increased food intake with reduced energy expenditure in rodents and humans in spite of obesity [Friedman 1998, Elmquist et al. 1998, Montague et al. 1997].

Leptin maintains normal intracellular lipid homeostasis despite wide variation in dietary fat. This function of leptin is similar to insulin that maintains tolerance to dietary carbohydrate despite wide variations in its intake [Lee et al. 2001]. On a very high 60% fat diet, adipocyte fat content for normal rats increases almost 150-fold, whereas that of pancreatic islets, liver, heart, and skeletal muscle rises less than 10-fold [Lee et al. 2001,

Unger 2003]. This low level of triglyceride deposition in nonadipocytes during the consumption of excess dietary fat is the result of leptin release in proportion to ingested excess TAG. Leptin exerts an antilipogenic, pro-oxidative action on its peripheral non-adipose target tissues [Unger 2003]. This is, in part, the result of lowering the expression of lipogenic transcription factors, such as SREBP-1c in liver, and PPARγ2 and lipogenic enzymes, such as acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), in fat [Zhou et al. 1998].

In addition to reduced synthesis of new ACC, the activity of ACC protein is decreased through AMPK-mediated phosphorylation of the enzyme [Minokoshi et al. 2002], and malonyl CoA formation is diminished. This not only reduces lipogenesis, but also enhances mitochondrial fatty acid oxidation by releasing carnitin palmitoyl transferase (CPT)-1 from inhibition by malonyl CoA. Leptin also enhances mitochondrial activity by upregulating a PPARγ coactivator (PGC)-1α [Kakuma et al. 2000] that induces mitochondrial biogenesis [Puigserver et al. 1998]. Thus leptin action may be required for normal liporegulation in tissues, just as insulin is required for normal glucoregulation, and leptin joins insulin and glucagon as a major regulator of nutrient homeostasis in plasma and tissues. Leptin and glucagon both antagonize insulin-mediated lipogenesis through downregulation of SREBP-1c. The liver is the principal target of glucagon, whereas leptin opposes insulin-mediated lipogenesis in extrahepatic tissues as well.

Any unoxidized FA surplus may enter pathways of non-oxidative metabolism. Esterification to TAGs appears to be a major metabolic avenue for diverting FA from entry into more harmful pathways. The accumulation of neutral fat probably does little harm to cells; in fact, esterification of FA may well be protective. As a result, adipose tissue is the safest place for accumulation of FAs as a neutral fat. Leptin is important in this buffering function of adipocytes via diverting FAs from other organs to the adipose depots [Unger 2003]. Lipotoxicity, accumulation of TAG in non fat-tolerable tissues, plays an important role in the pathogenesis of type 2 diabetes via damage to pancreatic β-cells (Unger 1995, Unger and Zhou 2001, Unger and Orci 2001, Unger 2003, Unger and Scherer 2010). Liver and muscle cells appear to have the highest tolerance for a lipid surplus because they both have mechanisms for converting surplus TAG. Liver cells can export TAG in the form of very low density lipoprotein (VLDL) and myocytes can increase TAG oxidization as the result of exercise. Pancreatic β-cells have neither of these options, which could explain their vulnerability to lipid overload.

Regulation of adipocyte differentiation by insulin and glucose

The composition of MDI regarding to the availability of glucose, insulin, and serum are known as important factors for differentiation of preadipocytes. A high glucose culture medium including 450 mg/dL glucose (4.5 g/L), which is comparible to the plasma glucose level of a diabetic mouse. Thus, the cells are growing and differentiating with high nutrient availability, and glucose is the major nutrient used by adipocytes to make fat. Glucose is required and sufficient for synthesis of TAG in adipocytes. Because

glucose is a polar molecule, its entrance into the cells depends on glucose transporters. Glucose transporter is provided by a family of highly related 12 transmembrane domain-containing proteins called the GLUT family. The GLUT family contains 13 known members and GLUT4 is the predominant glucose transporter isoform in adipocytes which is activated in response to insulin. Many studies indicate that insulin causes translocation of GLUT4 from intracellular stores to the plasma membrane, where they can catalyze glucose uptake into the cell. At least two pathways have been identified for the mechanism of GLUT4 translocation to the cell surface. One is the PI3K pathway [Kanzaki et al 2004] and the other is the Cbl–CAP–CrkII– C3G–TC10 pathway [Baumann et al 2000]. Most of evidences indicate that PI3K pathway is the main regulator of GLUT4 translocation in adipocytes fallowing activation by insulin signaling.

In the basal state, more than 90% of GLUT4 is sequestered in the intracellular compartments, while only 10% is present at PM [Satoh et al. 1993, Li et al. 2001]. Insulin shifts the steady-state distribution of GLUT4 by increasing the GLUT4 exocytosis rate which known as GLUT4 translocation. Approximately half of intracellular GLUT4 is located in “Glut4 storage vesicle” (GVS), the main target of insulin induced Glut4 translocation (Figure 2). The exocytosis of GLUT4 in response to insulin follows a complex intracellular trafficking pathway, passing through early and recycling endosomes before being resorted into GSVs [Bryant et al., 2002; Watson et al., 2004].

Studies on 3T3-L1 cells indicate that the main hormone to induce adipocyte differentiation is insulin [Gregoire et al. 1998], which works through the IGF-1 receptor

on the adipocyte cell membrane [Smith et al. 1998]. Following the binding of insulin with IGF-1 receptor, two signaling pathways, MAP kinases (MAPK) and phosphatidylinositol 3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), can be activated in 3T3-L1 cells. PI3K-PKB/Akt is an important intracellular pathway involved in the regulation of many cellular activities, including cell proliferation and apoptosis. It is also involved in the IGF-1 receptor signal that regulates cell growth and anti-apoptosis in many types of cells [Kulik et al. 1997, Blair et al. 1999, Barber et al. 2001, Peruzzi et al. 1999].

Based on several studies, an important function of the PI3K-PKB/Akt signaling pathway in adipogenesis has been established [Tomiyama et al. 1995, Magun et al. 1996, Kohn et al. 1996, Gagnon et al. 1999, Sorisky et al. 1996, Peng et al. 2003]. Inhibition of PI3K with wortmannin and LY294002 blocks adipocyte differentiation in 3T3-L1 cells [Tomiyama et al. 1995, Xu and Liao 2004]. The PI3K-PKB/Akt signaling pathway is important not only in the regulation of adipose tissue development but also in the development of other tissues originating from mesodermal cells. The results from PKB/Akt gene knockout in mice has also shown that the development of adipose tissue is impaired along with other abnormalities, including muscle, bone, and skin [Peng et al. 2003].

Insulin increases glucose uptake into adipocytes and muscle tissues through the PI3K-PKB/Akt pathway. This axis is mainly regulated by phosphorylation modification of proteins [Brown et al. 2001]. Binding of insulin to its tyrosine kinase receptor involves a cascade of intracellular phosphorylation events, including tyrosine phosphorylation of

insulin receptor substrate (IRS) proteins and activation of the PI3K-PKB/Akt pathway. The phosphorylation of IRS proteins and recruitment of PI3K result in the generation of PI (3,4,5) P3 which subsequently activates phosphoinoside-dependent kinase 1 (PDK1) and PKB and then atypical protein kinase C (PKC) (Figure 2.2). Increased PI (3,4,5) P3 produced from PI (4,5) P2 by class IA PI3K relays insulin signaling downstream to Akt and PKC [Brown et al. 2001]. In adipocytes, PI (4,5) P2 metabolism has also marked effects on GLUT4 endocytosis and intracellular vesicle trafficking due to changes in actin dynamics [Kanzaki et al. 2004].

Finally, these phosphorylated upstream molecules either directly or indirectly transduce GLUT4 translocation from GLUT4 specific vesicles (GSVs) to the plasma membrane (Figure 2.2). However translocation of GLUT4 to the cell surface does not mean that this transporter is able to elicit active glucose uptake into the cell [Funaki et al 2004].This is evident from experiments showing that exogenously delivered PI (3,4,5) P3, the product of PI3K, can augment plasma membrane GLUT4 content without increasing glucose uptake. Thus, PI3K suffices for GLUT4 translocation but may still be insufficient to stimulate glucose transport [Sweeney et al 2004]. Based on this scenario, it is required to check glucose uptake to make sure about the activity of GLUT4.

The activation of PI3K is also essential for the activation of Rac1, a Rho family of small G proteins involved in GLUT4 translocation [JeBailey et al. 2004, 2007]. The activation of Rac1 is important in the remodeling of actin, which is a necessary component for insulin-induced GLUT4 translocation [Khayat et al. 2000, Patel et al.

2006] (Figure 2.2). Small G proteins function as GDP/GTP-regulated molecules which are interconverted between an inactive GDP-bound form and an active GTP-bound form. In the active form, small G proteins interact with a large number of specific effector proteins to carry out their diverse physiological roles. The G proteins possess low intrinsic GTP hydrolysis, and their GDP/GTP cycling is highly regulated by three types of proteins; guanine-nucleotide exchange factor (GEF) catalyzes the exchange of GDP for GTP by transiently stabilizing the nucleotide-free protein [Schmidt & Hall 2002], GTPase-activating protein (GAP) accelerates the intrinsic GTPase activity to increase the GDP-bound form [Bernards & Settleman 2004], and guanine nucleotide dissociation inhibitors (GDIs) binds to the GDP-bound form of GTPases and prevents spontaneous and GEF-catalyzed release of nucleotide [Cormont and Marchand-Brustel 2001, Rizzo and Romero 1998].

Ras-related Rab GTP-binding proteins and Rho family, two families of G proteins, have been implicated in the regulation of intracellular vesicular trafficing including GLUT4 translocation.The Rab family plays a role in vesicle traffic, while the Rho family is mainly involved in actin remodeling and cytoskeletal organization. Insulin stimulates Rab4 activity and its physically associated KIF 3 (kinesin II in mice) mediating GLUT4 vesicles cargo transport along microtubules [Imamura et al 2003]. Mutant Rab4 lacking a prenylation site not only prevents GLUT4 translocation, but also inhibits the proximal cascades of insulin signals such as IRS-1, PI3K and PKB activation [Cormont et al 2001].

AS160, a new Rab-GAP protein, functions as a brake on GVSs to retain GLUT4 inside the cell by inactivating cognate Rab proteins [Sano et al. 2003, Eguez et al. 2005, Larance et al. 2005, Chen et al. 2012]. Insulin causes phosphorylation of AS160 at Akt phosphorylation sites resulting in its dissociation from GLUT4 vesicles [Larance et al. 2005], and the cognate Rab proteins now become active, helping GLUT4 translocation to the PM [Sakamoto and Holman 2008, Foley et al. 2011]. A AS160 mutant in which four of these phosphorylation sites are mutated to alanine (AS160-4P), a constitutively active form of AS160, inactivates cognate Rabs and inhibits GLUT4 translocation in adipocytes [Sano et al. 2003], muscle cells [Thong et al. 2007] and skeletal muscles [Kramer et al. 2006]. Therefore; the insulin-stimulated phosphorylation of AS160 seems to be essential for GLUT4 translocation.

The insulin signaling cascade bifurcates downstream of PI3K into an arm leading to the Akt/aPKC activation and another leading to Rac1 activation (Figure 2.2). It has been shown that neither dominant- negative mutants of Akt nor non-phosphorylatable AS160 prevents actin remodelling [Wang et al. 1998, Thong et al. 2007], and Rac1 knockdown does not alter Akt phosphorylation [JeBailey et al. 2007].

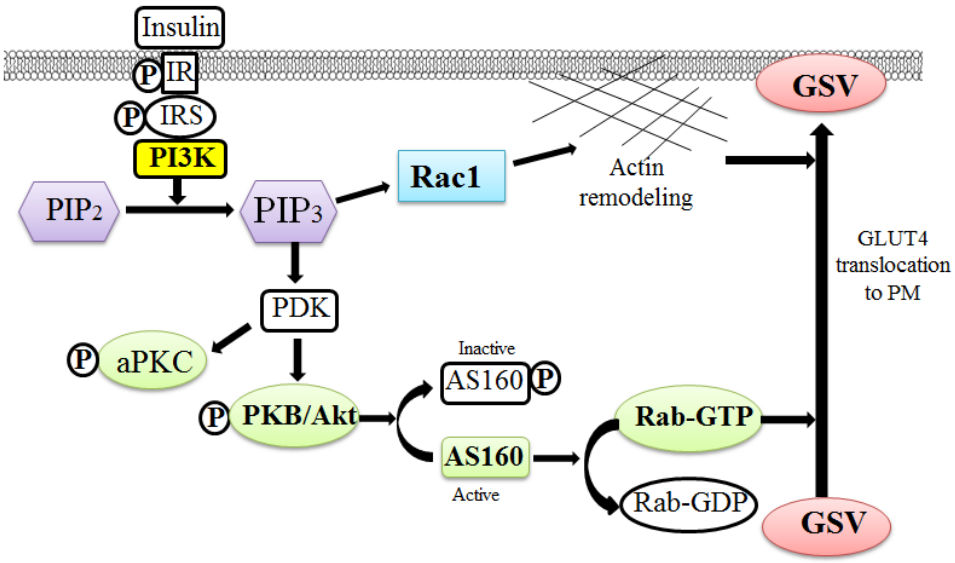

Figure 2.2 Insulin signaling pathway to induce the translocation of GLUT4. Insulin activates the IR-IRS-PI3K axis by phosphorylation. This signaling cascade bifurcates downstream of PI3K into an arm leading to activation of PKB/Akt and aPKC. Another arm results in activation of Rac1, causing actin remodeling. Activation of PKB/Akt cause phosphorylation and inactivation of AS 160 which inhibits translocation of GLUT4 by inactivating Rab proteins (Rab-GDP); thereby the level of active Rab (Rab-GTP) is increased, leading to GLUT4 translocation from intracellular to plasma membrane (PM).

Regulation of adipocyte differentiation by AMPK

As a key physiological energy sensor, AMP-actvated protein kinase (AMPK) is a major regulator of cellular and organismal energy homeostasis that coordinates multiple metabolic pathways to balance energy supply and demand and ultimately modulate cellular and organ growth [Hardie 2007]. AMPK is a serine/ threonine protein kinase and a member of AMPK-related kinase family that consists of 13 kinases in the human genome. Phosphorylation of their activation loops by upstream kinases, such as LKB1,

which was initially identified as a tumor suppressor [Lizcano et al. 2004, Bright et al. 2009], is required for the activation of AMPK-related kinases.

The AMPK holoenzyme is a trimer consisting of α subunit, β subunit, and γ subunit; α is the catalytic subunit and β and γ are regulatory subunits [Hardie et al. 1998]. In mammals, each subunit has multiple subtypes and expresses differentially in different tissues. The activity of AMPK is allosterically enhanced by AMP binding to the γ subunit. The binding of ATP or ADP to the γ subunit does not induce allosteric activation of AMPK. Binding of AMP to the γ subunit protects the activation loop from dephosphorylation by the phosphatases, therefore leading to AMPK activation. ADP may also play a regulatory role in AMPK activation [Lee et al. 2001]. Cellular concentrations of AMP or ADP are much lower than those of ATP [Kakuma et al. 2000, Zhou et al. 1997]. Therefore, AMPK is able to sense small changes in cellular energy charge by monitoring AMP and ADP. Thus, AMPK is able to maintain cellular energy homeostasis at a very constant level. A variety of cellular stresses including metabolic poisons that decrease ATP generation as well as pathologic cues such as nutrient starvation, ischemia, and hypoxia activate AMPK. Under these conditions, the activated AMPK phosphorylates many substrates that turn on alternative catabolic pathways to generate more ATP.

AMPK-dependent phosphorylation of ACC inhibits its enzyme activity to suppress malonyl-CoA synthesis, thereby relieving inhibition of fatty acid uptake into mitochondria and enhancing fatty acid oxidation. Thus, AMPK allows cells to utilize an

alternative source of energy such as lipids when the cells do not have access to carbohydrates, the preferred energy source. In addition to this metabolic switch, AMPK stimulates gene expression of GLUT4 as well as glucose uptake by inducing GLUT4 translocation through inhibition of TBC1D1 and AS160, two Rab-GTPase-activating protein (Rab-GAP) proteins [Park et al. 2014, Kristensen et al. 2014, Chavez et al. 2008]. AMPK phosphorylates and inhibits AS160, leading to Rab activation and increased plasma membrane localization of GLUT4 and glucose uptake.

Lipolysis describes the hydrolysis of TAGs. The importance of lipolysis to general metabolism became apparent when it is discovered that TAGs could not enter cells in its unhydrolyzed form [Whitehead, 1909]. The vascular lipolysis is responsible for the hydrolysis of lipoprotein-associated TAGs in the blood; and intracellular lipolysis catalyzes the breakdown of TAGs stored in intracellular lipid droplets for subsequent export of FAs (from adipose tissue) or their metabolism (in nonadipose tissue). Vascular TAG hydrolysis depends on lipoprotein lipase (LPL) and hepatic TAG lipase. Intracellular lipolysis of TAGs involves neutral (PH optimum around PH 7) and acid lipases present in lysosomes (PH optimum between PH 4-5). Well-characterized neutral TAG hydrolases include adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), whereas lysosomal acid lipase (LAL) is the most important lipase in lysosomes.

Lipolysis provides FAs in times of metabolic need and removes them when they are present in excess. FAs are essential substrates for energy production and the synthesis of

membrane lipids and lipids involved in cellular signaling. All organisms are able to synthesize FAs de novo from carbohydrate and/or protein metabolites. However, an oversupply of FAs is highly detrimental which results in disruption of the integrity of biological membranes, altering cellular acid-base homeostasis, and eliciting the generation of harmful bioactive lipids. These effects impair membrane function and induce endoplasmic reticulum (ER) stress, mitochondrial dysfunction, inflammation, and cell death. These deleterious effects are defined under the term “lipotoxicity” [Unger et al., 2010]. To prevent the detrimental consequences of lipotoxicity, adipocytes store nonesterified FAs in the form of inert TAGs. The carefully regulated balance of FA esterification and TAG hydrolysis creates an efficient buffer system, allowing FA flux without nonphysiological concentrations. Activation of AMPK which induces lipolysis can play a significant role in FAs turn over retaining the balance between TAG synthesis and hydrolysis in adipocytes.

Regulation of adipocyte differentiation by mevalonate

The mevalonate pathway or cholesterol synthesis pathway is an important cellular metabolic pathway that producing sterols, such as cholesterol, and several non-sterol (isoprenoid) products including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Figure 2.3) [Goldstein and Brown 1990, Zhang and Casey 1996]. The isoprenoid metabolites of mevalonate pathway, FPP and GGPP, play an important role in post-translational modification of signaling molecules, names prenylation [Zhang and Casey 1996]. Prenylation is a kind of post translational lipid

modification of proteins involving covalent attachment of farnesyl or geranylgeranyl. Most isoprenylated proteins are thought to serve as regulators of cell signaling and membrane trafficking. It is believed that prenylation is a required modification for small G proteins, particularly the Ras proteins which play crucial roles in signaling pathways controlling cell growth and differentiation [Lane et al. 2006, Willumsen et al. 1984, Clarke et al. 1988]. Faenesylation of the c-terminus of Ras proteins is required for their membrane localization and oncogenic activity [Jackson et al. 1990, Willumsen]. Prenylation results in increased functional activity of Ras and Ras-related G proteins, Rab, Rac1, and Rho by increasing their GTP-bound form.

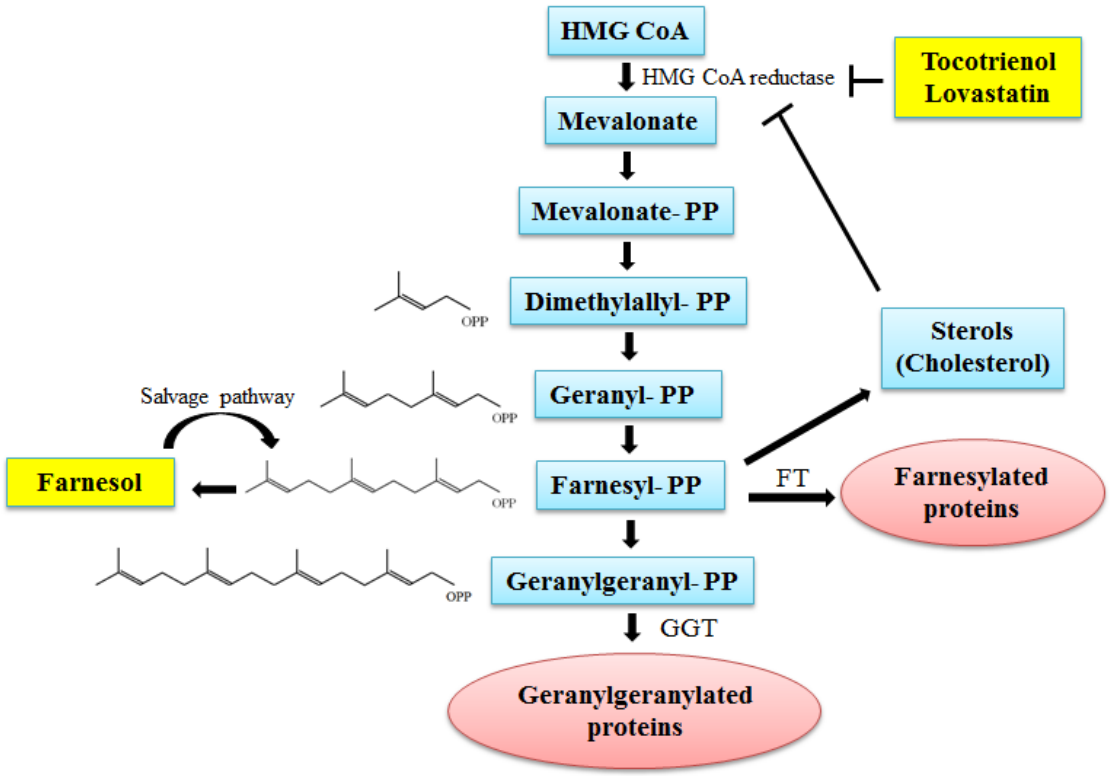

Figure 2.3 The mevalonate pathway. Mevalonate products are used for cholesterol and isoprenoid synthesis. HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; PP, pyrophosphate; FT, farnesyl transferase; GGT, geranylgeranyl transferase.

HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway, is regulated at the levels of transcription, translation, and degradation [Goldstein and Brown 1990]. The balance between mevalonate and cholesterol production is achieved by feedback regulation of HMG-CoA reductase and HMG-CoA synthase, two essential enzymes in the mevalonate pathway, and modulation of low density lipoprotein (LDL) receptors in the cell membrane [Goldstein and Brown 1990]. As a result of feedback

inhibition, the mevalonate pathway will be shut down when there are sufficient amounts of its products, cholesterol and isoprenoids (Figure 2.3). Upon supplementation of isoprenoid metabolites, the mRNA translation of HMG-CoA reductase was reduced [Goldstein and Brown 1990]. It was shown that farnesol, a 15-carbon sequiterpene and a FPP derivative of the mevalonate pathway, can induce degradation of HMG-CoA reductase [Correll et al. 1994]. However, more recently geranylgeraniol was found to induce degradation of HMG CoA reductase [Sever et al. 2003].

Statins, competitive inhibitors of HMG-CoA reductase, have been shown to inhibit adipocyte differentiation, suggested by mevalonate deprivation [Elfakhani et al. 2014]. It is thought that, by down regulating HMG CoA reductase activity, statins limit the pools of FPP and GGPP, thereby attenuating preadipocyte differentiation into adipocytes. However, the significance of the Ras signaling pathway in adipocyte differentiation is not clear.

Regarding to the significance of insulin signaling pathway and glucose uptake in the differentiation of adipocytes, even though mevalonate deprivation can suppress the translocation of GLUT4 by decreased prenylation of Rac1, Rho, and Rab proteins, however; phosphorylation/dephosphorylation modification of proteins has the major regulatory role in insulin induced GLUT4 translocation. On the other hand, the structure of GLUT4 protein includes several hydrophobic helixes required for its membrane bonding. Because GLUT4 protein is naturally membrane localized, it seems that prenylation is not a requirement for its membrane translocation. The role of mevalonate

deprivation in adipocyte differentiation cannot be properly explained by the prenylation modification of proteins. Intrestingly, mevalonate metabolites have been shown to regulate adipocyte functions as an endogenous PPARγ agonist. FPP induces the differentiation of 3T3-L1 cells as an endogenous agonist of PPARγ, and this effect is reversed by a PPARγ antagonist [Goto et al. 2011]. This is probably the more reasonable way to explain the role of mevalonate deprivation in adipocyte differentiation.

However, forced expression of PPARγ in preadipocytes does not cause differentiation without the addition of an exogenous ligand [Tontonoz et al. 1994]. This indicates that an endogenous PPARγ ligand may not be present in 3T3-L1 preadipocytes. The endogenous ligand must be produced in response to MDI during the course of early differentiation. The upregulation of ADD1/SREBP-1c gene expression that occurs during preadipocyte differentiation [Ericsson et al. 1997] is thought to lead to the production of endogenous PPAR ligands required for transcriptional activity [Kim et al. 1999].

Because there is not any evidence for the presence of endogenous PPARγ agonists inside the preadipocytes without addition of insulin, the role of mevalonate deprivation in adipocyte differentiation still remains a question.

d-δ-Tocotrienol and adipocyte differentiation

Vitamin Es are synthesized in plants from isoprenoids, the metabolites of mevalonate. Isoprenoids, the products of mevalonate pathway can inhibit HMG CoA reductase through feedback inhibition. Vitamin E has been an active research area since the first report as a micronutrient essential for reproduction in rats [Parker et al. 1993].

Vitamin Es include both tocotrienol and tocopherols which share a common chromonal ring [Traber and Manor 2012]. The difference is that the tocopherols have a saturated phytyl side chain, while the tocotrienols have the isoprenoid side chain with three double bonds [Mustacich et al. 2007] (Figure 2.4). The vitamin E family includes four tocopherols and four tocotrienols (designated as α-, β-, γ-, and δ-) found in food. Unlike other nutrients, the body cannot interconvert these forms, but only α-tocopherol meets human vitamin E requirements [Traber 2007].

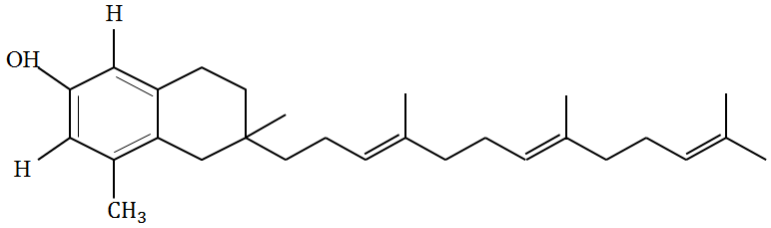

Figure 2.4 The structure of *d*-δ-tocotrienol.

Adminstration of a tocotrienol-rich fraction containing all four tocotrienol homologues and α-tocopherol to patients reduced serum total cholesterol and LDL cholesterol [Qureshi et al. 2001, Tan et al. 1991]. γ and δ-tocotrienols reduced triglycerides [Zaiden et al. 2010]. Additive effects were observed when these doses of tocotrienol-rich fraction and lovastatin were combined [Qureshi et al. 2001]. Furthermore, γ-tocotrienol inhibits the differentiation of 3T3-L1 preadipocytes in vitro [Uto-Kondo et al. 2009]. The expression of phosphorylated PKB/AKT protein was decreased by γ-tocotrienol during differentiation of 3T3-L1 adipocytes [Uto-Kondo et al.

2009], suggesting that the antiadipogenic effect of γ-tocotrienol is mediated by inhibition of PKB-Akt protein.

The effect of γ-tocotrienol on the proliferation of 3T3-L1 preadipocytes and cell cycle arrest has also been investigated. γ-tocotrienol, among other vitamin Es, has a potent anti-proliferative effect on 3T3-L1 cells [Wu et al. 2013]. It decreased the mitochondrial membrane potential, and increased cell apoptosis and cell cycle arrest at S phase. Furthermore, it suppressed the expression of PPARγ, and the activation and phosphorylation of Akt and ERK proteins in 3T3-L1 fibroblasts [Shu-Jing et al. 2013].

The effect of other tocotrienols on adipocyte differentiation and the significance of glucose uptake in the mechanism have not investigated.

trans, trans-Farnesol and adipocyte differentiation

Farnesol, a mevalonate-derived sesquiterpene, is a 15 carbon isoprenoid produced from FPP (Figure 2.3 and 2.5), is a metabolite of mevalonate pathway in plants.

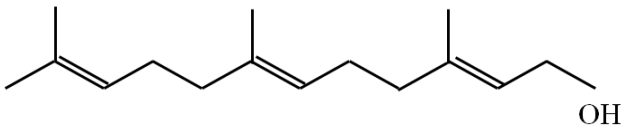

Figure 2.5 The structure of *trans, trans*-farnesol.

It has been shown that mammalian cells can also synthesize FPP and GGPP by a "salvage pathway" in addition of the de novo synthesis by mevalonate [Crick et al. 1997].

FPP is the precursor of almost all isoprenoids and is positioned at branch points leading to the synthesis of long chain isoprenoids (Figure 2.3).

Farnesol is primarily known as a quorum-sensing molecule (QSM) produced by fungus, *Candida albicans* [Hornby et al. 2001]. *C. albicans* is a polymorphic fungus that is capable of growing in yeast, hyphal, and pseudohyphal cell morphologies. Farnesol blocks the yeast-to-hyphal/pseudohyphal (filaments) switch [Hornby et al. 2001]. Several factors involved in the filamentation process are known to play a role in the *C. albicans* farnesol response. Ras1 of the cAMP pathway is a candidate for direct inhibition by farnesol [Davis-Hanna et al. 2008]. Mammalian cells can utilize free geranylgeraniol for protein isoprenylation and free farnesol for sterol biosynthesis and protein isoprenylation [Crick et al. 1994, Fliesler and Keller 1995]. Faenesylation of the Ras proteins results in their membrane localization and oncogenic activity [Jackson et al. 1990, Willumsen].

According to the previous studies, farnesol is known as an established promoter of HMG CoA reductase enzyme degradation that consequently starves cells of vital isoprenoids (FPP and GGPP), required for tumor growth [Cornell et al. 1994, Meigs et al. 1996, Server et al. 2003]. Several studies showed the suppressive effect of farnesol in tumor cells. For example, farnesol selectively induces apoptosis in leukemic cells from acute myeloid leukemia (AML) patients, but not primary monocytes [Rioja et al. 2000]. Farnesol, when used as an aerosol nebulizer, induced a 100% cell death in lung cancer [Wang et al. 2003]. *In vivo,* dietary farnesol inhibited the growth of pancreatic tumors in hamsters [Burke et al. 1997]. In another study conducted on a hepatocarcinogenesis rat

model, farnesol both inhibited cell proliferation and induced DNA damage [Ong et al. 2006]. The farnesol analog, farnesyl anthranilate, inhibited tumor growth through suppression of the HMG CoA reductase in both B16 cells and mice studies [Mo et al. 2000]. These suppressive effects of farnesol on tumor growth are explained mainly by its inhibitory effect on the mevalonate pathway via degradation of HMG CoA reductase.

In a study in which the effect of FPP on the differentiation of 3T3-L1 preadipocytes has been investigated, FPP activated PPARγ as an agonist during adipocyte differentiation [Goto et al. 2011]. FPP activated PPARγ in a dose dependent manner as an endogenous agonist ligand. The addition of FPP upregulated the mRNA expression of PPARγ target genes during adipocyte differentiation. Lovastatin, an HMG CoA reductase inhibitor, decreased the effect of FPP on PPARγ-target gene expressions [Goto et al. 2011]. These finding suggested that FPP might function as an endogenous PPARγ agonist and regulate gene expression in adipocytes. The mevalonate pathway has been shown to affect several nuclear hormone receptors such as PPARα, PPARγ, and LXR that regulate lipid and CHO metabolism [Forman et al. 1997, Argmann et al. 2005, Yano et al. 2007].

Interestingly, basil is one of the farnesol-rich herbs which benefited diabetic individuals due to hypoglycemic effect. A significant decrease in fasting and postprandial blood glucose levels on administration of holy basil leaves have been shown in a survey conducted in human subjects [Agrawal et al. 1996]. The anti-hyperglycemic and hypoglycemic effect of *Ocimum sanctum*, Holy Basil, has also been shown in diabetic

rats [Vats et al., 2001]. However, the mechanism of action is not clear. The hypoglycemic activity of basil (*Ocimum basillicum*) aqueous extract was shown by an *in vitro* study to be mediated via inhibition of α-glucosidase and α-amylase activities [El-Beshbishy and Bahashwan 2012]. These hypoglycemic effects of basil extract, as a rich source of farnesol, can be due to the increased glucose uptake in adipose tissue by farnesol. The effect of farnesol, a mevalonate-derived sesquiterpene, on the differentiation of preadipocytes and glucose uptake has not been investigated.

Summary

Activation of PPARγ promotes terminal differentiation of adipocytes through upregulation of adipogenic target genes. Following the binding of insulin with IGF-1 receptor, two signaling pathways, MAPK and PI3K-PKB/Akt, can be activated in 3T3-L1 cells. PI3K-PKB/Akt is an important intracellular pathway involved in the regulation of many cellular activities, including cell proliferation and apoptosis. In addition, the PI3K-PKB/Akt pathway is important not only in the regulation of adipose tissue development but also in the development of other tissues originating from mesodermal cells.

Increased adipocyte and muscle glucose uptake, stimulated by insulin, is mediated by the PI3K-PKB/Akt pathway. The activation of PI3K is also essential for the activation of Rac1, a Rho family of small G proteins involved in GLUT4 translocation. The activation of Rac1 is important for the remodeling of actin, which is a necessary component for insulin-induced GLUT4 translocation [Khayat et al. 2000, Patel et al. 2003]. Both Rab and Rho family of small G proteins are involved in GLUT4

translocation. AS160 is a new Rab-GAP protein which functions as a brake on GVSs to retain GLUT4 inside the cell [Sano et al. 2007, Eguez et al. 2005, Larance et al. 2005]. Insulin inactivates AS160 by phosphorylation of its Akt phosphorylation sites [Larance et al. 2005].

The mevalonate pathway produces the sterols, such as cholesterol, and non-sterol products including different isoprenoids, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Most isoprenylated proteins serve as regulators of cell signaling and membrane trafficking [Lane and Beese 2006, Willumsen et al. 1984]. Ras proteins are known as oncogenic factors in tumor cells, functioning as molecular switches in signal transduction pathways leading to cell growth and proliferation [Lane and Beese 2006, Willumsen et al. 1984,Clarke et al. 1988]. It is believed that mevalonate metabolites, isoprenoids, are required for the prenylation and activation of Ras and Ras-related small G proteins [Lane and Beese 2006, Willumsen et al. 1984]. However, the significance of the Ras signaling pathway in the differentiation of adipocytes is a subject of controversy.

It has been shown that FPP, a mevalonate metabolite, induces the differentiation of 3T3-L1 cells as an endogenous agonist of PPARγ [Goto et al. 2011], which may explain the role of mevalonate deprivation in adipocyte differentiation.Tocotrienols have been shown to inhibit the mevalonate pathway by suppression of HMG CoA reductase. γ-Tocotrienol inhibits differentiation of 3T3-L1 preadipocytes by decreasing phosphorylation of PKB/Akt protein [Uto-kondo 2009]. The mevalonate pathway has

been shown to affect several nuclear hormon receptors such as PPARα, PPARγ, and LXR that regulate lipid and CHO metabolism [Forman et al. 1997, Argmann et al. 2005, Yano et al. 2007].

The effects of *d*-δ-tocotrienol, a suppressor of HMG CoA reductase, and *trans, trans*-farnesol, a metabolite of mevalonate, on adipocyte differentiation can be explained by PI3K-PKB/Akt signaling pathway which may consequently be related to glucose uptake. Glucose uptake is regulated by the expression of GLUT4 protein in PM of adipocytes. Investigating the effect of these compounds on both intracellular TAG accumulation and glucose uptake in adipocytes is important for their potential use in obesity and insulin resistance conditions.

CHAPTER III

METHODOLOGY

Chemicals

d-δ-Tocotrienol was a gift from American River Nutrition Inc.(ARN), and *trans, trans*-farnesol was purchased from Sigma-Aldrich (St. Louis, MO). Lovastatin was a gift from Merck Research Laboratories (Rahway, NJ). The list of chemicals is summarized in Table 3.1. Dr. Russell DeBose-Boyd, UT Southwestern Medical Center (Dallas, TX) kindly provided antibody against HMG-CoA reductase. Antibodies against calnexin, the endoplasmic reticulum protein marker, and β-actin as the non-membrane protein marker were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against GLUT4, PPARγ, and phosphorylated PKB/Akt (Thr 172) were from Millipore (MA), and phosphorylated AMPK was purchased from Santa Cruz (TX). PCR Primer sets were designed and synthesized by Bio-Synthesis, Inc. (Lewisville, TX) (Table 3.2).

Table 3.1 List of chemicals

Chemicals	Defnition and function	Dose	Provider
d-δ-tocotrienol	Suppressor of HMG CoA reductase (Vitamin E)	0-10 µM/L	American River Nutrition Inc.
trans, trans-farnesol	Metabolite of mevalonate pathway	0-75 µM/L	Sigma, USA
Insulin	Hormone	10 mg/mL	Sigma Aldrich, St. Luis, MO
Lovastatin	Inhibitor of HMG CoA reductase (Statins)	1.25 µM/L	Gift from Merck Research Laboratories, Rahway, NJ
Rosiglitazone	Agonist of PPARγ	1 µM/L	Sigma, USA
GW9662	Antagonist of PPARγ	10 µM/L	Sigma, USA
Ly294002	PI3K inhibitor	5 and 10 µM/L	Sigma, USA
Dimethyl sulfoxide	Solvent	0.1%, v/v	DMSO, ATCC
Ethanol	Solvent	0.1%, v/v	Sigma, USA

Table 3.2 Primer sequences and accession numbers. Primer sequences (forward and reverse) and GenBank accession numbers used in the quantitative real-time polymerase chain reaction (real-time qPCR)

GENE	ACCESSION #	PRIMER SEQUENCE
Pparγ	NM_001127330 & NM_011146	5'-AGAGGGCCAAGGATTCATGACCAGG-3' 5'-TTCAGCTTGAGCTGCAGTTCCAGGG-3'
LPL	NM_008509	5'-TCCCTTCACCCTGCCCGAGGT-3' 5'-CGATGACGAAGCTGGGGCTGCT-3'
Fabp4	NM_024406	5'-GTGTGATGCCTTTGTGGGAACCTGG-3' 5'-TGCGGTGATTTCATCGAATTCCACG-3'
AdipoQ	NM_009605	5'-CGGCAGCACTGGCAAGTTCTACTGC-3' 5'-TTGTGGTCCCCATCCCCATACACCT-3'
Pref1	NM_001190703	5'-CCGTGCCAGAACGGGGGCAC-3'5'- CGGGGGTCAGGCGGTAGGTGA-3'
HMG CoA R	NM_008255	5'-GCCAGTGGTGCGTCTTCCACG-3'5'- CATGCCCATGGCGTCCCCCG-3'
GLUT4	NM_009204	5'-GAACCCCCTCGGCAGCGAGT-3' 5'-ATCCGGTCCCCCAGGACCTTGC-3'
RPL22	NM_009079	5'-GCGACTTTAACTGGGCTGCTGCT-3' 5'-GCCCACCACCCAGCCTCTCG-3'

Pparγ: peroxisome proliferator-activated receptor γ; *LPL*: lipoprotein lipase; *Fabp4*: fatty acid-binding protein 4; AdipoQ: adiponectin; *Pref1*: preadipocyte factor1; *HMG CoA R*:3-hydroxy-3-methylglutaryl coenzyme A reductase; *GLUT4*: glucose transport protein4; *RPL22*: ribosomal protein L22

Cell culture and treatment preparation

Cell culture: Murine 3T3-442A preadipocytes, purchased from Dr. Howard Green (Harvard Medical School), were cultured in 6 wells plates (5×10^3 cells/well/ 2mL medium) containing maintenance medium, Dulbecco's modified Eagle's medium (DMEM), adjusted by American Type Culture Collection (ATCC, Manassas, VA),

supplemented with 10% bovine calf serum (BCS, Fisher Scientific Company LLC, Houston, TX) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) at 37° C in a humidified atmosphere of 10% CO_2. At ~100% confluency, the medium was replaced by differentiating medium (DM), DMEM +10% fetal bovine serum (FBS, Fisher Scientific Company) and 1% penicillin/streptomycin (GIBCO) supplemented by 10 µg/ml insulin (Sigma Aldrich, St. Luis, MO) for 24 h, which then was replaced by DM ± treatments. Treatments: *d*-δ-tocotrienol (0-10 µM/L), *trans, trans*-farnesol (0-75 µM/L), lovastatin (0-1.25 µM/L), and rosiglitazone (1 µM/L) up to day 7-8 of differentiation. Control cells were incubated in medium containing dimethyl sulfoxide (DMSO, ATCC; 0.1%, v/v). Supplemental treatments of 10 mg/mL insulin, 100 µM/L mevalonolactone, and 1 µmol/L rosiglitazone, an agonist of PPARγ, were added with and without *d*-δ-tocotrienol; and GW9662 (10 µmol/L), an antagonist of PPARγ, and Ly294002 (10 µmol/L), a PI3K inhibitor, were added with and without *trans, trans*-farnesol to examine how the effects of *d*-δ-tocotrienol or farnesol on differentiation of 3T3-F442A preadipocytes rescue by activation of different signaling pathways. The study design is shown as follows (Figure 3.1).

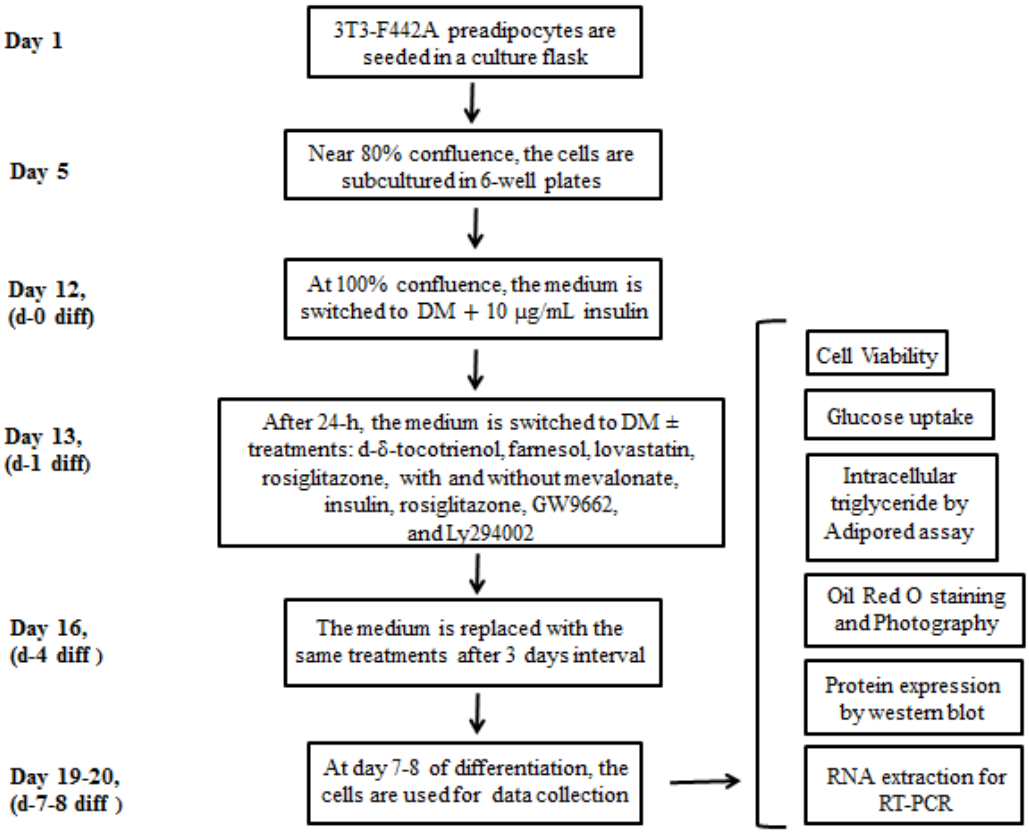

Figure 3.1 The study design.

Oil red O staining

On day 7-8 of differentiation, cells were rinsed with phosphate buffer solution (PBS) twice and fixed in 1 mL of 10% formalin per well at room temperature for 1 h. Cells were then rinsed with deionized water and stained with 0.5 mL of 0.3% freshly filtered Oil Red O working solution per well at room temperature for 30 min [Ramírez-Zacaríaset al. 1992] to visualize cellular lipid accumulated in fat droplets. The cells were

then washed with 1 mL deionized water per well for three times before photomicrography of monolayer cells, taken with Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

Cell viability

Cell viability was measured by the percentage of dead cells in the proliferation phase on day 7-8 of differentiation after cells were treated with different doses of compounds. Two days after murine 3T3-F442A cells were inoculated into 6-well plates, *d*-δ-tocotrienol was added to the growth medium and cells were incubated for 48- and 72-h. Floaters were collected and combined with detached monolayer cells following trypsinization. Viable and dead cells were counted by Trypan Blue staining with a hemocytometer. In a separate experiment, differentiated adipocytes following 6-7 days of incubation in differentiation medium, including treatments, *d*-δ-tocotrienol or *trans, trans*-farnesol, were trypsinized and counted for viable and dead cells.

Intracellular triglyceride measurement

Intracellular lipid content was measured at day 7 of differentiation using AdipoRed kit (Lonza, Walkersville, MD) according to the manufacturer's instructions. AdipoRed contains hydrophilic Nile Red stain and allows the quantification of intracellular lipid droplets [Greenspan et al. 1985]. After differentiated cells are rinsed with 2 mL Phosphate Buffer Solution (PBS), 5 mL PBS and 140 µL of AdipoRed reagent were added to each well for 15 min; and, the plates were positioned in a Tecan Infinite

M200 microplate reader (Tecan Systems Inc., Salzburg, Austria) to measure the fluorescence at excitation wavelength of 485 nm and the emission wavelength of 572 nm.

Glucose uptake

Murine 3T3-F442A preadipocytes were cultured and induced to differentiate as described above. On day 4 of differentiation, the medium was changed and cells continued incubation until day 8 when an aliquot of medium was sampled for the measurement of glucose concentration using a Stanbio Glucose LiquiColor kit® (Stanbio Laboratory, Boerne, TX). The difference in glucose concentration between fresh medium and medium from day 8 was considered as cellular glucose uptake.

RNA extraction and preparation

Total cellular RNA was extracted, as previously reported [Elfakhani et al. 2014], using 1 ml of TRIzol reagent (Invitrogen) per plate. Chloroform was added equal to 20% of volume and samples were centrifuged for 1 hour at 4000 RPM and 4°C. Aqueous RNA phase was collected and mixed with equal volume of isopropanol and kept at -20°C. After 24 hours, samples were centrifuged for 1 hour at 4000 RPM and 4°C. Pellets were collected and mixed with equal volume of ethanol followed by another centrifugation. Ethanol was decanted and pellets were air dried and dissolved in Tris-EDTA (1 mmol Tris-0.1 mmol EDTA, pH 7). Concentration of total RNA extracts was determined spectrophotometrically using optical density 260:280 ratio and samples were diluted with Tris-EDTA to reach the final concentration of 0.5 µg/µL. These quantified RNA samples were then used to perform agarose gel electrophoresis on a 1.5% agarose

gel (Tris-acetate [TAE] buffer) at 80 V for 60-90 min. Gels were soaked in 0.5 µg/mL ethidium bromide for 1 hour on a shaker and visualized by Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA). Total RNA was reverse transcribed into complementary DNA (cDNA) by using Oligo $(dT)_{20}$ primer and SuperScript® III First-Strand Synthesis System kit (Invitrogen) according to the manufacturer's instruction. cDNA samples were diluted by 25-fold with 25 µg/mL of acetylated bovine serum albumin and stored at -20°C.

Quantitative Real-time Polymerase Chain Reaction (Real-time qPCR)

Real-time qPCR was performed, as previously reported [Elfakhani et al. 2014], on 25 µL of the PCR mixture containing 6 µL cDNA sample, 13 µL iQ™ SYBER® Green Supermix (Bio-Rad), 3 µL primer, and 3 µL DI water. Using the Bio-Rad iQ™5 PCR Detection System and Optical System software, the cDNA was denatured at 95°C for 3 minutes followed by 40 cycles of PCR (94°C for 30 s, 60°C for 25 s, 72°C for 25 s, and 78°C for 9 s). Ribosomal protein L22 (RPL22) was used as an internal control to normalize mRNA levels [de Jonge et al. 2007].

Fractionation of membrane and non-membrane proteins

Protein fractionation was performed following 7-8 day incubation with the treatments as previously reported [Fernandes et al. 2013]. Basically, pooled cell pellets were obtained from two 6 well plates by scraping cells into medium and centrifuging at 1258 × g for 5 min at 4°C. Cell pellets were washed in phosphate-buffered saline (PBS) and resuspended in 0.5 m1 of buffer (10 mM HEPES-KOH, pH 7.4), mixed with the

protease inhibitor cocktail (1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Pefabloc, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml ALLN, and, 10 μg/ml aprotinin). Cell suspension was homogenized by passing through a 22G1 needle 30 times followed by short time sonication on ice (3 × 5 seconds) and centrifugation at 1000 × g for 10 min at 4°C. Supernatant was used to obtain the membrane fraction by centrifugation at 20,000 × g for 15 min at 4°C. The pellet was used as membrane fraction. Acetone was added to the supernatant to precipitate the non-membrane proteins, kept for 30 min at -20°C, and centrifuged at 20,000 × g for 15 min at 4 °C. Both the membrane and non-membrane pellets were resuspended in SDS lysis buffer (10 mM Tris-HCl [pH 6.8], 1% (w/v) SDS, 100 mM NaCl, and 1 mM EGTA). Protein concentration of each extract was measured using the BCA™ Protein Assay Kit (Pierce, USA). All extracts were then mixed with an equal volume of buffer (62.5 mM Tris-HCl, pH 6.8, 15% [w/v] SDS, 8 mol/L urea, 10% [v/v] glycerol, and 100 mmol/L dithiothreitol) and 1/6 volume of the 4 × SDS loading buffer (150 mmol/L Tris-HCL, pH 6.8, 12% [w/v] SDS, 30% glycerol, 6% β-mercaptoethanol, and bromphenol blue) and stored at -80 °C.

Western-blot analysis

All fractions were incubated at 37°C for 20 min prior to loading to an 8% SDS polyacrylamide gel. Gels were run at 150 V for 2 h and proteins were then transferred onto a polyvinyl difluoride (PVDF) membrane at 100 mA overnight. Membranes were incubated in blocking solution (5% non-fat dry milk-PBS) for 2 h at room temperature.

After rinsing with PBS, membranes were incubated in the primary antibody-PBST solution at 4°C overnight. Membranes were then washed with PBS containing 0.1% Tween-20 (PBST) for 20 min and incubated with secondary antibody (horseradish peroxidase linked (Cell Signaling Technology, 1/3000 in PBST) for 1 h at room temperature followed by another 20-min wash with PBST. Subsequently, membranes were exposed to SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to manufacturer's protocol and photographed by the Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA). Molecular weight of protein bands were identified using Precision StrepTactin-HRP (Bio-Rad) protein standards. Analysis and quantification of the bands were performed using the Quantity One™ software. A non-specific IgG (Cell Signaling Technology) was used for background control.

Statistics

Experiments were repeated 2-3 times in duplicates or triplicates. One-way analysis of variance (ANOVA) was performed to assess the differences in means between groups using Prism® 4.0 software (GraphPad Software Inc., SanDiego, CA) and SPSS-19. Levels of significance were considered as $P \leq 0.05$.

CHAPTER IV

trans, trans-FARNESOL INDUCES THE DIFFERENTIATION OF MURINE 3T3-F442A PREADIPOCYTES BY INCREASING GLUCOSE UPTAKE

A Paper to Be Submitted For Publication

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Running title: Farnesol induces adipocyte differentiation

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Abbreviations:

FABP4: fatty acid-binding protein 4; **GLUT4**: glucose transport protein 4; **HMG CoA**: 3-hydroxy-3-methylglutaryl coenzyme A; **LPL**: lipoprotein lipase; PI3K-PkB/Akt: phosphatidyl-inositol 3 kinase-protein kinase B/Akt; **PPARγ**: peroxisome proliferator-activated receptor γ

Key words:

trans, trans-farnesol, 3T3-F442A, adipocyte, differentiation, mevalonate, lovastatin, LY294002, PI3K, HMG CoA reductase, PPARγ, FABP4, GLUT4, adiponectin

Abstract

The mevalonate pathway is required for adipocyte differentiation. We investigated the effect of mevalonate-derived *trans, trans*-farnesol (farnesol) on differentiation of murine 3T3-F442A preadipocytes. Adipo-Red assay and oil Red O staining were used to monitor effects of an 8-day incubation with 25 - 75 μmol/L farnesol. Farnesol dose-dependently increased glucose uptake and intracellular triglyceride content without affecting cell viability. GW9662 (10 μmol/L), an antagonist of peroxisome proliferator-activated receptor γ (PPARγ), and LY294002 (10 μmol/L), a phosphatidyl-inositol 3 kinase (PI3K) inhibitor, reversed the effects of farnesol on cellular lipid content. Farnesol increased the mRNA and protein levels of PPARγ and glucose transport protein 4 (GLUT4). The mRNA levels for fatty acid binding protein 4 (FABP4), lipoprotein lipase (LPL), and adiponectin were also upregulated. Farnesol did not affect the protein or mRNA level of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in the mevalonate pathway. Farnesol may induce adipocyte differentiation via upregulation of PPARγ, PI3K and adipogenic genes.

Introduction

Obesity is characterized by an excess of fat tissue in the body. Long term positive energy balance results in the expansion of white adipose tissue (WAT) or obesity. WAT is the major energy reserve in the human body; in periods of energy excess WAT stores triacylglycerol (TAG) which is mobilized during energy deprivation. Because of its crucial impact for development of obesity, abnormal regulation of adipocyte differentiation and lipogenesis is also linked to important pathological conditions such as obesity, type 2 diabetes, and lipodystrophy.[1,2] At the cellular level, obesity is characterized by an increase in the number and size of adipocytes.[2,3] Excess accumulation of TAG in adipocytes produces hypertrophic adipocytes which become insulin resistant and dysfunctional. This condition develops to metabolic energy imbalance in the whole body system leading to chronic metabolic disorders. Therefore, evaluating the effect of different nutrients on lipogenesis may help to find a new strategy to control obesity and its related metabolic disorders.

Most of our current understandings on the molecular mechanisms of adipocyte differentiation have come from *in vitro* studies on established preadipocyte cell lines, 3T3-L1 and 3T3-F442A.[2,3,4,5] Adipocyte differentiation is a well-orchestrated multi-step process involving several genes.[3] Two transcriptional factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα),[2,3] are the major regulators of adipogenesis and sit at the core of the adipogenic cascade. Sterol regulatory element binding protein (SREBP) is another key lipogenic transcription factor

that is nutritionally regulated by glucose and insulin.[6,7,8] Insulin or insulin growth factor-1 (IGF1) are the main hormones required for the differentiation of these established cell lines.[2,9] Insulin can induce the translocation of glucose transport protein 4 (GLUT4) vesicles from the cytoplasm to the plasma membrane (PM) by the phosphatidyl-inositol 3 kinase-protein kinase B/Akt (PI3K-PKB/Akt) pathway.[10,11,12] This results in increased glucose uptake which provides the main source of energy for TAG synthesis in adipocytes.

Statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, inhibit adipocyte differentiation.[13,14,15,16,17,18,19] It is suggested that by inhibiting HMG CoA reductase activity, the rate limiting enzyme in mevalonate pathway, statins limit pools of farnesyl pyrophosphate (FPP), a mevalonate-derived metabolite, thereby attenuating preadipocyte differentiation into adipocytes.

One of the potential roles of FPP in adipocyte differentiation is protein prenylation. It is believed that prenylation is a required modification for small G proteins, particularly the Ras proteins which play crucial roles in signaling pathways controlling cell growth and differentiation.[20,21,22] Prenylation of proteins involves covalent attachment of a farnesyl or geranylgeranyl moiety, the isoprenoid products of the mevalonate pathway. Farnesylation of the c-terminus of Ras proteins is required for their membrane localization and oncogenic activity.[21,23] Prenylation results in increased functional activity of Ras and Ras-related G proteins, Rab, Rac1, and Rho, by increasing their GTP-bound form. However, the significance of the Ras signaling pathway in

adipocyte differentiation is not clear. Accordingly, the essential need of mevalonate products, FPP and GGPP, for adipocyte differentiation may need to be explained through different mechanisms.

Farnesol, a mevalonate-derived sesquiterpene, is a 15-carbon isoprenoid which is produced from FPP. Mammalian cells can utilize free geranylgeraniol for protein isoprenylation and free farnesol for sterol biosynthesis and protein isoprenylation.[24,25] There is one study which investigated the effect of FPP on the differentiation of 3T3-L1 preadipocytes. It has been shown that FPP can activate PPARγ as an agonist during adipocyte differentiation.[16] These findings suggested that FPP might function as an endogenous PPARγ agonist and regulate gene expression in adipocytes. However, forced expression of PPARγ in preadipocytes does not cause differentiation without the addition of an exogenous ligand.[26] This indicates that an endogenous PPARγ ligand may not be present in 3T3-L1 preadipocytes. It is suggested that the upregulation of ADD1/SREBP-1c gene expression during preadipocyte differentiation leads to the production of endogenous PPAR ligands required for transcriptional activity.[27] SREBP1c expression and activity, via cleavage and nuclear translocation, are acutely responsive to insulin,[8,28,29,30] indicating that the contribution of SREBP1c in production of an endogenous PPARγ ligand in adipocyte differentiation depends on insulin.

In this study we evaluated the effect of farnesol on 3T3-F442A preadipocyte differentiation and the expression of adipogenic genes including PPARγ, GLUT4, FABP4, LPL, and adiponectin. We also investigated the effect of farnesol on protein and

gene expression of HMG CoA reductase to see if farnesol has any effect on rate limiting enzyme of mevalonate pathway. According to early studies, farnesol is known to degrade HMG CoA reductase,[31,32,33,34,35] though a later study suggested geranylgeraniol as the mediator of HMG CoA reductase degradation.[33] We further determined whether the effect of farnesol is mediated by upregulation of PI3K and PPARγ which induce glucose uptake via membrane translocation of GLUT4 protein.

Materials and methods

Culture and Oil Red O staining of 3T3-F442A cells

Murine 3T3-F442A cells purchased from Dr. Howard Green (Harvard Medical School) were cultured in 6-well (5×10^3 cells/2 mL medium/well) plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS, Fisher Scientific Company LLC, Houston, TX) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY) at 37^{o}C in a humidified atmosphere of 10% CO_2. Upon reaching ~100% confluency, the 3T3-F442A cells were switched from the maintenance medium above to DMEM supplemented with 10% fetal bovine serum (FBS, Fisher Scientific Company), 10 µg/mL insulin (Sigma Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (GIBCO) and incubated for 24 h; cells were then changed to differentiation medium (DMEM with 10% FBS) supplemented with rosiglitazone (1 µmol/L), *trans, trans,* farnesol (0-75 µmol/L), with and without presence of GW9662 (10 µmol/L), an antagonist of peroxisome proliferator activated receptor γ (PPARγ), and LY294002 (10 µmol/L), a phosphatidyl-inositol 3 kinase (PI3K) inhibitor, (Sigma, USA).

Lovastatin (1.25 μmol/L) was a gift from Merck Research Laboratories (Rahway, NJ). Control cells were incubated in medium containing dimethyl sulfoxide (DMSO, ATCC) (0.1%, v/v). Cells were continued in medium without insulin – unless noted otherwise – for an additional 6-7 days until approximately 70-80% of the cells differentiated into adipocytes with lipid droplets. Cells were rinsed twice with phosphate buffer solution (PBS) and fixed in 1 mL of 10% formalin per well at room temperature for 1 h. Cells were then rinsed with water and stained with 0.5 mL of 0.3% fresh Oil red O per well at room temperature for 30 min[36] to visualize cellular neutral lipids. The cells were then washed 3X with 1 mL water per well before photomicrographs of representative fields of monolayers of 3T3-L1 cells were made with a Nikon Eclipse TS 100 microscope (Nikon Corporation, Tokyo, Japan) equipped with a Nikon Coolpix 995 digital camera (Nikon Corporation).

AdipoRed™ assay for measuring intracellular triglyceride content

Eight days after the induction of differentiation, lipid content was quantified using an AdipoRed™ Assay kit (Lonza, Walkersville, MD) according to manufacturer's instructions. AdipoRed is a reagent that contains the hydrophilic Nile Red stain and allows the quantification of intracellular lipid droplets.[37] Differentiated cells were rinsed with 2 mL phosphate buffer solution (PBS) and then to each well, 5 mL PBS and 140 μL of AdipoRed reagent were added. After 10-15 min. of incubation, the plates were positioned in a Tecan Infinite M200 microplate reader (Tecan Systems Inc., Salzburg,

Austria**)** and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 572 nm.

Cell viability assay

Following 6-7 days incubation of murine 3T3-F442A cells in differentiation medium including 0-75 µmol/L farnesol, floaters were collected and combined with monolayer cells detached following trypsinization. Viable and dead cells were counted by trypan blue staining and a hemocytometer.

Glucose uptake

Murine 3T3-F442A preadipocytes were cultured and induced to differentiate as described above. On day 4 of differentiation, the medium was changed and cells continued incubation until day 8 when an aliquot of medium was sampled for the measurement of glucose concentration using a Stanbio Glucose LiquiColor kit® (Stanbio Laboratory, Boerne, TX). The difference in glucose concentration between fresh medium and medium from day 8 was considered as cellular glucose uptake.

Western-blot

Protein fractionation was performed following 6-7 days incubation with the test agents as previously reported.[38] 3T3 cells were lysed with HNTG lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol and 1% Triton X-100, pH 7.5) and homogenized with a dounce homogenizer. The lysate was transferred to a microfuge tube, incubated at 4 ºC for 30 min with rotation and centrifuged at 18,000 g at 4 ºC for 20 min. After the top fat portion was removed, the supernatant was transferred to a new tube for protein

content determination with the BCA™ Protein Assay Kit (Pierce) before being loaded to a SDS-PAGE gel.

RNA extraction and real-time qPCR

Following 7-8 day incubation of murine 3T3-F442A cells with treatments, total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The concentration and purity of the isolated total RNA was determined spectrophotometrically using OD260:280 ratio. The integrity of the purified total RNA was verified by detecting a 2:1 ratio for the 28S:18S ribosomal RNA (rRNA) using gel electrophoresis. Samples were run on a 1.5% agarose gel (Tris-acetate (TAE) buffer) at 80 volts for 90 min. and visualized by Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA) following the addition of 0.5 μg/ml ethidium bromide. The mRNA expression levels of PPARγ, SREBP-1c, aP2, leptin and adiponectin were analyzed by reverse transcription (RT) followed by polymerase chain reaction (PCR). 2 μg of total RNA in a 20 μl reaction buffer were reverse transcribed into cDNA using an Oligo $(dT)_{20}$ primer and SuperScript® III First-Strand kit (Invitrogen, Grand Island, NY) following the manufacturer's instructions. cDNA was diluted by 20-fold with RNAse free water, and 6 μl of diluted cDNA were amplified in a 25 μl PCR solution containing 250 nM of both forward and reverse primers of the gene and iQ™SYBR® Green Supermix (Bio-Rad). Primers were designed using Vector NTI Advance version 11 software (Invitrogen). The sequences for the primers are listed in Table 4.1.The cDNA was denatured at 95°C for 3 minutes followed by 40 cycles of PCR (94°C for 30 s, 60°C for

25 s, 72^{o}C for 25 s, and 78^{o}C for 9 s) by means of an iQ^{TM}5 multi-color Real-Time PCR Detection System (Bio-Rad) with Bio-Rad iQ5 Optical System Software (version 2.1). The mRNA levels of all the genes were normalized using ribosomal protein L22 (RPL22) as internal control by using the ΔC_T method. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistics

Experiments were repeated 2-3 times in duplicates or triplicates. One-way analysis of variance (ANOVA) was performed to assess the differences in means between groups. Prism® 4.0 software (GraphPad Software Inc., San Diego, CA) and SPSS-19 were used. Levels of significance were considered as $P \leq 0.05$.

Results

The effect of farnesol on the differentiation of murine 3T3-F442A cells is shown in Figure 4.1-A (A-D). Farnesol induced a concentration-dependent increase in the number of lipid droplets that were stained by Oil Red O and the total amount of visible lipids. Rosiglitazone (1 μmol/L), an agonist of PPARγ, increased the number of lipid droplets (Figure 4.1:E), while GW9662 (10 μmol/L), an antagonist of PPARγ, reduced the number of lipid droplets (Figure 4.1:F). LY294002, an inhibitor of PI3K, suppressed differentiation of preadipocytes (Figure 4.1:G).

These visual effects were confirmed by measuring the amount of intracellular TAG accumulation following 7-8 day differentiation of 3T3-F442A preadipocytes using AdipoRed™ (Figure 4.1-B). Farnesol dose-dependently increased cellular TAG content

($P \leq 0.05$). As expected, GW9662 (10 μmol/L) and LY294002 decreased cellular TAG content whereas rosiglitazone had an opposite effect ($P \leq 0.01$) (Figure 4.1-B).

GW9662 (10 μmol/L) reversed the effect of farnesol (25 and 50 μmol/L) significantly ($P \leq 0.05$) (Figure 4.2-A), suggesting that farnesol-induced adipocyte differentiation may be mediated by PPARγ activation. LY294002, 5 and 10 μmol/L, decreased intracellular TAG accumulation significantly ($P \leq 0.01$) and reversed the effect of farnesol 25 and 50 μmol/L significantly ($P \leq 0.01$) (Figure 4.2-B), suggesting that PI3K signaling may be involved in farnesol effect.

There was no effect of cytotoxicity following 8-day incubation of 3T3-F442A cells with farnesol (0 - 75 μmol/L) when the total number of cells (Figure 4.3-A) and the % of dead cells were assessed with Trypan Blue staining (Figure 4.3-B), suggesting the effect of farnesol on cellular triacylglycerol was not related to cytotoxicity.

To clarify whether farnesol has any effect on glucose uptake, the amount of glucose consumption by cells during differentiation was measured. The difference of glucose concentration in media from day 4 to day 8 of differentiation was measured via Stanbio Glucose LiquiColor kit® and used as glucose uptake. Figure 4.4 shows that farnesol increased glucose uptake significantly and dose dependently ($P \leq 0.05$), concomitant to farnesol-induced cellular triacylglycerol content.

Furthermore, to see whether the inducing effect of farnesol on glucose uptake can be explained by the protein expression of membrane associated GLUT4, the amount of GLUT4 protein was measured in the membrane fraction of adipocytes. Farnesol induced

(Figure 4.5-A) the expression of GLUT4 protein, while lovastatin (1.25 µmol/L) reduced the expression of GLUT4 protein and rosiglitazone (1 µmol/L) induced it. The protein expression of PPARγ and phosphorylated protein kinase B/Akt (p-PKB/Akt) was measured in non-membrane fraction of 3T3-F442A adipocytes. Farnesol uregulated the protein expression of PPARγ, while lovastatin (1.25 µmol/L) reduced it and rosiglitazone (1 µmol/L) induced it (Figure 4.5-B). Farnesol did not change the expression of p-PKB/Akt protein (data not shown).

Next, the underlying mechanism regarding the role of mevalonate pathway was investigated. To differentiate if farnesol serving as the triggering molecule for HMG CoA reductase degradation, as known in previous studies in tumor cells, the amount of HMG CoA reductase protein in the membrane fraction of adipocytes was measured following 7 day incubation with farnesol 0-75 µmol/L. Figure 4.5-C shows that farnesol did not have any effect on HMG CoA reductase protein, while lovastatin (1.25 µmol/L) induced it as a compensatory reaction.

The effect of farnesol on the expression of PPARγ and its target genes, GLUT4, adiponectin, FABP4, and LPL which are also known as insulin sensitizing genes, and the expression of HMG CoA reductase and Pref1 were detected in differentiated and undifferentiated cells. Following 8 days incubation of 3T3-F442A cells with farnesol 0-75 µmol/L, the cells were collected for RNA extraction and RT-PCR. Undifferentiated 3T3-F442A preadipocytes were collected 4 days after seeding at about 70% confluence. Farnesol upregulated the expression of PPARγ (Figure 4.6-A), GLUT4 (Figure 4.6-B),

adiponectin (Figure 4.6-C), and FABP4 (Figure 4.6-D) significantly and dose dependently ($P \leq 0.01$), while these genes were expressed at very low baseline levels in undifferentiated cells. The LPL mRNA expression also showed an upward trend (Figure 4.6-G). The expression of Pref1 mRNA, a marker of undifferentiated preadipocytes, was down regulated significantly ($P < 0.01$) in differentiated cells treated with farnesol, while it was highly expressed in undifferentiated cells (Figure 4.5-E). Farnesol 0-75 µmol/L had no effect on mRNA expression level of HMG CoA reductase (Figure 4.6-G).

Table 4.1 Primer sequences (forward and reverse) and GenBank accession numbers used in the quantitative real-time polymerase chain reaction (real-time qPCR)

GENE	ACCESSION #	PRIMER SEQUENCE
Pparγ	NM_001127330 & NM_011146	5'-AGAGGGCCAAGGATTCATGACCAGG-3' 5'-TTCAGCTTGAGCTGCAGTTCCAGGG-3'
LPL	NM_008509	5'-TCCCTTCACCCTGCCCGAGGT-3' 5'-CGATGACGAAGCTGGGGCTGCT-3'
Fabp4	NM_024406	5'-GTGTGATGCCTTTGTGGGAACCTGG-3' 5'-TGCGGTGATTTCATCGAATTCCACG-3'
Adiponectin	NM_009605	5'-CGGCAGCACTGGCAAGTTCTACTGC-3' 5'-TTGTGGTCCCCATCCCCATACACCT-3'
Pref1	NM_001190703	5'-CCGTGCCAGAACGGGGGCAC-3' 5'-CGGGGGTCAGGCGGTAGGTGA-3'
HMG CoA R	NM_008255	5'-GCCAGTGGTGCGTCTTCCACG-3' 5'-CATGCCCATGGCGTCCCCCG-3'
GLUT4	NM_009204	5'-GAACCCCCTCGGCAGCGAGT-3' 5'-ATCCGGTCCCCCAGGACCTTGC-3'
RPL22	NM_009079	5'-GCGACTTTAACTGGGCTGCTGCT-3' 5'-GCCCACCACCCAGCCTCTCG-3'

Pparγ: peroxisome proliferator-activated receptor γ; *LPL*: lipoprotein lipase; *FABP4*: fatty acid-binding protein 4; *Pref1*: preadipocyte factor1; *HMG CoA R*:3-hydroxy-3-methylglutaryl coenzyme A reductase; *GLUT4*: glucose transport protein4; *RPL22*: ribosomal protein L22.

1-A

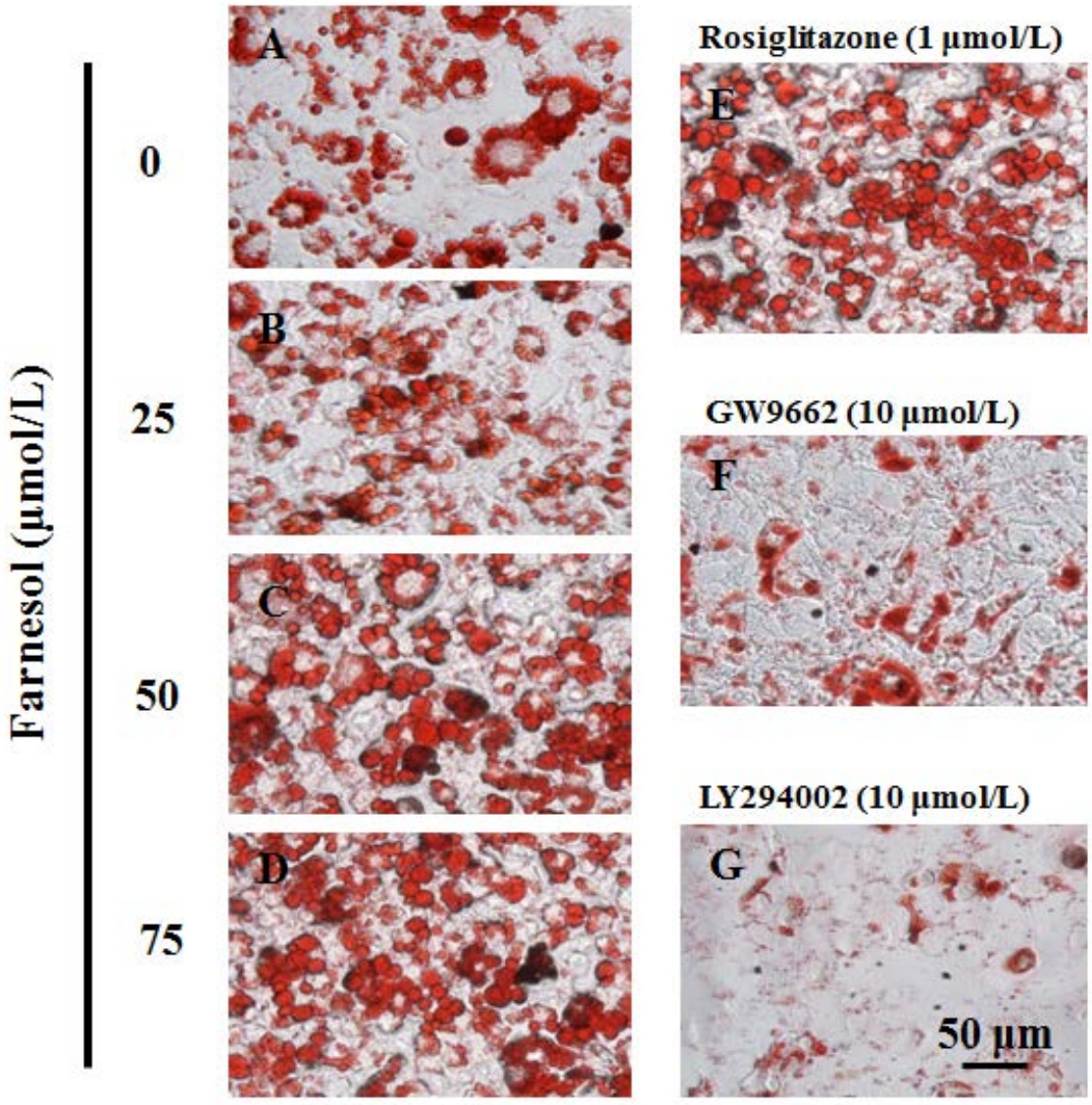


Figure 4.1-A Oil Red O staining and photomicrography of 3T3-F442A adipocytes. Following 8 day incubation with farnesol (0-75 µmol/L A-D), rosiglitazone (1 µmol/L E), GW9662 (10µmol/L F), and LY294002 (10 µmol/L G), the cells were collected for Oil Red O staining and photomicrography.

1-B

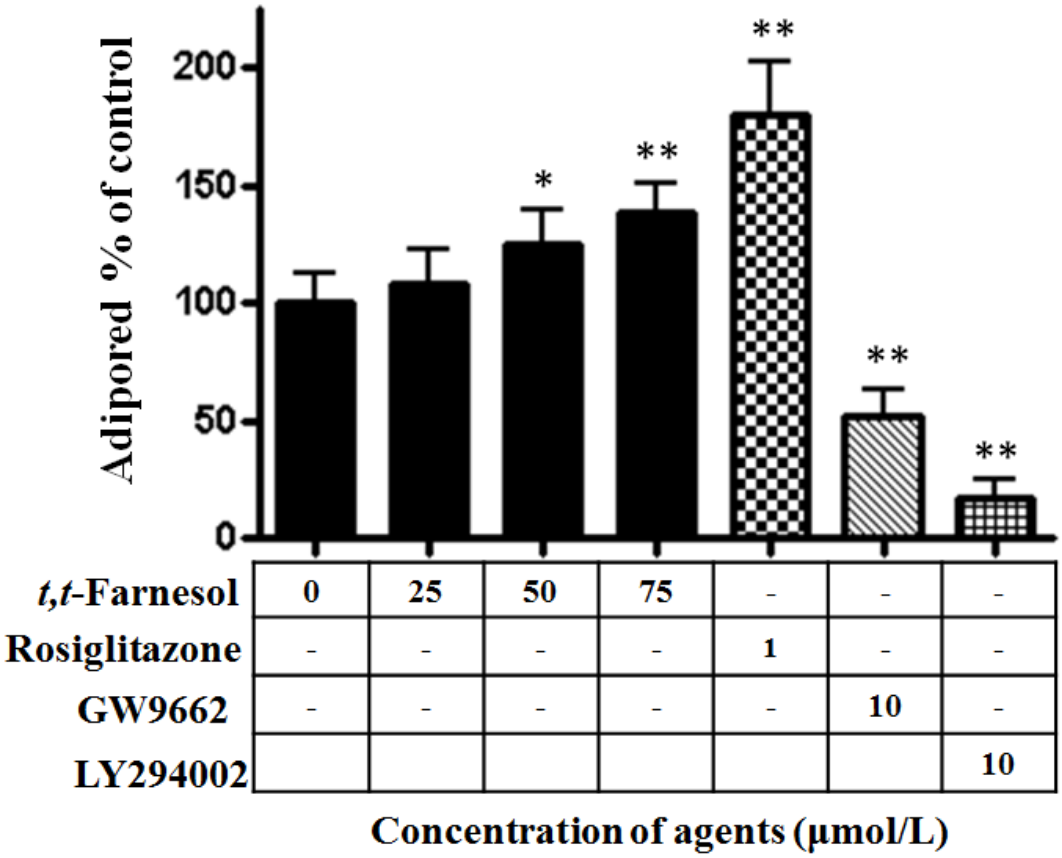


Figure 4.1-B The effect of *t,t*-farnesol on the intracellular triglyceride content of 3T3-F442A adipocytes. Following 8 day incubation of cells with *t,t*-farnesol (0-75 µmol/L), rosiglitazone (1µmol/L), an agonist of PPARγ, and GW9662 (10 µmol/L), an antagonist of PPARγ, the intracellular triglyceride content was measured by Adipored assay (*$P \leq 0.05$; **$P \leq 0.01$).

2-A

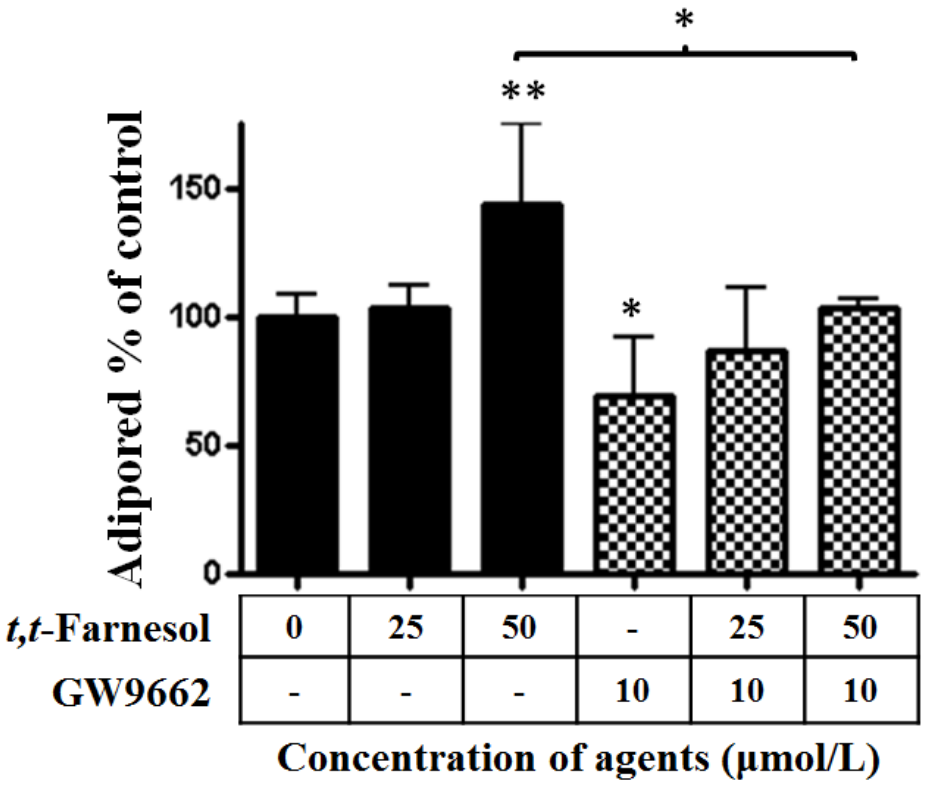


Figure 4.2-A The effect of GW9662 on *t,t*-farnesol-mediated induction of intracellular triglyceride content of 3T3-F442A adipocytes. Following 8 day incubation of 3T3-F442A cells with *t,t*-farnesol (0- 50 µmol/L) in the presence or absence of GW9662 (10µmol/L), intracellular triglyceride accumulation were measured by Adipored assay. Rosiglitazone (1µmol/L) was used as a positive control (*$P \leq 0.05$; **$P \leq 0.01$).

2-B

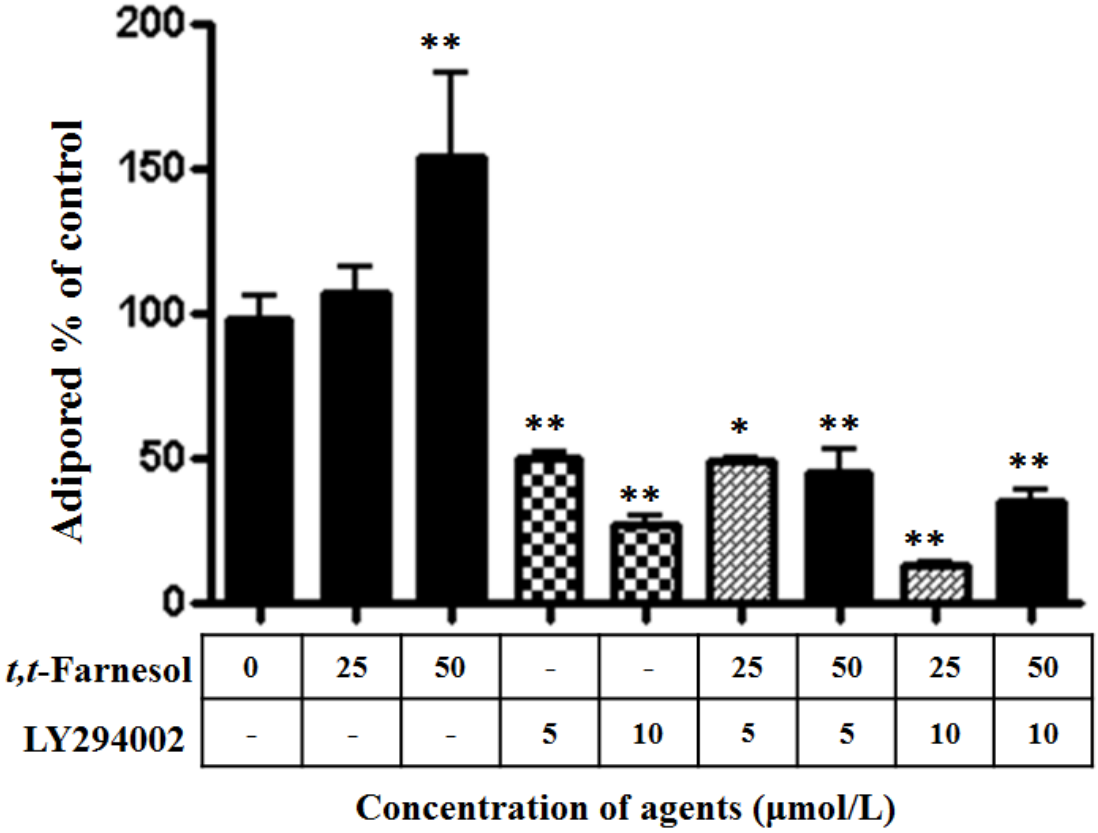
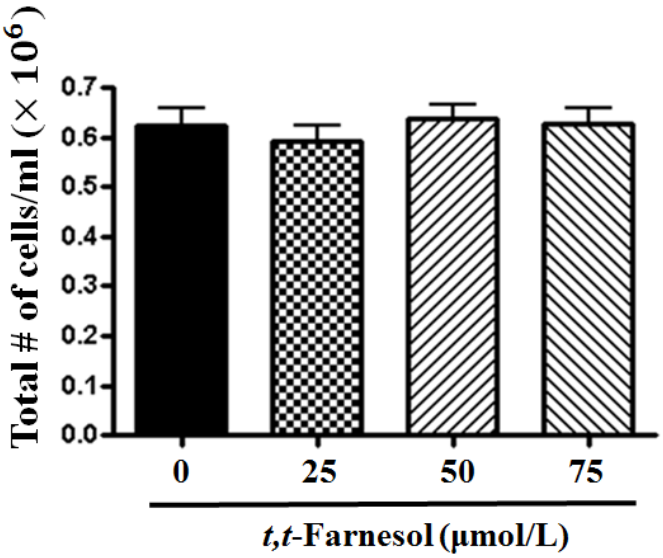


Figure 4.2-B The effect of LY294002, a PI3K inhibitor, on *t,t*-farnesol-mediated induction of intracellular triglyceride content of 3T3-F442A adipocytes. Following 8 day incubation of cells with *t,t*-farnesol (0- 50 µmol/L) with and without LY294002 (0- 10 µmol/L), intracellular triglyceride content was measured by Adipored assay (*$P \leq 0.05$; **$P \leq 0.01$).

3-A



3-B

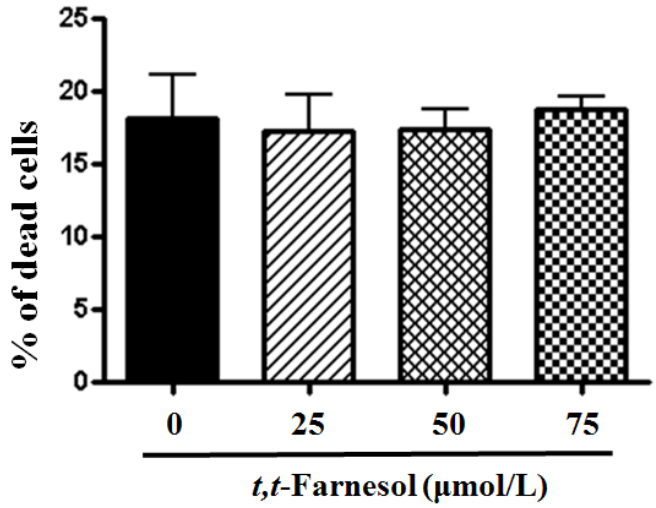


Figure 4.3 The effect of an 8-d incubation with *t,t*-farnesol on viability of 3T3-F442A adipocytes. Following 8 day incubation of cells with *t,t*-farnesol (0 - 75 µmol/L), the total cell count (3-A) and the % of dead cells (3-B) were measured by Trypan Blue staining.

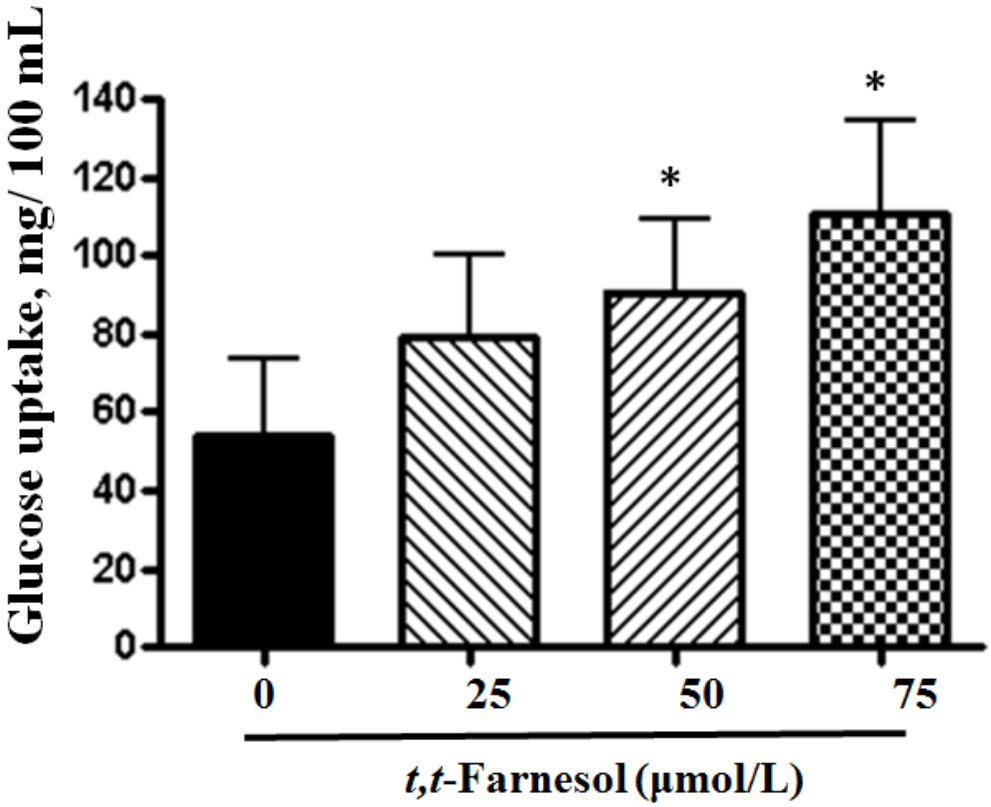
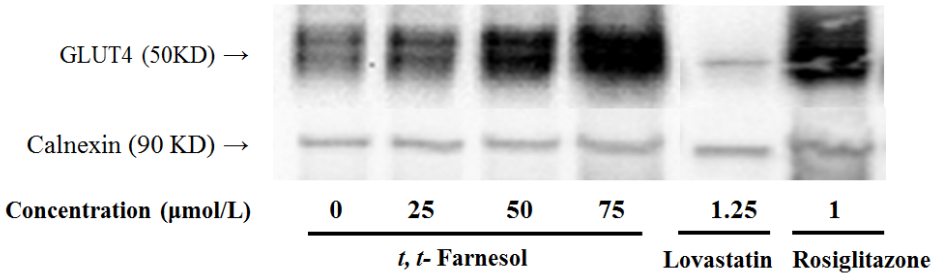
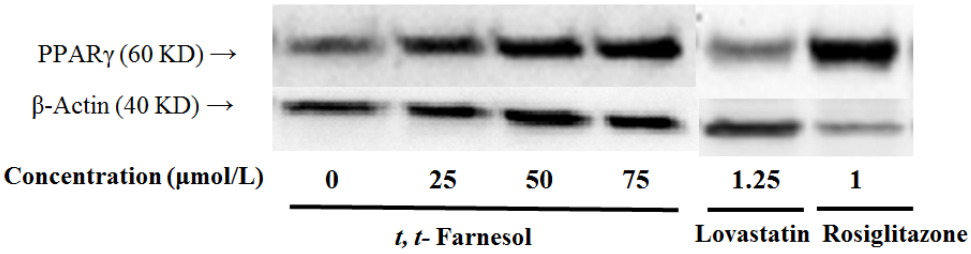


Figure 4.4 The effect of *t,t*-farnesol on glucose uptake during differentiation of the 3T3-F442A adipocytes. Following 8 day incubation of cells with *t,t*-farnesol (0-75 µmol/L), glucose uptake was calculated by difference of glucose concentration in media between day 8 and day 4 of differentiation (*$P \leq 0.05$).

5-A



5-B



5-C

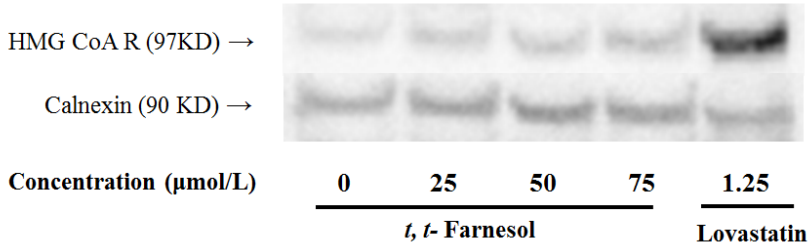
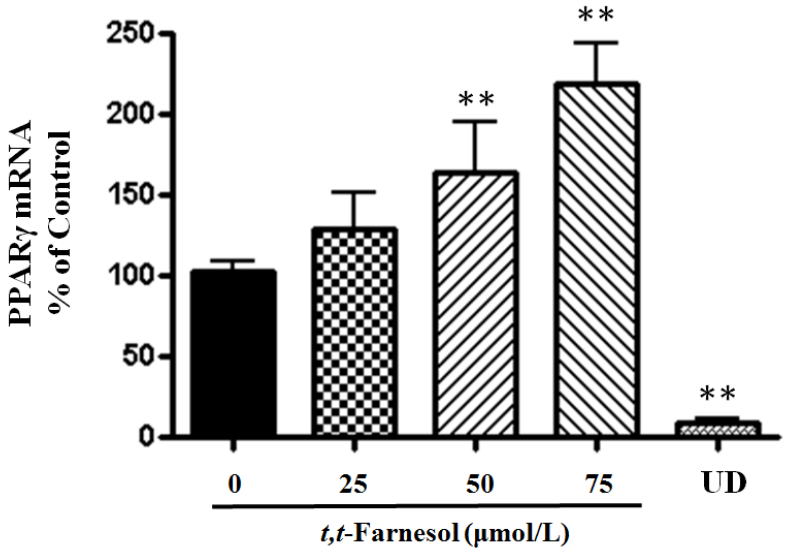
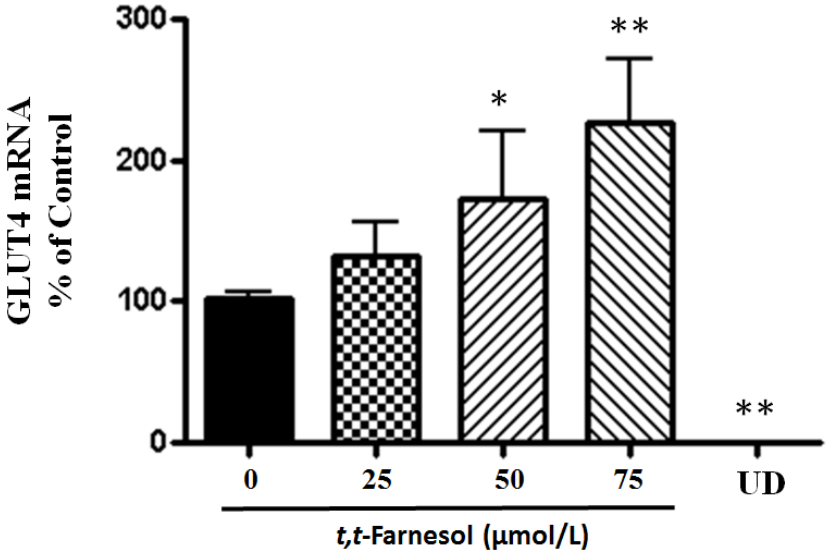


Figure 4.5 The effect of *t,t*-farnesol on the protein content of GLUT4 (5-A), PPARγ (5-B), and HMG CoA reductase (5-C) in 3T3-F442A adipocytes. Following 8 day incubation of cells with *t,t*-farnesol (0-75 µmol/L), the cells were collected for subcellular protein fractionation and western-blot analysis. The expression of GLUT4 (5-A) and HMG CoA reductase (5-C) proteins were measured in membrane fraction and that of PPARγ protein (5-B) was measured in the non-membrane fraction.

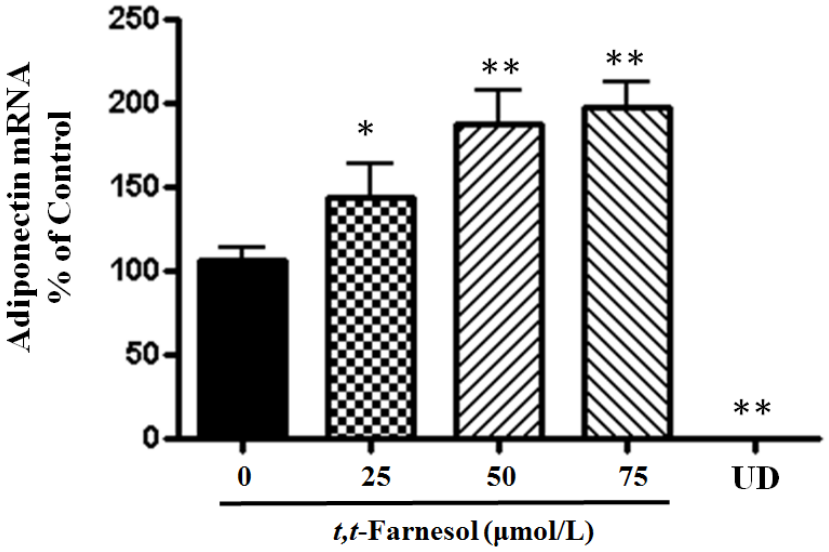
6-A


PPARγ mRNA
% of Control
250
200
150
100
50
0
**
**
**
0
25
50
75
UD
t,t-Farnesol (µmol/L)

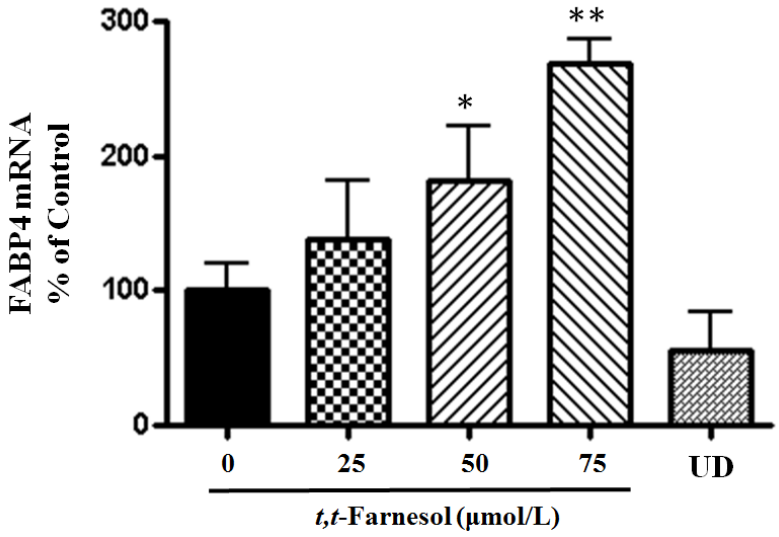
6-B


GLUT4 mRNA
% of Control
300
200
100
0
*
**
**
0
25
50
75
UD
t,t-Farnesol (µmol/L)

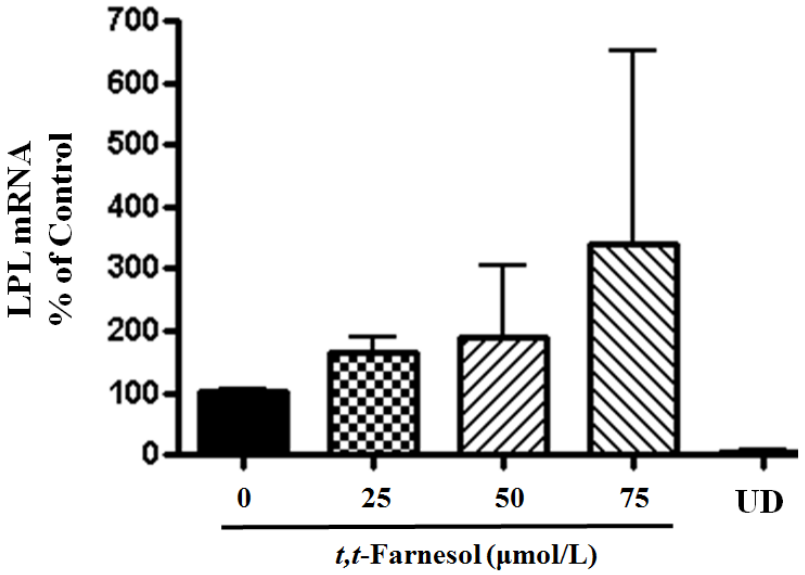
6-C


Adiponectin mRNA
% of Control
250
200
150
100
50
0
*
**
**
**
0
25
50
75
UD
t,t-Farnesol (µmol/L)

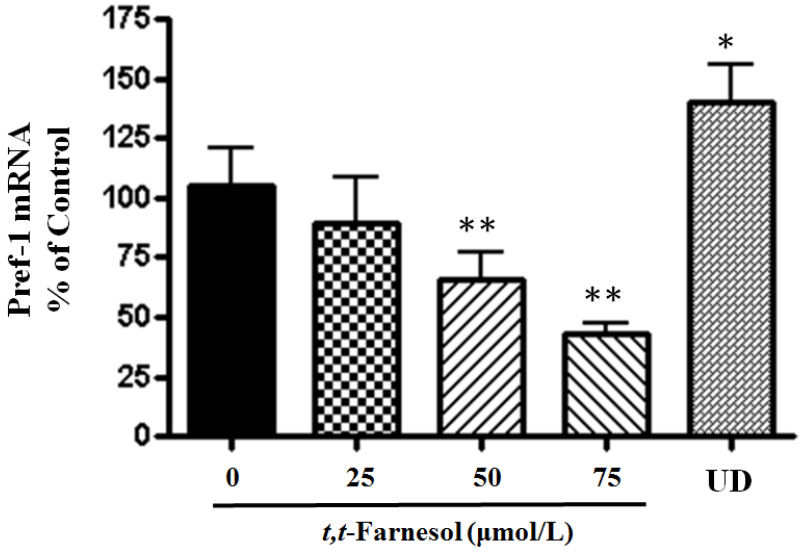
6-D


FABP4 mRNA
% of Control
300
200
100
0
*
**
0
25
50
75
UD
t,t-Farnesol (µmol/L)

6-E


LPL mRNA
% of Control
700
600
500
400
300
200
100
0
0
25
50
75
UD
t,t-Farnesol (µmol/L)

6-F


Pref-1 mRNA
% of Control
175
150
125
100
75
50
25
0
**
**
*
0
25
50
75
UD
t,t-Farnesol (µmol/L)

6-G

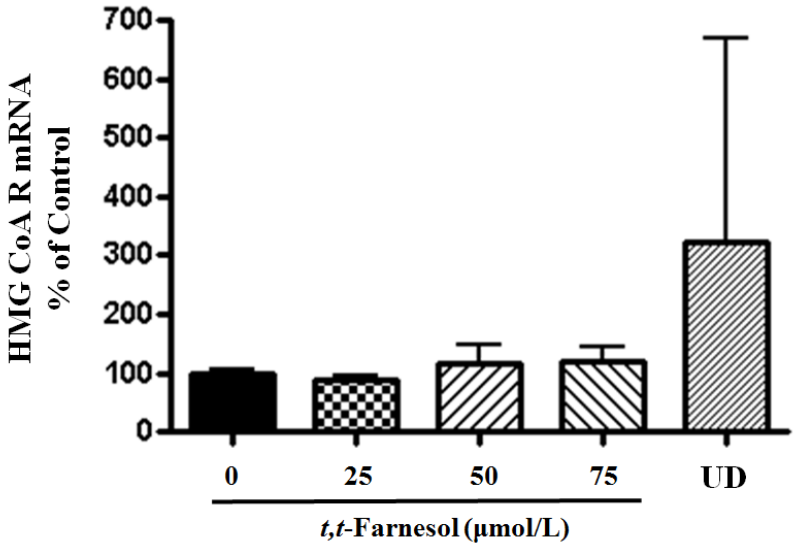


Figure 4.6 The effect of *t,t*- farnesol on the expression of adipogenic genes and HMG COA reductase gene in 3T3-F442A adipocytes and preadipocytes. Following 8 day incubation of 3T3-F442A cells with farnesol (0-75 µmol/L), the cells were collected for RNA extraction and RT-PCR to measure the gene expression of PPARγ (6-A), GLUTT4 (6-B), Adiponectin (6-C), FABP4 (6-D), LPL (6-E), Pref1 (6-F), and HMG CoA reductase (6-G) mRNA levels in 3T3-F442A adipocytes and undifferentiated preadipocytes (UD) . Undifferentiated preadipocytes were collected at 70% confluence to investigate the expression of these genes in undifferentiated cells (*$P \leq 0.05$; **$P \leq 0.01$).

Discussion

It is generally recognized that in 3T3-L1 or 3T3-F442A cells, the main hormone to induce adipocyte differentiation is insulin, working via the IGF-1 receptor on the adipocyte cell membrane.[2,39,40] After hormonal stimulation, post-confluent growth-arrested preadipocytes reenter the cell cycle[41,42] and undergo several rounds of mitosis, referred to as mitotic clonal expansion.[3] This mitosis is necessary to unwind DNA, allowing transcription factors to access regulatory response elements in genes responsible for the adipocyte phenotype.[3] After exiting the cell cycle, the cells lose their fibroblastic morphology, accumulate triglyceride, and acquire the appearance of mature adipocytes.[9,41]

Following the binding of insulin with IGF-1 receptor, two signaling pathways, MAPK and PI3K-PKB/Akt, can be activated in 3T3-L1 cells. The MAPK signaling pathway in adipocyte differentiation is a subject of controversy.[43,44,45,46,47,48] PI3K-PKB/Akt pathway is involved in the regulation of many cellular activities, including cell proliferation and apoptosis.[49,50,51] Inhibition of PI3K with wortmannin and LY294002 blocks adipocyte differentiation in 3T3-L1 cells.[52,53] The results from PKB/Akt gene knockout in mice have shown that the development of adipose tissue is impaired along with other abnormalities, including muscle, bone, and skin.[54] These evidences indicate that the PI3K-PKB/Akt pathway is important not only in the regulation of adipose tissue development but also in the development of other tissues originating from mesodermal cells.

Increased adipocyte and muscle tissue glucose uptake by insulin is mediated by the PI3K-PKB/Akt pathway.[55] In the basal state, more than 90% of GLUT4 is sequestered in the intracellular compartments, while only 10% is present at PM.[56] The intracellular GLUT4 is located in "Glut4 storage vesicle" (GVS), which is the main target of insulin induced GLUT4 translocation.[57]

Our results that farnesol induces adipocyte differentiation (Figure 1-A), intracellular TAG accumulation (Figure 1-B), and glucose uptake (Figure 4) suggest that farnesol may act through the insulin activated PI3K and GLUT4 upregulation. Consistently, the expression of GLUT4 in adipocyte membrane fraction was increased by farnesol 25-75 μmol/L dose dependently (Figure 4.5-A). Our observation that LY294002, an inhibitor of PI3K, and GW9662, an antagonist of PPARγ, reversed the effect of farnesol on cellular lipid content (Figure 4.2-A and B) supports the hypothesis that differentiation of adipocytes is coordinated by PI3K and PPARγ signaling pathways and that they are promoted by farnesol. As we showed, the expression of PPARγ and GLUT4 mRNA were upregulated by farnesol 25-75 μmol/L significantly (Figure 4.6-A and B).

Interestingly, basil is a farnesol rich herb which have hypoglycemic effect benefited diabetic individuals. A significant decrease in fasting and postprandial blood glucose levels on administration of "Holy Basil" leaves has been shown in human subjects.[58] The anti-hyperglycemic and hypoglycemic effect of Holy Basil has also been shown in diabetic rats.[59] Our results have shown for the first time that farnesol induces

adipocyte differentiation by increasing glucose uptake explaining the underlying mechanism of the hypoglycemic effect of basil extract as a rich source of farnesol.

Activation of PPARγ promotes terminal differentiation of adipocytes through upregulation of adipogenic target genes.[26] There are several reports indicating a relation between the activation of PPARγ pathway and insulin sensitivity that resulted in increased glucose uptake. Thiazolidodions (TZDs) exert their biological effects on insulin sensitivity through binding to PPARγ.[60] Mutations in PPARγ in both rodents and humans are associated with insulin resistance.[61,62,63] In this study, we used rosiglitazone, an agonist of PPARγ, as the positive control and GW9662, an antagonist of PPARγ, as the negative control. Our results indicate that rosiglitazone (1 µmol/L) induces adipocyte differentiation (Figure 4.1-A:E) and intracellular fat content (Figure 4.1-B), while GW9662 suppressed adipocyte differentiation (Figure 4.1-A:F) and the intracellular fat content (Figure 4.1-B).

The production of bioactive molecules, named adipokines, derived from adipose tissue, is another potential mechanism which explains the cross talk between the PPARγ pathway and insulin sensitivity. Multiple studies have shown that plasma levels of adiponectin, an adipokine selectively released by adipocytes,[64] are correlated with adipose tissue mass and insulin sensitivity.[65] Consistent with this information, farnesol was shown to upregulate the expression of adiponectin in a dose dependent manner (Figure 4.6-C). FABP4 (also named αP2) and LPL are other target genes for PPARγ related to insulin sensitizing activity.[66,67,68] Farnesol upregulated the expression of

FABP4 and LPL (Figure 4.6-D and E). Furthermore, downregulation of Pref1, a marker of preadipocytes, by farnesol confirmed its differentiation-stimulating effect (Figure 4.6-F). Research methodology validated the low expression of adipogenic genes in undifferentiated preadipocytes (Figure 4.6:A-E).

Evidence showed that farnesol, as a metabolite of mevalonate pathway, induced degradation of HMG CoA reductase, resulting in feedback inhibition of the pathway.[31,32,33,34,35,69,70,71,72] However, all previous evidence came from studies in tumor cells, including lung cancer,[70] pancreatic tumor,[71] hepatocarcinoma,[72] acute myeloid leukemia (AML),[69] and myeloma (both B16 cells and mice).[34] These suppressive effects of farnesol on tumor growth are explained mainly by its inhibitory effect on mevalonate pathway via degradation of HMG CoA reductase.

In contrast to observations in tumor cells, farnesol has no effect on either protein or gene expression of HMG CoA reductase in 3T3-F442A murine adipocytes (Figure 4.5-B). This result is consistent with previous studies showing lack of farnesol effect in non-tumor cells and our observation that farnesol is non-toxic in adipocytes. Our results show that farnesol may induce adipocyte differentiation via activation of the PI3K signaling pathway and PPARγ, independent of HMG CoA reductase, the rate limiting enzyme in the mevalonate pathway. Because of the role PI3K-PKB/Akt signaling plays in stimulating tumor growth, farnesol may have inhibited tumor cell growth by downregulating the PI3K-PKB/Akt pathway and consequently glucose uptake, which is

an essential nutrient for cancer cell growth. Farnesol-induced glucose and energy starvation may have lead to apoptosis contributing to tumor suppression.

However, our results show that farnesol increases glucose uptake and the expression of GLUT4 in membrane of 3T3-F442A adipocytes which may be the opposite of its effect on tumor cells. This indicates that farnesol may divert the energy source from tumor cells to the adipocytes, *in vivo*. As a result, the extra energy stores as TAG in adipocytes instead of using for tumor growth. In other words, farnesol may stimulate adipocyte's buffering function to protect the organism against accumulation of TAG in non fat-tolarable cells. Lipotoxicity is an accumulation of lipids in non fat-tolerable tissues leading to cell dysfunction which plays an important role in the pathogenesis of type 2 diabetes by degradation of pancreatic β-cells.[73,74,75,76,77] It is possible that farnesol, by inducing this defense mechanism, protects the organism against lipotoxicity and glucotoxicity. The potential mechanism underlying the differential effect of farnesol on tumor cells and adipocytes is unknown.

In summary, the results from this study support our hypothesis that farnesol induces the differentiation of adipocytes. As a component of plant foods, farnesol and related compounds may provide a novel approach in the intervention of insulin resistance and other obesity-related disorders.

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References

1. Moller D, Flier J. Insulin resistance--mechanisms, syndromes, and implications. *N Engl J Med* 1991;**325**:938-48
2. Gregoire F, Smas C, Sul H. Understanding adipocyte differentiation. *Physiol Rev* 1998;**78**:783-809
3. Tang QQ, Lane MD. Adipogenesis: from stem cell to adipocyte. *Annu Rev Biochem* 2012;**81**:715–36
4. Rosen E, Spiegelman B. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 2000;**16**:145-71
5. Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* 1975;**5**:19-27
6. Horton JD. Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochem Soc Trans* 2002;**30**:1091-95
7. Radhakrishnan Y, Maile L, Ling Y, Graves L, Clemmons D. Insulin-like growth factor-I stimulates Shc-dependent phosphatidylinositol 3-kinase activation via Grb2-associated p85 in vascular smooth muscle cells. *J Biol Chem* 2008;**283**:16320-31
8. Brown M, Goldstein J. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 1997;**89**:331-40
9. Green H, Kehinde O. Sublines of mouse 3T3 cells that accumulate lipid. *Cell* 1974; **1**:113-6
10. Kulik G, Klippel A, Weber M. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol* 1997;**17**:1595-606
11. Wang Q, Somwar R, Bilan P, Liu Z, Jin J, Woodgett J, Klip A. Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* 1999;**19**:4008-18
12. Ishiki M, Randhawa V, Poon V, Jebailey L, Klip A. Insulin regulates the membrane arrival, fusion, and C-terminal unmasking of glucose transporter-4 via distinct phosphoinositides. *J Biol Chem* 2005;**280**:28792-802
13. Brown M, Faust J, Goldstein J, Kaneko I, Endo A. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J Biol Chem* 1978;**253**:1121-8

14. Nakanishi M, Goldstein J, Brown M. Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J Biol Chem* 1988;**263**:8929-37
15. Nishio E, Tomiyama K, Nakata H, Watanabe Y. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor impairs cell p4fferentiation in cultured adipogenic cells (3T3-L1). *Euro J Pharmacol* 1996;**301**:203-6
16. Goto T, Nagai H, Egawa K, Kim YI, Kato S, Taimatsu A, Sakamoto T, Ebisu S, Hohsaka T, Miyagawa H, Murakami S, Takahashi N, Kawada T. Farnesyl pyrophosphate regulates adipocyte functions as an endogenous PPARγ agonist. *J Biochem* 2011;**438**:111-9
17. Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control. *Diabetologia* 2006;**49**:1881-92
18. Nicholson A, Hajjar D, Zhou X, He W, Gotto A, Han J. Anti-adipogenic action of pitavastatin occurs through the coordinate regulation of PPARgamma and Pref-1 expression. *Br J Pharmacol* 2007;**151**:807-15
19. Elfakhani M, Torabi S, Hussein D, Mills N, Verbeck G, Mo H. Mevalonate deprivation mediates the impact of lovastatin on the differentiation of murine 3T3-F442A preadipocytes. *Exp Biol Med* 2014;**239**:293-301
20. Lane K, Beese L. Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J Lipid Res* 2006;**47**:681-99
21. Willumsen B, Christensen A, Hubbert N, Papageorge A, Lowy D. The p21 ras C-terminus is required for transformation and membrane association. *Nature* 1984;**310**:583-6
22. Clarke S, Vogel J, Deschenes R, Stock J. Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc Natl Acad Sci* U S A 1988;**85**:4643-7
23. Jackson J, Cochrane C, Bourne J, Solski P, Buss J, Der C. Farnesol modification of Kristen-ras exon 4B protein is essential for transformation. *Proc Nati Acad Sci* U S A 1990;**87**:3042-6
24. Crick D, Andres D, Waechter C. Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. *Biochem Biophys Res Commun* 1997;**237**:483-7
25. Fliesler, S., & Keller, R. Metabolism of [3H] farnesol to cholesterol and cholesterogenic intermediates in the living rat eye. *Biochem Biophys Res Commun* 1995;**210**:695-702
26. Tontonoz P, Hu E, Graves R, Budavari A, Spiegelman B. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 1994;**8**:1224-34

27. Kim J, Wright H, Wright M, Spiegelman B. ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand. *Proc. Nati Acad. Sci* U S A 1998;**95**:4333-7
28. Kim J, Sarraf P, Wright M, Yao K, Mueller E, Solanes G, Lowell B, Spiegelman B. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 1998;**101**:1-9
29. Kim J, Spiegelman B. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev* 1996;**10**:1096-107
30. Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie* 2004;**86**:839-48
31. Correll C, Ng L, Edwards, P. Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol Chem* 1994;**269**:17390-3
32. Meigs T, Roseman D, Simoni R. Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. *J Biol Chem* 1996;**271**:7916–22
33. Sever N, Song B, Yabe D, Goldstein J, Brown M, DeBose-Boyd R. Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem* 2003;**278**:52479–90
34. Mo H, Tatman D, Jung M, Elson C. Farnesyl anthranilate suppresses the growth, in vitro and in vivo, of murine B16 melanomas. *Cancer Lett* 2000;**157**:145-53
35. Mo H, Elson CE. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. *Exp Biol Med* (Maywood) 2004;**229**:567–85
36. Ramirez-Zacarias JL, Castro-Munozledo F, Kuri-Harcuch W. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry* 1992;**97**:493–7
37. Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* 1985;**100**:965–73
38. Fernandes NV, Yeganehjoo H, Katuru R, DeBose-Boyd R, Morris LL, Michon R, Yu ZL, Mo H. Geranylgeraniol suppresses the viability of human DU145 prostate carcinoma cells and the level of HMG CoA reductase. *Exp Biol Med* 2013;**238**:1265–74
39. MacDougald O, Lane M. Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem* 1995;**64**:345-73
40. Smith P, Wise L, Berkowitz R, Wan C, Rubin C. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem* 1988; **263**:9402-8
41. Student A, Hsu R, Lane M. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem* 1980;**255**:4745-50

42. Davis L, Zur Nieden N. Mesodermal fate decisions of a stem cell: the Wnt switch. *Cell Mol Life Sci* 2008;**65**:2658-74
43. Engelman J, Lisanti M, Scherer P. Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J Biol Chem* 1998;**273**:32111-20
44. Font de Mora J, Porras A, Ahn N, Santos E. Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. *Mol Cell Biol* 1997;**17**:6068-75
45. Qiu Z, Wei Y, Chen N, Jiang M, Wu J, Liao K. DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J Biol Chem* 2001; **276**:11988-95
46. Boney C, Gruppuso P, Faris R, Frackelton A. The critical role of Shc in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. *Mol Endocrinol* 2000;**14**:805-13
47. Tang Q, Otto T, Lane M. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci* U S A 2003;**100**:44-9
48. Prusty D, Park B, Davis K, Farmer S. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. *J Biol Chem* 2002; **277**:46226-32
49. Blair L, Bence-Hanulec K, Mehta S, Franke T, Kaplan D, Marshall J. Akt-dependent potentiation of L channels by insulin-like growth factor-1 is required for neuronal survival. *J Neurosci* 1999;**19**:1940-51
50. Barber A, Nakamura M, Wolpert E, Reiter C, Seigel G, Antonetti D, Gardner T. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem* 2001;**276**:32814-21
51. Peruzzi F, Prisco M, Dews M, Salomoni P, Grassilli E, Romano G, Calabretta B, Baserga R. Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol* 1999;**19**:7203-15
52. Tomiyama K, Nakata H, Sasa H, Arimura S, Nishio E, Watanabe Y. Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells. *Biochem Biophys Res Commun* 1995;**212**:263-69
53. Xu J, Liao K. Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J Biol Chem* 2004;**279**, 35914-22.
54. Peng X, Xu P, Chen M, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen W, Crawford S, Coleman K, Hay N. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev* 2003;**17**:1352-65

55. Watson R, Khan A, Furukawa M, Hou J, Li L, Kanzaki M, Okada S, Kandror K, Pessin J. Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is GGA dependent. *EMBO J* 2004;**23**:2059-70
56. Satoh S, Nishimura H, Clark A, Kozka I, Vannucci S, Simpson I, Quon M, Cushman S, Holman G. Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *J Biol Chem* 1993;**268**:17820-9
57. Chen Y, Lippincott-Schwartz J. Insulin triggers direct trafficking to the cell surface of sequestered GLUT4 storage vesicles marked by Rab10. *Small Gtpases* 2013;**4**:193-7
58. Agrawal P, Rai V, Singh R. Randomized placebo-controlled, single blind trial of holy basil leaves in patients with noninsulin-dependent diabetes mellitus. *Int J Clin Pharm Ther* 1996;**34**:406-9
59. Vats V, Grover J, Rathi S. Evaluation of anti-hyperglycemic and hypoglycemic effect of Trigonella foenum-graecum Linn, Ocimum sanctum Linn and Pterocarpus marsupium Linn in normal and alloxanized diabetic rats. *J Endocrinol* 2002;**79**:95-100
60. Lehmann J, Moore L, Smith-Oliver T, Wilkison W, Willson T, Kliewer S. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* 1995;**270**:12953-6
61. Barroso I, Gurnell M, Crowley V, Agostini M, Schwabe J, Soos M, O'Rahilly S. Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 1999;**402**:880-3
62. Agostini M, Schoenmakers E, Mitchell C, Szatmari I, Savage D, Smith A, Rajanayagam O, Semple R, Luan J, Bath L, Zalin A, Labib M, Kumar S, Simpson H, Blom D, Marais D, Schwabe J, Barroso I, Trembath R, Wareham N, Nagy L, Gurnell M, O'Rahilly S, Chatterjee K. Non-DNA binding, dominant-negative, human PPARgamma mutations cause lipodystrophic insulin resistance. *Cell Metabolism* 2006;**4**:303-11
63. He W, Barak Y, Hevener A, Olson P, Liao D, Le J, Nelson M, Ong E, Olefsky J, Evans R. Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc. Natl. Acad. Sci.* U S A 2003;**100**:15712-7
64. Bouskila M, Pajvani U, Scherer P. Adiponectin: a relevant player in PPARgamma-agonist-mediated improvements in hepatic insulin sensitivity. *Int J Obesity* 29 Suppl 2005;**1**:517-23
65. Hu E, Liang P, Spiegelman B. AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 1996;**271**:10697-703
66. Hotamisligil G, Johnson R, Distel R, Ellis R, Papaioannou V, Spiegelman B. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science* 1996;**274**:1377-9

67. Coe N, Simpson M, Bernlohr D. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J Lipid Research* 1999;**40**:967-972
68. Scheja L, Makowski L, Uysal K, Wiesbrock S, Shimshek D, Meyers D, Morgan M, Parker R, Hotamisligil G. Altered insulin secretion associated with reduced lipolytic efficiency in aP2-/- mice. *Diabetes* 1999;**48**:1987-94
69. Rioja A, Pizzey A, Marson M, Thomas N. Preferential induction of apoptosis of leukemic cells by farnesol. *FEBS Letters* 2000;467:291-5
70. Wang Z, Chen H, Roa W, Finlay W. Farnesol for aerosol inhalation: Nebulization and activity against human lung cancer cells. *J Pharm Pharm Sci* 2003;**6**:95-100
71. Burke Y, Stark M, Roach S, Sen S, Crowell P. Inhibition of pancreatic cancer growth by the dietary isoprenoids farnesol and geraniol. *Lipids* 1997;**32**:151-6
72. Ong T, Heidor R, de Conti A, Dagli M, Moreno F. Farnesol and geraniol chemopreventive activities during the initial phases of hepatocarcinogenesis involve similar actions on cell proliferation and DNA damage, but distinct actions on apoptosis, plasma cholesterol and HMGCoA reductase. *Carcinogenesis* 2006;**27**:1194-203
73. Unger R. Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes* 1995;**44**:863-8
74. Unger R, Zhou Y. Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50 Suppl 2001;1:S118-S121
75. Unger R, Orci L. Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J* 2001;**15**:312-21
76. Unger R. The physiology of cellular liporegulation. *Annu Rev Physiol* 2003;**65**:333-47
77. Unger R, SchererP. Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity. *Trends Endocrinol Metab TEM* 2010;**21**:345-52

CHAPTER V

RESULTS (*d*-δ-TOCOTRIENOL)

The effect of *d*-δ-tocotrienol on differentiation of 3T3-F442A murine preadipocytes

Suppression of adipogenesis is one of the potential approaches in obesity prevention. In a previous study, we showed that lovastatin, a competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, inhibited adipocyte differentiation and adipogenic genes [Elfakhani et al. 2014]. Other statins have been shown to inhibit adipocyte differentiation through mevalonate deprivation [Brown et al. 1978, Nakanishi et al. 1988, Nishio et al. 1996, Goto et al. 2011, Nakata et al. 2006, Nicholson et al. 2007, Elfakhani et al. 2014]. Isoprenoids, the products of mevalonate pathway in plants, can down-regulate HMG CoA reductase through feedback inhibition [Goldstein and Brown 1990]. It is suggested that by down regulating HMG CoA reductase activity, the rate limiting enzyme in mevalonate pathway, pools of farnesyl pyrophosphate (FPP), the metabolite of mevalonate, will be limited, thereby attenuating preadipocyte differentiation into adipocytes.

Vitamin Es are synthesized in plants from isoprenoids and include four tocopherols and four tocotrienols (designated as α-, β-, γ-, and δ-). The tocotrienols have the farnesol side chain with three double bonds. Adminstration of a tocotrienol-rich fraction containing all four tocotrienol homologues to patients reduced serum cholesterol

[Qureshi et al. 2001, Tan et al. 1991, Parker et al. 1993]. γ and δ-Tocotrienols reduced triglycerides [Zaiden et al. 2010]. Additive effects were observed when tocotrienol-rich fraction and lovastatin were combined [Qureshi et al.2001]. Furthermore, γ-tocotrienol inhibits the differentiation of 3T3-L1 preadipocytes *in vitro*, suggested by decreased phosphorylation of PKB-Akt [Uto-kondo et al. 2009]. The effect of other tocotrienols on adipocyte differentiation and the significance of glucose uptake in the mechanism were not clear.

We investigated the effect of *d*-δ-tocotrienol on differentiation of 3T3-F442A preadipocytes and the underlying mechanism. The purpose of this study was to evaluate the impact of *d*-δ-tocotrienol on adipocyte differentiation, intracellular triglyceride content, glucose uptake, and the expression of GLUT4 protein in murine 3T3-F442A preadipocytes. The results of this study may help to consider the risk/benefits of using this compound in clinical conditions such as insulin resistance and obesity.

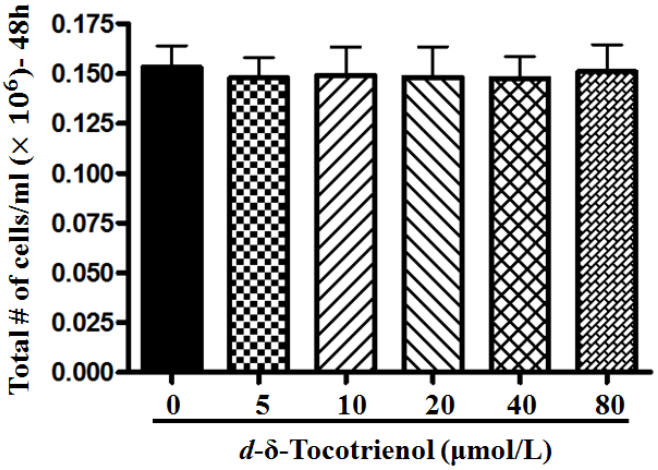
Our results are listed as following:

Viability

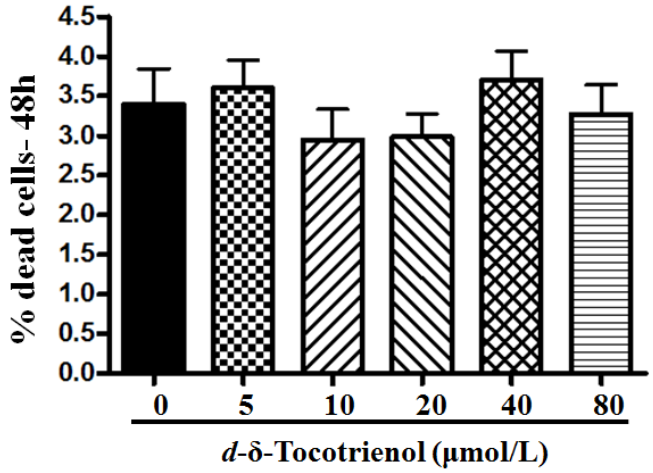
Following 48-h incubation of 3T3-F442A cells with increasing concentrations of *d*-δ-tocotrienol (0 - 80 μmol/L), cell populations (Figure 5.1-A) and the percentage of dead cells (Figure 5.1-B) were determined using Trypan Blue staining. No changes in cell viability was shown, suggesting that the adipogenesis-suppressive activity of *d*-δ-tocotrienol was independent of cytotoxicity. Lack of *d*-δ-tocotrienol toxicity was also demonstrated in 7-day incubations with d-δ-tocotrienol 0-10 μmol/L and α-tocopherol

10µmol/L via unchanged total number of cells (Figure 5.1-C) and the percentage of dead cells (Figure 5.1-D).

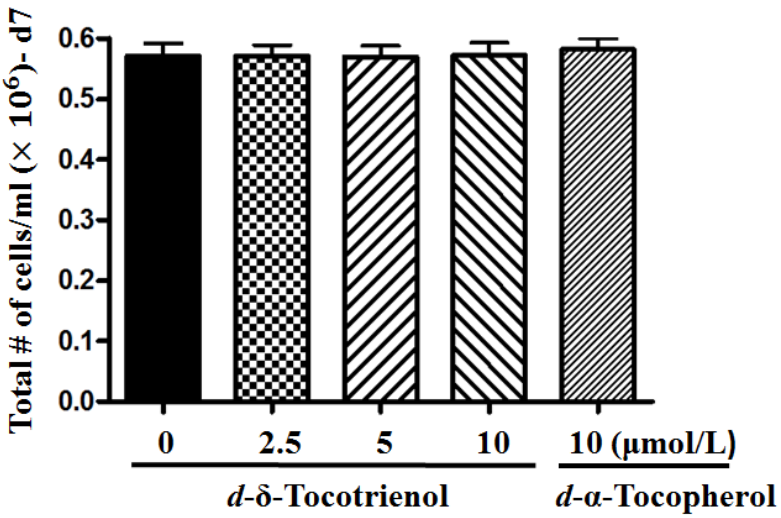
1-A



1-B



1-C



1-D

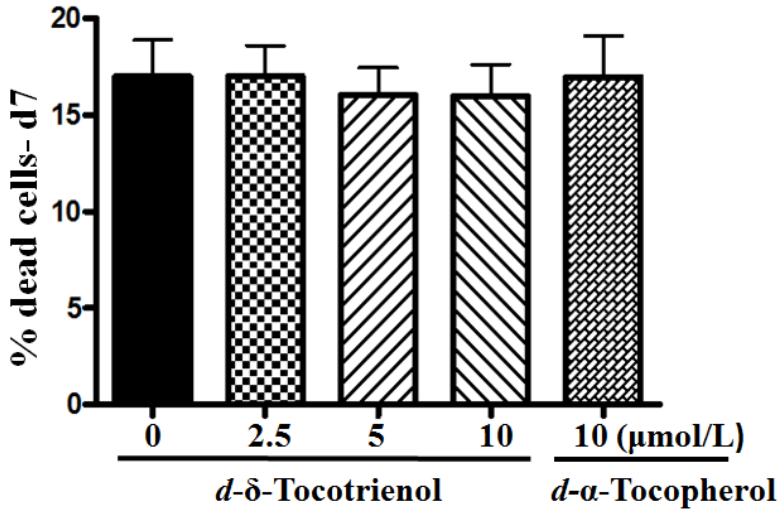


Figure 5.1 The effect of *d*-δ-tocotrienol on viability of 3T3-F442A preadipocytes. Following 48-h incubation of preadipocytes with *d*-δ-tocotrienol (0-80 µmol/L), total cell count (1-A) and the % of dead cells (1-B) were measured by Trypan Blue staining. The viability of adipocytes were also measured on differentiation period. Following 7-day incubation of 3T3-F442A adipocytes with *d*-δ-tocotrienol (0 - 10 µmol/L) and *d*-α-tocopherol (10 µmol/L), total cell count (1-C) and the % of dead cells (1-D) were measured by Trypan Blue staining.

Oil Red O staining and photomicrography

Following 7-day differentiation of 3T3-F442A preadipocytes treated by *d*-δ-tocotrienol (0-10 μmol/L) and α-tocopherol (10 μmol/L), the cells were stained by Oil Red O. The images from cells showed d-δ-tocotrienol induced a dose-dependent decrease in differentiation of adipocytes, while α-tocopherol did not have any effect on differentiation (Figure 5.2-A).

2-A

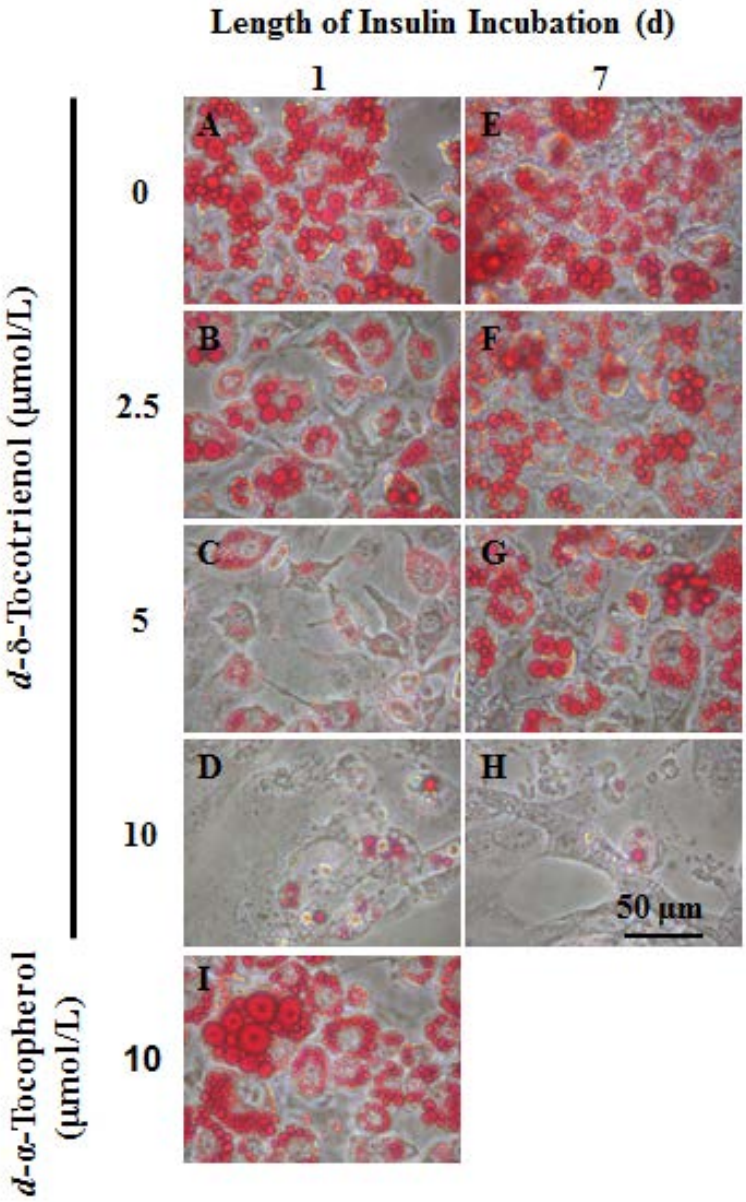


Figure 5.2-A Photomicrographs of 3T3-F442A adipocytes stained with Oil Red O. Following 7-day incubation of cells with *d*-δ-tocotrienol (0-10 μmol/L) and *d*-α-tocopherol (10 μmol/L), the cells were washed and stained with Oil Red. *d*-δ-Tocotrienol dose-dependently inhibited the differentiation of preadipocytes incubated with insulin for 1 (A-D) or 7 days (E-H)

Intracellular triglyceride accumulation

The effect of *d*-δ-tocotrienol on intracellular TAG accumulation (adipogenesis) during differentiation of 3T3-F442A preadipocytes with and without insulin (10 omg/mL), rosiglitazone (1 µmol/L) and mevalonate (100 µmol/L) was measured at day 7-8 of differentiation by AdipoRed Assay. *d*-δ-Tocotrienol (2.5-10 µmol/L) decreased adipogenesis significantly in a dose dependent manner ($P \leq 0.05$). Longer incubation with insulin (10 mg/mL) attenuated the effect of 2.5 and 5 µmol/L *d*-δ-tocotrienol, but not that of 10 µmol/L *d*-δ-tocotrienol (Figure 5.2-B). Lovastatin 1.25 µmol/L decreased intracellular TAG accumulation significantly ($P \leq 0.01$). Rosiglitazone (1 µmol/L) reversed the effect of *d*-δ-tocotrienol, but not that of lovastatin, on adipogenesis. In contrast, mevalonate (100 µmol/L) reversed the effect of lovastatin, but not that of *d*-δ-tocotrienol (10 µmol/L) (Figure 5.3).

2-B

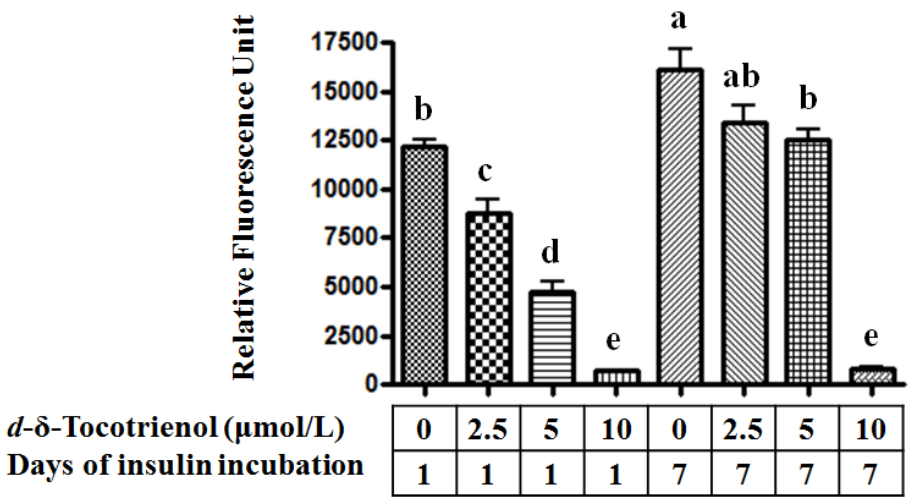


Figure 5.2-B The effect of *d*-δ-tocotrienol on the intracellular triglyceride content of 3T3-F442A adipocytes. Following 7-d incubation with *d*-δ-tocotrienol (0-10 µmol/L) and 1- or 7-day incubation with insulin (10 µmol/L), intracellular triglyceride accumulation was measured by Adipored assay. Means that are not denoted with a common letter are significantly different ($P \leq 0.05$).

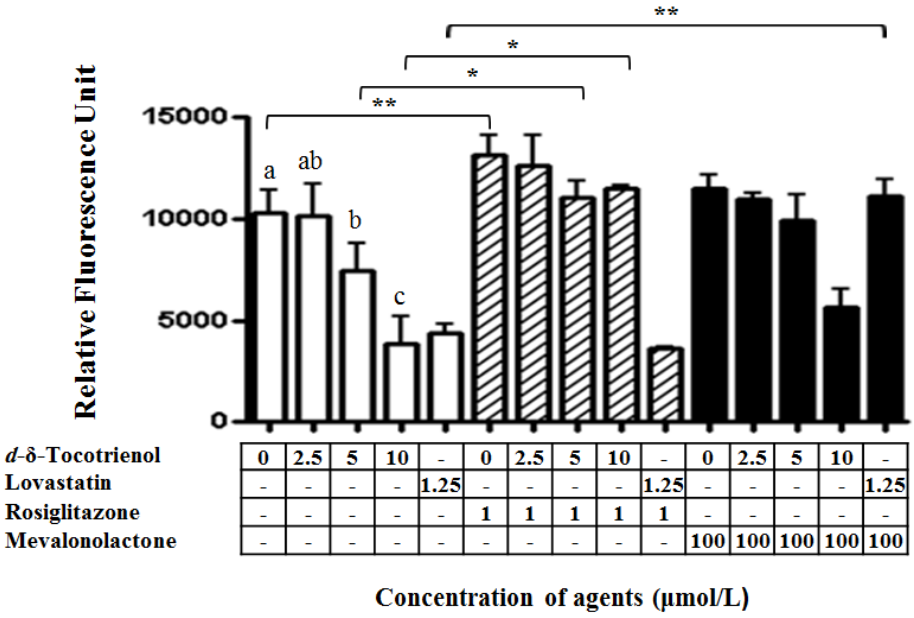


Figure 5.3 The effect of rosiglitazone and mevalonolactone on *d*-δ-tocotrienol- and lovastatin-mediated suppression of intracellular triglyceride content in 3T3-F442A adipocytes. Following 7 day incubation of 3T3-F442A adipocytes with *d*-δ-tocotrienol (0-10 µmol/L), lovastatin (1.25 µmol/L), rosiglitazone (1 µmol/L), and mevalonolactone (100 µmol/L), the intracellular triglyceride accumulation was measured by Adipored assay. Means that are not denoted with a common letter are significantly different (*P ≤ 0.05; **P ≤ 0.01).

Glucose uptake

The effect of *d*-δ-tocotrienol on glucose uptake was measured during differentiation of 3T3-F442A preadipocytes by difference in medium glucose concentration in between d4 and d8 of differentiation. *d*-δ-Tocotrienol (2.5-10 µmol/L),

dose dependently decreased glucose uptake, and lovastatin (1.25 µmol/L) significantly decreased glucose uptake ($P \leq 0.01$). Rosiglitazone (1 µmol/L) reversed the effect of *d*-δ-tocotrienol, but not that of lovastatin, on glucose uptake. In contrast, mevalonate 100 µmol/L reversed the effect of lovastatin, but not that of *d*-δ-tocotrienol (Figure 5.4).

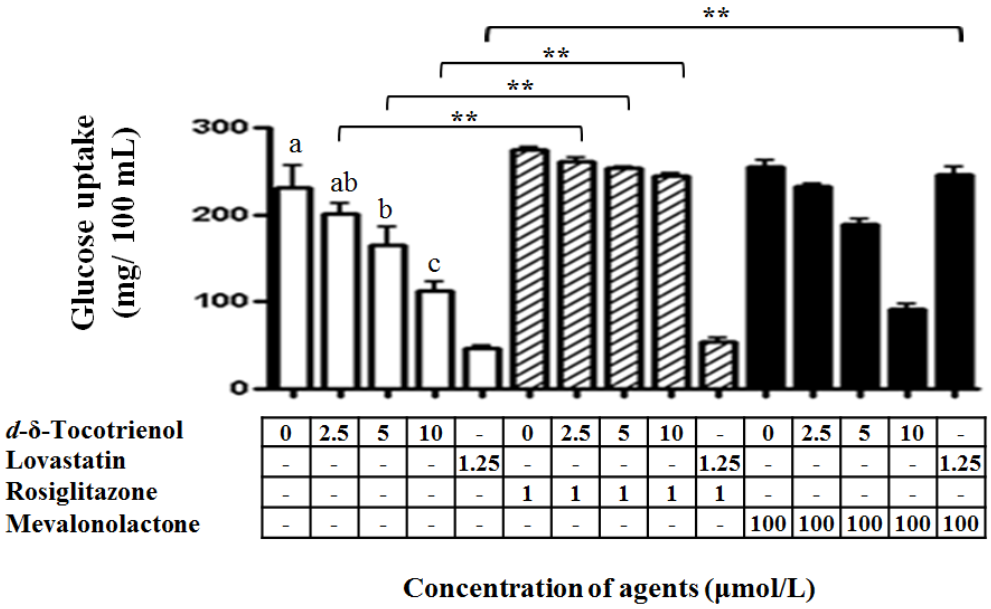
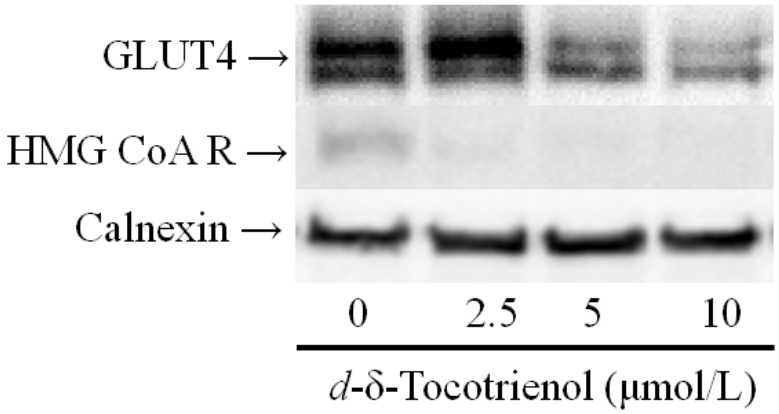


Figure 5.4 The effect of rosiglitazone and mevalonolactone on *d*-δ-tocotrienol- and lovastatin-mediated suppression of glucose uptake in 3T3-F442A adipocytes. Following 7 day incubation of 3T3-F442A adipocytes with *d*-δ-tocotrienol (0-10 µmol/L), lovastatin (1.25 µmol/L), rosiglitazone (1 µmol/L), and mevalonolactone (100 µmol/L), the glucose uptake were measured by difference in medium glucose concentration between day 8 and day 4 of differentiation. Within the tocotrienol group, means that are not denoted with a common letter are significantly different ($P < 0.05$). (*$P \leq 0.05$; **$P \leq 0.01$).

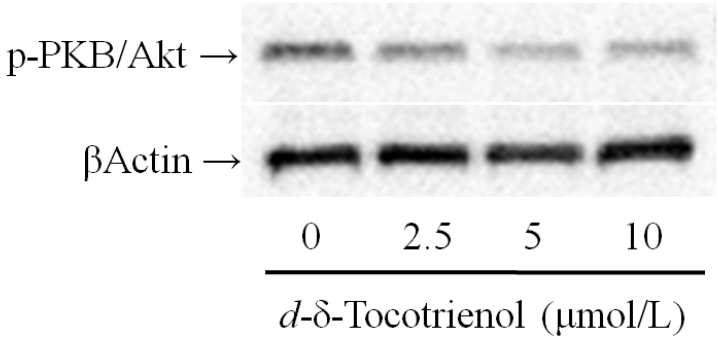
Protein content

Following 7-8 days incubation of 3T3-F442A cells with *d*-δ-tocotrienol(0-10 µmol/L), cells were collected for subcellular protein fractionation. The membrane fraction was used to measure the expression of HMG CoA reductase and GLUT4 proteins, and the non-membrane fraction was used to measure the expression of phosphorylated PKB/Akt (p-PKB/Akt) and phosphorylated AMPK (pAMPK) proteins. *d*-δ-Tocotrienol (2.5-10 µmol/L) reduced the expression of both HMG CoA reductase and GLUT4 proteins in the membrane fraction (Figure 5.5-A), and reduced pPKB/Akt and pAMPK proteins in the non membrane fraction (Figure 5.5-B and C).

5-A



5-B



5-C

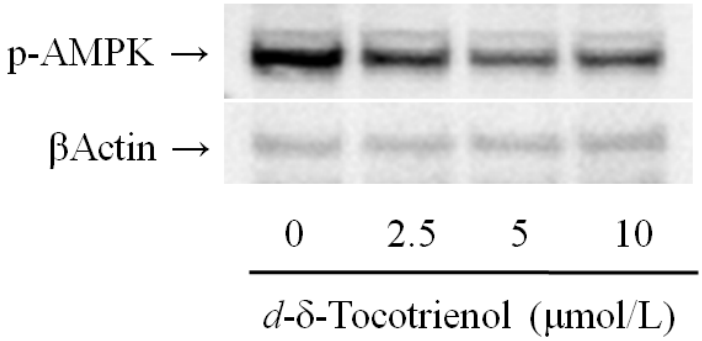


Figure 5.5 The effect of *d*-δ-tocotrienol on the protein content of GLUT4 and HMG CoA reductase (5-A), protein kinase-B/Akt (p-PKB/Akt) (5-B), and phosphorylated AMPK (p-AMPK) (5-C) in 3T3-F442A adipocytes. Following 8 day incubation of cells with *d*-δ-tocotrienol (0-10 μmol/L), the cells were collected for subcellular protein fractionation and western-blot analysis. The expression of GLUT4 and HMG CoA reductase (5-A) proteins were measured in membrane fraction, and that of p-PKB/Akt (5-B) and p-AMPK proteins (5-C) were measured in the non-membrane fraction. Calnexin and β-actin were used as loading controls for membrane and non-membrane fractions, respectively.

CHAPTER VI

SUMMARY AND CONCLUSION

In this study, we investigated the effect of *d*-δ-tocotrienol and *trans, trans*-farnesol (farnesol) on differentiation of 3T3-F442A preadipocytes and intracellular TAG accumulation for the first time. We found that *d*-δ-tocotrienol (2.5-10 μmol/L) significantly suppressed the differentiation and intracellular TAG accumulation of 3T3-F442A adipocytes in a concentration-dependent manner. In contrast, farnesol (25-75 μmol/L) significantly and dose dependently induced the differentiation of 3T3-F442A adipocytes.

The stimulatory effect of insulin and the contrasting effects of d-δ-tocotrienol and farnesol on 3T3-F442A differentiation suggest two possible mechanisms of action for these agents as shown in the following model:

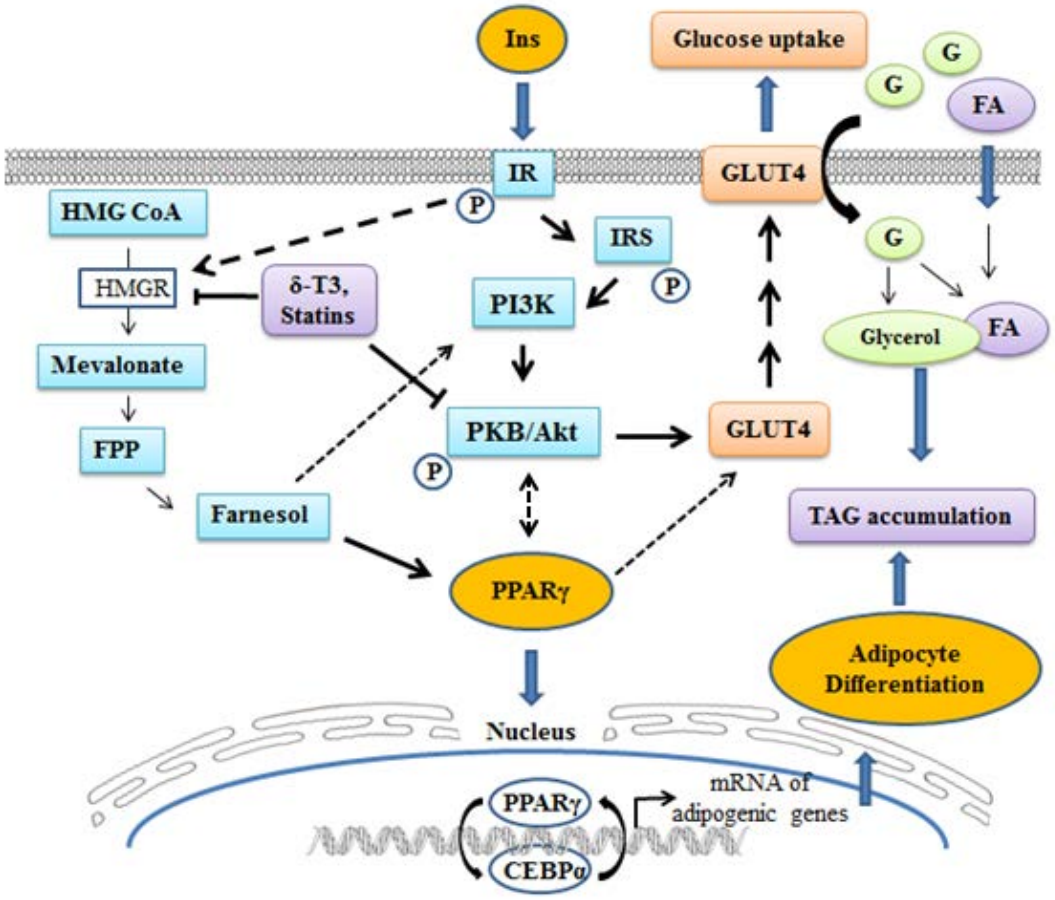


Figure 6.1 Hypothesized model. Insulin (Ins) activates the insulin receptor- insulin receptor substrate- phosphatidylinositol 3-kinase (IR-IRS-PI3K) axis by phosphorylation which results in glucose transporter 4 (GLUT4) translocation from intracellular to plasma membrane and increased glucose uptake. Glucose produces FAs and glycerol, required for TAG synthesis. *d*-δ-Tocotrienol (δ-T3) and statins down-regulate both HMG CoA reductase (HMGR) and PKB/Akt proteins. Farnesol activates peroxisome proliferator-activated receptor γ (PPARγ). PPARγ and CCAAT/enhancer binding protein α (CEBPα) activate each other and induce the expression of adipogenic genes. PKB/Akt, protein kinase B/Akt; G, glucose; FPP, farnesyl pyrophosphate. ⟶ based on cited references, ⇢ hypothesized.

In one possible mechanism, we consider that FPP and its product, farnesol, induces differentiation of preadipocytes by activation of PPARγ, the master regulator of adipogenesis. Activation of PPARγ pathway then results in activation of the PI3K-PKB/Akt pathway which induces translocation of the GLUT4 protein to plasma membrane and consequently increases glucose uptake. *d*-δ-Tocotrienol, as a suppressor of HMG CoA reductase, down-regulated the mevalonate pathway resulting in a decrease of FPP and farnesol and hence reduced activation of PPARγ. This possibility would support an essential role of the mevalonate pathway in adipocyte differentiation.

A second possibility is that *d*-δ-tocotrienol suppresses the differentiation of preadipocytes by directly inhibiting the PI3K-PKB/Akt axis. Similarly, in this model, farnesol would induce differentiation of preadipocytes by direct activation of PI3K-PkB/Akt axis. If this mechanism is correct, insulin could induce the PI3K-PKB/Akt pathway upstream to PPARγ, and activate anabolic pathways involved in TAG synthesis and cholesterol synthesis at the cytoplasmic level. Thus, the mevalonate pathway or cholesterol synthesis pathway could be activated by the insulin-PI3K pathway. This explanation supports the essential role of insulin-PI3K pathway in adipocyte differentiation.

Althernatively, both mechanisms could work together to regulate adipocyte differentiation. In this cooperative action, the PPARγ pathway and PI3K-PKB/Akt pathway work together to regulate adipogenesis in adipocytes.

If the first mechanism is correct, we would expect that both mevalonate derived products affect PPARγ pathway. Consistently, farnesol increased the mRNA expression and the protein level of PPARγ. This mechanism is further supported by farnesol-mediated upregulation of the PPARγ target genes, including GLUT4, FABP4, adiponectin, and LPL. Moreover, GW9662 (10 μmol/L), an antagonist of PPARγ partially reversed the effects of farnesol, 25 and 50 μmol/L, on cellular lipid content ($P \leq 0.05$). These results are consistent with Goto's study showing that FPP functions as an endogenous PPARγ agonist in 3T3-L1 preadipocytes [Goto et al. 2011]. Furthermore, rosiglitazone (1 μmol/L), an agonist of PPARγ, reversed the effects of *d*-δ-tocotrienol on cellular lipid content and glucose uptake, while supplemental mevalonate did not show the reversal effect. Even though *d*-δ-tocotrienol suppressed the expression of HMG CoA reductase, our results showed that farnesol did not affect both gene and protein expression of HMG CoA reductase. This result is inconsistent with previous evidences [Correll et al. 1994, Meigs et al. 1996] that farnesol induces HMG CoA reductase degradation, but is consistent with a more recent study showing that geranylgeraniol, rather than farnesol, induces reductase degradation [Sever et al. 2003].

If the second mechanism is correct, we would expect that these agents affect the markers of PI3K pathway and finaly glucose uptake. Consistently, there was a positive correlation between the amount of intracellular TAG accumulation and glucose uptake for both *d*-δ-tocotrienol and farnesol. Up- and down- regulation of intracellular TAG accumulation induced by farnesol and *d*-δ-tocotrienol, respectively, were accompanied with changes in glucose uptake, accordingly. Our results showed that *d*-δ-tocotrienol decreased glucose uptake by downregulating the protein levels of pAkt and GLUT4, while farnesol induced glucose uptake by increasing the protein level of GLUT4. Furthermore, the differentiation-suppressive activity of *d*-δ-tocotrienol was attenuated by insulin, whereas LY294002, an inhibitor of PI3K, completely reversed the effect of farnesol on intracellular lipid content ($P \leq 0.01$).

Our findings clearly suggest that PI3K pathway mediates the effects of farnesol and *d*-δ-tocotrienol on adipocyte differentiation. The role of the mevalonate pathway is less clear. The fact that mevalonate reversed the effect of lovastatin, but not that of *d*-δ-tocotrienol, suggests that *d*-δ-tocotrienol may have multifaceted impact, including those related to HMG CoA reductase and PPARγ, on the adipocyte differentiation. This hypothesis may be further supported by the tocotrienol-mediated suppression of HMG CoA reductase. Further studies are required to reveal the mechanisms underlying mevalonate deprivation and suppression of adipocyte differentiation.

REFERENCES

Aagaard MM, Siersback R, Mandrup S. (2011). Molecular basis for gene-specific transcription by nuclear receptors. *Biochim Biophys Acta* 1812:824-35

Adams M, Reginato M, Shao D, Lazar M, Chatterjee V. (1997). Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. *J Biol Chem.* 272:5128-32

Agostini M, Schoenmakers E, Mitchell C, Szatmari I, Savage D, Smith A, Chatterjee K. (2006). Non-DNA binding, dominant-negative, human PPARgamma mutations cause lipodystrophic insulin resistance. *Cell Metabolism* 4(4):303-11

Agrawal P, Rai V, Singh RB. (1996). Randomized placebo-controlled, single blind trial of holy basil leaves in patients with noninsulin-dependent diabetes mellitus. *Int J Clin Pharmacol Ther.* 34(9):406-9

Ailhaud G, Grimaldi P, Négrel R. (1992). Cellular and molecular aspects of adipose tissue development. *Annu Rev Nutr.* 12:207-33

Akira S, Isshiki H, Kishimoto T, et al. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* 6:1897-906

Altiok S, Xu M, Spiegelman B. (1997). PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. *Genes Dev.* 11:1987-98

Antras J, Hilliou F, Redziniak G, Pairault J. (1989). Decreased biosynthesis of actin and cellular fibronectin during adipose conversion of 3T3-F442A cells.Reorganization of the cytoarchitecture and extracellular matrix fibronectin. *Biol Cell.* 66:247-54

Aratani Y, Kitagawa Y. (1988). Enhanced synthesis and secretion of type IV collagen and entactin during adipose conversion of 3T3-L1 cells and production of unorthodox laminin complex. *J Biol Chem.* 263:16163-9

Argmann C, Edwards J, Huff M, et al. (2005). Regulation of macrophage cholesterol efflux through hydroxymethylglutaryl-CoA reductase inhibition: a role for RhoA in ABCA1-mediated cholesterol efflux. *J Biol Chem.* 280(23):22212-21

Barber A, Nakamura M, Gardner T, et al. (2001). Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem.* 276(35):32814-21

Barroso I, Gurnell M, Crowley V, Agostini M, Schwabe J, Soos M, O'Rahilly S. (1999). Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature*. 402(6764):880-3

Baumann C, Ribon V, Saltiel A, et al. (2000). CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature.* 407(6801):202-207

Bennett C, Longo K, Wright W, Suva L, Lane T, et al. (2005). Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci. USA.* 102:3324-29

Berg A, Combs T, Scherer P. (2002). ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab.* 13(2):84-9

Berger J, Patel H, Moller D, et al. (2000). A PPARgamma mutant serves as a dominant negative inhibitor of PPAR signaling and is localized in the nucleus. *Mol Cell Endocrinol.* 162(1-2):57-67

Bernards A, Settleman J. (2004). GAP control: regulating the regulators of small GTPases. *Trends in Cell Biology.* 14(7):377-85

Beutler B, Greenwald D, Cerami A, et al. (1985). Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature.* 316(6028):552-4.

Bjorntorp P. (1974). Effects of age, sex, and clinical conditions on adipose tissue cellularity in man. *Metabolism.* 23:1091–102

Blair L, Bence-Hanulec K, Mehta S, Franke T, Kaplan D, Marshall J. (1999) Akt-dependent potentiation of L channels by insulin-like growth factor-1 is required for neuronal survival. *J Neurosci*. 19(6):1940-51

Boden G, Homko C, Mozzoli M, Showe L, Nichols C, Cheung P. (2005). Thiazolidinediones upregulate fatty acid uptake and oxidation in adipose tissue of diabetic patients. *Diabetes.* 54 (3):880-885.

Boney C, Gruppuso P, Faris R, Frackelton A. (2000). The critical role of Shc in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. *Mol Endocrinol.* 14(6):805-13

Boström P, Andersson L, Olofsson S, et al. (2007). SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nat Cell Biol.* 9(11):1286-93

Bouskila M, Pajvani U, Scherer P. (2005). Adiponectin: a relevant player in PPARgamma-agonist-mediated improvements in hepatic insulin sensitivity. *Int J Obesity.* 29 Suppl 1:517-23

Bowers RR, Lane MD. (2008). Wnt signaling and adipocyte lineage commitment. *Cell Cycle.* 7:1191-6

Bowers R, Kim J, Otto T, Lane M. (2006). Stable stem cell commitment to the adipocyte lineage by inhibition of DNA methylation: role of BMP-4 gene. *PROC Natl Acad Sci. USA.*103:13022-27

Bright N, Thornton C, Carling D. (2009). The regulation and function of mammalian AMPK-related kinases. *Acta Physiologica.* 196(1):15-26

Brown M, Goldstein J. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell.* 89:331-40

Brown M, Faust J, Goldstein J, Kaneko I, Endo A. (1978). Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. *J Biol Chem.* 253(4):1121-8

Brun R, Kim J, Hu E, Altiok S, Spiegelman B. (1996). Adipocyte differentiation: a transcriptional regulatory cascade. *Curr Opin Cell Biol.* 8:826-832.

Burdi A, Poissonnet C, Garn S, Lavelle M, Sabet M, Bridges P. (1985). Adipose tissue growth patterns during human gestation: a histometric comparison of buccal and gluteal fat depots. *Int J Obes.* 9(4):247-56.

Calvo J, Rodbard D, Katki A, Chernick S, Yanagishita M. (1991). Differentiation of 3T3-L1 preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. *J Biol Chem.* 266:11237-44

Cao Z, Umek R, McKnight S. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* 5:1538-52

Chang C, Chen T, Lei H, Chen D, Lee S. (1990). Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell. Biol.* 10:6642-53

Chao L, Marcus-Samuels B, Reitman M, et al. (2000). Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. *J Clin Investig.* 106(10):1221-8

Chavez J, Roach W, Keller S, Lane W, Lienhard G. (2008). Inhibition of GLUT4 Translocation by Tbc1d1, a Rab GTPase-activating Protein Abundant in Skeletal Muscle, Is Partially Relieved by AMP-activated Protein Kinase Activation. *J Biol Chem.* 283(14):9187-95

Chen S, Murphy J, Toth R, Campbell D, Morrice N, Mackintosh C. (2008). Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. *J Biochem.* 409(2):449-59

Chen Y, Lippincott-Schwartz J. (2013). Insulin triggers surface-directed trafficking of sequestered GLUT4 storage vesicles marked by Rab10. *Small Gtpases*. 4(3):193-7

Christy R, Kaestner K, Geiman D, Lane M. (1991). CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc Natl Acad Sci.* USA. 88:2593-7

Clarke S, Vogel J, Deschenes R, Stock J. (1988). Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc Natl Acad Sci* USA. 85(13):4643-7

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, et al. (1998). A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature.* 392:398-401

Crick D, Andres D, Waechter C. (1997). Novel salvage pathway utilizing farnesol and geranylgeraniol for protein isoprenylation. *Biochem Biophys Res Commun.* 237(3):483-7

Collins J, Blanchard S, Tong W, et al. (1998). N-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. 2. Structure-activity relationship and optimization of the phenyl alkyl ether moiety. *J Med Chem.* 41(25):5037-54

Correll C, Ng L, Edwards, P. (1994). Identification of farnesol as the non-sterol derivative of mevalonic acid required for the accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol Chem.* 269(26):17390-3

Cormont M, Le Marchand-Brustel Y. (2001). The role of small G-proteins in the regulation of glucose transport (review). *Mol.Membr.Biol.* 18(3):213-20

Cornelius P, MacDougald O, Lane M. (1994). Regulation of adipocyte development. *Annu Rev Nutr.*14:99-129.

Cowherd R, Lyle R, McGehee R. (1999). Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol.*10(1):3-10.

Cristancho A, Lazar M. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol.*12(11):722-34

Crick D, Waechter C, Andres D. (1994). Utilization of geranylgeraniol for protein isoprenylation in C6 glial cells. *Biochem Biophys Res Commun.* 205(1):955-61

Darlington G, Ross S, MacDougald O. (1998).The role of C/EBP genes in adipocyte differentiation. *J Biol Chem.* 273(46):30057-60

Date T, Doiguchi Y, Nobuta M, Shindo H. (2004). Bone morphogenetic protein-2 induces differentiation of multipotent C3H10T1/2 cells into osteoblasts, chondrocytes, and adipocytes in vivo and in vitro. *J. Orthop Sci.* 9:503-8

Davies S, Sim A, Hardie D. (1990). Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur J Bioch. FEBS.* 187(1):183-90

Davis L, Zur Nieden N. (2008). Mesodermal fate decisions of a stem cell: the Wnt switch. *Cell Mol Life Sci. CMLS.* 65(17):2658-74

Davis-Hanna A, Piispanen A, Stateva L, Hogan D. (2008).Farnesol and dodecanol effects on the Candida albicans Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol Microbiol.* 67(1):47-62

de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ, ter Elst A. (2007). Evidence based selection of housekeeping genes. *PLoS One*;2:e898

Deslex S, Negrel R, Vannier C, Etienne J, Ailhaud G. (1987). Differentiation of human adipocyte precursors in a chemically defined serum-free medium. *Int J Obesity.*11:19-27

Duggan S. (2012). Pitavastatin: a review of its use in the management of hypercholesterolaemia or mixed dyslipidaemia. *Drugs.* 72(4):565-584

Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. (2004). SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie.* 86(11):839-48

Eguez L, Lee A, McGraw T, et al. (2005). Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell Metab.* 2(4):263-72

El-Beshbishy H, Bahashwan S. (2012). Hypoglycemic effect of basil (Ocimum basilicum) aqueous extract is mediated through inhibition of α-glucosidase and α-amylase activities: an in vitro study. *Toxicology And Industrial Health.* 28(1):42-50

Elfakhani M, Torabi S, Hussein D, Mills N, Verbeck G, Mo H. (2014). Mevalonate deprivation mediates the impact of lovastatin on the differentiation of murine 3T3-F442A preadipocytes. *Exp Biol Med.* 239(3):293-301

Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. (1998). Unraveling the central nervous system pathways underlying responses to leptin. *Nat Neurosci.* 1:445-49

Engelman J, Lisanti M, Scherer P. (1998). Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. *J Biol Chem.* 273(48):32111-20

Faust I, Miller W, Sclafani A, Aravich P, Triscari J, Sullivan A. (1984). Diet-dependent hyperplastic growth of adipose tissue in hypothalamic obese rats. *Am J Physiol.* 247:R1038-R1046

Fernandes N, Yeganehjoo H, Mo H, et al. (2013). Geranylgeraniol suppresses the viability of human DU145 prostate carcinoma cells and the level of HMG CoA reductase. *Exp Biol Med.* 238(11):1265-74

Fève B, Emorine L, Briend-Sutren M, Lasnier F, Strosberg A, Pairault J. (1990). Differential regulation of beta 1- and beta 2-adrenergic receptor protein and mRNA levels by glucocorticoids during 3T3-F442A adipose differentiation. *J Biol Chem.* 265:16343-9

Font de Mora J, Porras A, Ahn N, Santos E. (1997). Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. *Mol Cell Biol.* 17(10):6068-75

Forman B, Ruan B, Chen J, Schroepfer G, Evans R. (1997). The orphan nuclear receptor LXRalpha is positively and negatively regulated by distinct products of mevalonate metabolism. *Proc Natl Acad Sci. USA.* 94(20):10588-93

Foster L, Yaworsky K, Trimble W, Klip A. (1999). SNAP23 promotes insulin-dependent glucose uptake in 3T3-L1 adipocytes: possible interaction with cytoskeleton. *Am. J. Physiol.* 276(5 Pt 1):C1108-C1114

Friedman JM, Halaas JL. (1998). Leptinand the regulation of body weight im mammals. *Nature.* 395:763-70

Fu Y, Luo N, Klein R, Garvey W. (2005). Adiponectin promotes adipocyte differentiation, insulin sensitivity, and lipid accumulation. *J Lipid Res.* 46(7):1369-79

Funaki M, Randhawa P, Janmey P. (2004). Separation of insulin signaling into distinct GLUT4 translocation and activation steps. *Mol Cell Biol.* 24(17):7567-77

Gagnon A, Chen C, Sorisky A. (1999). Activation of protein kinase B and induction of adipogenesis by insulin in 3T3-L1 preadipocytes: contribution of phosphoinositide-3,4,5-trisphosphate versus phosphoinositide-3,4-bisphosphate. *Diabetes.* 48(4):691-8

Gastaldelli A, Harrison S, Cusi K, et al. (2009). Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. *Hepatology.* 50(4):1087-93

Geraghty K, Chen S, MacKintosh C, et al. (2007). Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *J Biochem.* 407(2):231-41

Goldstein J, Brown M. (1990). Regulation of the mevalonate pathway. *Nature.* 343(6257):425-30

Goto T, Nagai H, Kawada T, et al. (2011). Farnesyl pyrophosphate regulates adipocyte functions as an endogenous PPARγ agonist. *J Biochem.* 438(1):111-9

Green H, Kehinde O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. *Cell.* 1:113-6

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

Green H, Meuth M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell.* 3(2):127-33

Greenspan P, Mayer E, Fowler S. (1985). Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol.* 100(3):965-73

Green H, Kehinde O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J Cell Physiol.* 101(1):169-71

Greenberg A, Egan J, Wek S, Moos M, Londos C, Kimmel A. (1993). Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes. *Proc Natl Acad Sci.* USA 90(24):12035-9

Grégoire F, Smas C, Sul H. (1998). Understanding adipocyte differentiation. *Physiol Rev.* 78(3):783-809

Grégoire F, Genart C, Hauser N, Remacle C. (1991). Glucocorticoids induce a drastic inhibition of proliferation and stimulate differentiation of adult rat fat cell precursors. *Exp Cell Res.* 196:270-8

Grégoire F, Johnson P, Greenwood M. (1995). Comparison of the adipoconversion of preadipocytes derived from lean and obese Zucker rats in serum-free cultures. *Int J Obes Relat Metab Disorders.* 19:664-70

Grégoire F, Todoroff G, Hauser N, Remacle C. (1990). The stroma-vascular fraction of rat inguinal and epididymal adipose tissue and the adipoconversion of fat cell precursors in primary culture. *Biol Cell.* 69(3):215-22

Goldstein J, Brown M. (1990). Regulation of the mevalonate pathway. *Nature.* 343(6257): p. 425-30.

Goto T, Nagai H, Kawada T, et al. (2011). Farnesyl pyrophosphate regulates adipocyte functions as an endogenous PPARγ agonist. *J Biochem.* 438(1):111-9

Hardie D. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature Reviews. Mol Cell Biol.* 8(10):774-85

Hardie D, Carling D, Carlson M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem.* 67:821-55

Hauner H, Röhrig K, Petruschke T. (1995). Effects of epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) on human adipocyte development and function. *Eur J Clin Invest.* 25:90-6

Hauner H, Entenmann G, Pfeiffer E, et al. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest.* 84:1663-70

He W, Barak Y, Evans R, et al. (2003). Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc Natl Acad Sci. USA* 100(26):15712-7

Herranz P, de Lucas R, Pérez-España L, Mayor M. (2008).Lipodystrophy syndromes. *Dermatol Clin.* 26(4):569

Hirsch J and Batchelor B. (1976). Adipose tissue cellularity in human obesity. *Clin Endocrinol Metab*. 5: 299–311

Hornby J, Jensen E, Nickerson K, et al. (2001). Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol. *Appl Environ Microbiol*. 67(7):2982-92

Horton JD. (2002). Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. *Biochem Soc Trans.* 30:1091-95

Hotamisligil G, Shargill N, Spiegelman B. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science.* 259(5091):87-91

Hu E, Liang P, Spiegelman B. (1996). AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem.* 271(18):10697-703

Hu E, Tontonoz P, Spiegelman B. (1995). Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad.Sci. USA.* 92:9856-60

Hu E, Kim J, Sarraf P, Spiegelman B. (1996). Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma. *Science.* 274(5295):2100-3

Hwang CS, Loftus TM, Mandrup S, Lane MD. (1997). Adipocyte differentiation and leptin expression. *Annu Rev Cell Dev Biol.* 13:231-59

Ibrahimi A, Sfeir Z, Magharaie H, Amri E, Grimaldi P, Abumrad N. (1996). Expression of the CD36 homolog (FAT) in fibroblast cells: effects on fatty acid transport. *Proc Natl Acad Sci.* USA 93:2646-51

Ishiki M, Randhawa V, Poon V, Jebailey L, Klip A. (2005). Insulin regulates the membrane arrival, fusion, and C-terminal unmasking of glucose transporter-4 via distinct phosphoinositides. *J Biol Chem.* 280(31):28792-802

Jackson J, Cochrane C, Bourne J, Solski P, Buss J, Der C. (1990). Farnesol modification of Kristen-ras exon 4B protein is essential for transformation. *Proc Nati Acad Sci.* USA. 87:3042-6

JeBailey L, Rudich A, Huang X, Di Ciano-Oliveira C, Kapus A, Klip A. (2004). Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. *Mol Endocrinol.* 18(2):359-72

JeBailey L, Wanono O, Niu W, Roessler J, Rudich A, Klip A. (2007). Ceramide- and oxidant-induced insulin resistance involve loss of insulin-dependent Rac-activation and actin remodeling in muscle cells. *Diabetes.* 56(2):394-403

Jäger S, Handschin C, St-Pierre J, Spiegelman B. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. *Proc Nati Acad Sci.* USA. 104(29):12017-22

Jones F, Prediger E, Bittner D, De Robertis E, Edelman G. (1992). Cell adhesion molecules as targets for Hox genes: neural cell adhesion molecule promoter activity is modulated by cotransfection with Hox-2.5 and -2.4. *Proc Natl Acad Sci.* USA. 89(6):2086-90

Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. (2006). Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J. Clin. Invest.*116(7):1784-92

Kakuma T, Wang Z, Pan W, Unger R, Zhou Y. (2000). Role of leptin in peroxisome proliferator-activated receptor gamma coactivator-1 expression. *Endocrinol.* 141(12):4576-82

Kakuma T, Lee Y, Unger R, et al. (2000).Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. *Proc Natl Acad Sci.* USA. 97(15):8536-41

Kane S, Sano H, Lienhard G, et al. (2002). A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem.* 277(25):22115-8

Kang S, Bennett C, Gerin I, Rapp L, Hankenson K, Macdougald O. (2007). Wnt signaling stimulates osteoblastogenesis of mesenchymal precursors by suppressing CCAAT/enhancer-binding protein alpha and peroxisome proliferator-activated receptor gamma. *J Biol Chem.* 282:14515-24

Kanzaki M, Mora S, Hwang J, Saltiel A, Pessin J. (2004). Atypical protein kinase C (PKCzeta/lambda) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. *J Cell Biol.* 164(2):279-90

Kanzaki M, Pessin J. (2003). Insulin signaling: GLUT4 vesicles exit via the exocyst. *Curr Biol.*13(14):R574-R576

Kawakami M, Murase T, Shibata S, et al. (1987). Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis in 3T3-L1 cells. *J Biochem.* 101(2):331-38

Kelly I, Han T, Walsh K, Lean M. (1999). Effects of a thiazolidinedione compound on body fat and fat distribution of patients with type 2 diabetes. *Diabetes Care.* 22(2):288-93

Keller H, Dreyer C, Medin J, Mahfoudi A, Ozato K, Wahli W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci.* USA. 90(6):2160-4

Kim J, Spiegelman B. (1996). ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.* 10:1096-107

Kim J, Spotts G, Spiegelman B, et al. (1995). Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. *Mol Cell Biol.* 15:2582-8

Kim J, Wright H, Wright M, Spiegelman B. (1998). ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand. *Proc Nati Acad Sci.* USA. 95(8):4333-7

Kim J, Sarraf P, Spiegelman B, et al. (1998). Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest.* 101(1):1-9

Kirkland J, Hollenberg C, Gillon W. (1990).Age, anatomic site, and the replication and differentiation of adipocyte precursors. *Am J Physiol.* 258(Cell Physiol. 27):C206-C210

Kliewer S, Umesono K, Mangelsdorf D, Evans R. (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature.* 355(6359):446-9

Kliewer S, Sundseth S, Lehmann J, et al. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci.* USA. 94(9):4318-23

Kliewer S, Lenhard J, Willson T, Patel I, Morris D, Lehmann J. (1995). A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell.* 83(5):813-9

Kohn A, Summers S, Birnbaum M, Roth R. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem.* 271(49):31372-8

Koo S, Flechner L, Montminy M, et al. (2005). The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature.* 437(7062):1109-11

Kristensen J, Treebak J, Schjerling P, Goodyear L, Wojtaszewski J. (2014). Two weeks of metformin treatment induces AMPK-dependent enhancement of insulin stimulated glucose uptake in mouse soleus muscle. *Am J Physiol Endocrinol Metab*. 306(10):1099-109

Kubota N, Terauchi Y, et. a, et al. (1999). PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell.* 4(4):597-609

Kulik G, Klippel A, Weber M. (1997).Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. *Mol Cell Biol.* 17(3):1595-606

Kuri-Harcuch W, Wise L, Green H. (1978). Interruption of the adipose conversion of 3T3 cells by biotin deficiency: differentiation without triglyceride accumulation. *Cell.*14:53-9

Khayat Z, Tong P, Yaworsky K, Bloch R, Klip A. (2000). Insulin-induced actin filament remodeling colocalizes actin with phosphatidylinositol 3-kinase and GLUT4 in L6 myotubes. *J Cell Sci.* 113(2):279-90

Kramer H, Witczak C, Taylor E, Fujii N, Hirshman M, Goodyear L. (2006). AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem.* 281(42):31478-85

Kusminski C, Shetty S, Orci L, Unger R, Scherer P. (2009). Diabetes and apoptosis: lipotoxicity. *Apoptosis.*14(12):1484-95

Kuerschner L, Moessinger C, Thiele C. (2008). Imaging of lipid biosynthesis: how a neutral lipid enters lipid droplets. *Traffic.* 9(3):338-52

Landschulz W, Johnson P, Adashi E, Graves B, McKnight S. (1988).Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* 2(7):786-800

Landschulz W, Johnson P, McKnight S. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science.*243:1681-8

Lane M, Tang Q, Jiang M. (1999). Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem Biophys Res Commun*. 266(3):677-83

Lane K, Beese L. (2006). Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J Lipid Res*. 47(4):681-99

Lane K, Beese L. (2006). Thematic review series: lipid posttranslational modifications. Structural biology of protein farnesyltransferase and geranylgeranyltransferase type I. *J Lipid Res.* 47(4):681-99

Lane M, Lin F, MacDougald O, Vasseur-Cognet M. (1996).Control of adipocyte differentiation by CCAAT/enhancer binding protein alpha (C/EBP alpha). *Int J Obesity Related Metab Disorders.* 20 Suppl 3:S91-S96

Lai E, Rosen O, Rubin C. (1982). Dexamethasone regulates the beta-adrenergic receptor subtype expressed by 3T3 L1 preadipocytes and adipocytes. *J Biol Chem.* 257:6691-6

Larance M, Ramm G, James D, et al. (2005). Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. *J Biol Chem.* 280(45):37803-13

Lee Y, Wang M, Kakuma T, Wang Z, Babcock E, et al. (2001). Liporegulation in diet-induced obesity: the antisteatotic role of hyperleptinemia. *J Biochem.* 276:5629-35

Lehmann J, Moore L, Smith-Oliver T, Wilkison W, Willson T, Kliewer S. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem.* 270(22):12953-6

Leonardini A, Laviola L, Perrini S, Natalicchio A, Giorgino F. (2009). Cross-Talk between PPARgamma and Insulin Signaling and Modulation of Insulin Sensitivity. *PPAR Research*. :818-945

Li Y, Xu S, Zang M, et al. (2011). AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metab.*13(4):376-88

Liliom K, Tsukahara T, Tsukahara R, Zelman-Femiak M, Swiezewska E, Tigyi G. (2006). Farnesyl phosphates are endogenous ligands of lysophosphatidic acid receptors: inhibition of LPA GPCR and activation of PPARs. Biochimica Et Biophysica Acta (BBA)-*Mol. Cell Biol Lipids.*1761(12):1506-14

Lin F, Lane M. (1992). Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev.* 6:533-44

Lin F, Lane M. (1994). CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc Natl Acad Sci.* USA. 91:8757-61

Litthauer D, Serrero G. (1992). The primary culture of mouse adipocyte precursor cells in defined medium. *Comp Biochem Physiol A Physiol*. 101:59-64

Lizcano J, Göransson O, Alessi D, et al. (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* 23(4):833-43

MacDougald O, Lane M. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Annu Rev Biochem.* 64:345-73

Maggs D, Buchanan T, Shulman G, et al. (1998). Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med.* 128(3):176-85

Magun R, Burgering B, Sorisky A, et al. (1996). Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. *Endocrinology*. 137(8):3590-3

Mandrup S, Lane M. (1997). Regulating adipogenesis. *J Biol Chem.* 272:5367-70

Martin G, Schoonjans K, Lefebvre A, Staels B, Auwerx J. (1997). Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *J Biol Chem.* 272(45):28210-7

Masuzaki H, Ogawa Y, et al. (1995). Human obese gene expression.Adipocyte-specific expression and regional differences in the adipose tissue. *Diabetes.*44:855-8

Mäuser W, Perwitz N, Meier B, Fasshauer M, Klein J. (2007). Direct adipotropic actions of atorvastatin: differentiation state-dependent induction of apoptosis, modulation of endocrine function, and inhibition of glucose uptake. *Eur J Pharmacol.* 564(1-3):37-46

McGarry J, Mannaerts G, Foster D. (1977). A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest.* 60(1):265-70

McGee S, van Denderen B, Hargreaves M, et al. (2008).AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. *Diabetes.* 57(4):860-7

Meigs, T. E., Roseman, D. S., & Simoni, R. D. (1996). Regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation by the nonsterol mevalonate metabolite farnesol in vivo. *J Biol Chem.* 271(14), 7916-22

Miller W, Faust I, Hirsch J. (1984). Demonstration of de novo production of adipocytes in adult rats by biochemical and radioautographic techniques. *J Lipid Res.* 25(4):336-47

Minokoshi Y, Kim Y, Kahn B, et al. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature.* 415(6869):339-43

Mo H, Tatman D, Jung M, Elson C. (2000). Farnesyl anthranilate suppresses the growth, in vitro and in vivo, of murine B16 melanomas. *Cancer Letters*, 157(2), 145-53

Mo H, Elson C. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. (2004). *Exp Biol Med.* (Maywood, N.J.);229(7):567-85

Montague C, Farooqi I, Whitehead J, Soos M, Rau H, et al. (1997). Congenital leptin deficiency is associated with severe early onset obesity in humans. *Nature.* 387:903-8

Mori Y, Murakawa Y, Ikeda Y, et al. (1999). Effect of troglitazone on body fat distribution in type 2 diabetic patients. *Diabetes Care.* 22(6):908-12

Morrison R, Farmer S. (2000). Hormonal signaling and transcriptional control of adipocyte differentiation. *J Nutr.* 130(12):3116S-3121S

Murphy D. (2001).The biogenesis and functions of lipid bodies in animals, plants and microorganisms.*Prog. Lipid Res.* 40(5):325-438

Nishio E, Tomiyama K, Nakata H, Watanabe Y. (1996). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor impairs cell differentiation in cultured adipogenic cells (3T3-L1). *Euro J Pharmacol.* 301(1-3):203-6

Nakanishi M, Goldstein J, Brown M. (1988). Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. *J Biol Chem.* 263(18):8929-37

Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. (2006). Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control. *Diabetologia.* 49(8):1881-92

Nicholson A, Hajjar D, Zhou X, He W, Gotto A, Han J. (2007). Anti-adipogenic action of pitavastatin occurs through the coordinate regulation of PPARgamma and Pref-1 expression. *Br J Pharmacol.*151(6):807-15

Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. (2006). Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control. *Diabetologia*. 49(8):1881-92

Nawrocki A, Rajala M, Scherer P, et al. (2006). Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem.* 281(5):2654-60

Nishio E, Tomiyama K, Nakata H, Watanabe Y. (1996). 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor impairs cell p4fferentiation in cultured adipogenic cells (3T3-L1). *Euro J Pharmacol.* 301(1-3):203-6

Nicholson A, Hajjar D, Zhou X, He W, Gotto A, Han J. (2007). Anti-adipogenic action of pitavastatin occurs through the coordinate regulation of PPARgamma and Pref-1 expression. *Br J Pharmacol.* 151(6):807-15

Ntambi J, Young-Cheul K. (2000).Adipocyte differentiation and gene expression. *J Nutr.* 130(12):3122S-3126S

Nolan J, Ludvik B, Beerdsen P, Joyce M, Olefsky J. (1994). Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med.* 331(18):1188-93

Park S, Kim M, Ahn J, Lee S, Lee J, Eum W, Choi S, Kwon H. (2014). The Stimulatory Effect of Essential Fatty Acids on Glucose Uptake Involves Both Akt and AMPK Activation in C2C12 Skeletal Muscle Cells. Korean J Physiol Pharmacol. 18(3):255-61

Poissonnet C, LaVelle M, Burdi A. (1988). Growth and development of adipose tissue. *J Pediatr.* 113:1-9

Poissonnet C, Burdi A, Bookstein F. (1983). Growth and development of human adipose tissue during early gestation. *Early Hum. Dev.* 8:1-11

Patel YM, Lane MD. (2000). Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J Biol Chem.* 275:17653-60

Patel N, Rudich A, Khayat Z, Garg R, Klip A. (2003). Intracellular segregation of phosphatidylinositol-3,4,5-trisphosphate by insulin-dependent actin remodeling in L6 skeletal muscle cells. *Mol Cell Biol.* 23(13):4611-26

Pajvani U, Hawkins M, Scherer P, et al. (2004). Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem.* 279(13):12152-62

Prusty D, Park B, Davis K, Farmer S. (2002).Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. *J Biol Chem.* 277(48):46226-32

Puigserver P, Wu Z, Park C, Graves R, Wright M, Spiegelman B. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell.* 92(6):829-39

Peruzzi F, Prisco M, Baserga R, et al. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol.* 19:7203-15

Peng X, Xu P, Hay N, et al. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev.*17(11):1352-65

Parker R, Pearce B, Clark R, Gordon D, Wright J. (1993).Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol Chem.* 268(15):11230-8

Payne V, Au W, Rochford J, et al. (2009). C/EBP transcription factors regulate SREBP1c gene expression during adipogenesis. *Biochem J.* 425(1):215-23

Pairault J, Green H. (1979). A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc Natl Acad Sci.* USA. 76:5138-42

Prins JB and O'Rahilly S. (1997). Regulation of adipose cell number in man. *Clin Sci.* (Lond.) 92:3–11

Qureshi AA, Sami SA, Salser WA, Khan FA.(2001). Synergistic effect of tocotrienol-rich fraction (TRF25) of rice bran and lovastatin on lipid parameters in hypercholesterolemic humans. *J Nutr Biochem.* 12(6): 318-29

Qiu Z, Wei Y, Chen N, Jiang M, Wu J, Liao K. (2001). DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J Biol Chem.* 276(15):11988-95

Rosen E, Spiegelman B. (2000). Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol.* 16:145-71

Rosen E, Sarraf P, Mortensen R, et al. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell.*4(4):611-7

Rosen E, Hsu C, Wang X, Sakai S, Freeman M, Gonzalez F, Spiegelman B. (2002). C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev.* 16(1):22-6

Robelin J, Barboiron C, Jailler R. (1985). [Cellularity of different adipose deposits in growing cattle]. *Reprod Nutr Dev.* 25:211-4

Ramírez-Zacarías J, Castro-Muñozledo F, Kuri-Harcuch W. (1992). Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochem.* 97(6):493-7

Ricote M, Li A, Willson T, Kelly C, Glass C. (1998). The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature.* 391(6662):79-82

Rizzo M, Romero G. (1998). The role of G proteins in insulin signalling. *J Basic Clin Physiol Pharmacol.* 9(2-4):167-95

Ren D, Collingwood T, Rebar E, Wolffe A, Camp H. (2002). PPARgamma knockdown by engineered transcription factors: exogenous PPARgamma2 but not PPARgamma1 reactivates adipogenesis. *Genes Dev.* 16(1):27-32

Rangwala S, Lazar M. (2004). Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. *Trends Pharmacol Sci.* 25(6):331-6

Sano H, Eguez L, McGraw T, et al. (2007). Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metab.* 5(4):293-303

Sano H, Kane S, Lienhard G, et al. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem.* 278(17):14599-602

Satoh S, Nishimura H, Holman G, et al. (1993). Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *J Biol Chem.* 268(24):17820-9

Schmidt A, Hall A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* 16(13):1587-609

Schmidt A, Hall A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev.* 16(13):1587-609

Schoonjans K, Peinado-Onsurbe J, Auwerx J, et al. (1996). PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* 15(19):5336-48

Sears I, MacGinnitie M, Kovacs L, Graves R. (1996). Differentiation-dependent expression of the brown adipocyte uncoupling protein gene: regulation by peroxisome proliferator-activated receptor gamma. *Mol Cell Biol.* 16(7):3410-9

Seo J, Moon H, Kim J, et al. (2004). Adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c regulates mouse adiponectin expression. *J Biol Chem.* 279(21):22108-17

Serino M, Menghini R, Federici M, et al. (2007). Mice heterozygous for tumor necrosis factor-alpha converting enzyme are protected from obesity-induced insulin resistance and diabetes. *Diabetes.* 56(10):2541-546

Serrero G, Mills D. (1987). Differentiation of newborn rat adipocyte precursors in defined serum-free medium. *In Vitro Cell Dev Biol.*23(1):63-6

Sever N, Song B, Yabe D, Goldstein J, Brown M, DeBose-Boyd R. (2003). Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem.* 278:52479-90

Shang W, Yang Y, Zhou L, Jiang B, Jin H, Chen M. (2008).Ginsenoside Rb1 stimulates glucose uptake through insulin-like signaling pathway in 3T3-L1 adipocytes. *J Endocrinol.*198(3):561-9

Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F, Kahn BB. (1993). Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *J Biol Chem.* 268:22243-46

Sims, E. A. et al. Experimental obesity in man. (1968). *Trans Assoc Am Physicians.* 81:153–70

Smas C, Sul H. (1995). Control of adipocyte differentiation. *Biochem. J.* 309:697-710

Smas C, Chen L, Sul H. (1997). Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. *Mol Cell Biol.*17:977-88

Smith P, Wise L, Berkowitz R, Wan C, Rubin C. (1988). Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem.* 263(19):9402-8

Spalding K, Arner E, Westermark P, Bernard S, Buchholz B, Bergmann O, Blomqvist L, Hoffstedt J, &... Arner P. (2008). Dynamics of fat cell turnover in humans. *Nature.* 453(7196):783-787.

Spalding K, Bhardwaj R, Buchholz B, Druid H, Frisen J. (2005). Retrospective birth dating of cells in humans. *Cell.* 122:133–43

Spiegelman B, Choy L, Hotamisligil G, Graves R, Tontonoz P. (1993). Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. *J Biol Chem.* 268(10):6823-6

Spiegelman B, Green H. (1981). Cyclic AMP-mediated control of lipogenic enzyme synthesis during adipose differentiation of 3T3 cells. *Cell.* 24:503-10

Spiegelman B, Ginty C. (1983). Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes.*Cell.* 35:657-66

Spiegelman B, Farmer S. (1982). Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. *Cell.* 29(1):53-60

Spiegelman B, Frank M, Green H. (1983). Molecular cloning of mRNA from 3T3 adipocytes.Regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. *J Biol Chem.* 258(16):10083-9

Spiegelman B, Ginty C. (1983). Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell.* 35(3 Pt 2):657-66

Spiegelman B, Green H. (1981).Cyclic AMP-mediated control of lipogenic enzyme synthesis during adipose differentiation of 3T3 cells. *Cell* 24 (2): 503-10

Spinella-Jaegle S, Rawadi G, Kawai S, Gallea S, Faucheu C, et al. (2001). Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *J Cell Sci.* 114:2085-94

Student A, Hsu R, Lane M. (1980).Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem.* 255(10):4745-50

Stark W, Blaskovich M, Davies P, et al. (1998). Inhibiting geranylgeranylation blocks growth and promotes apoptosis in pulmonary vascular smooth muscle cells. *Am J Physiol.* 275(1 Pt 1):L55-L63

Stephens J, Pekala P. (1991). Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. *J Biol Chem.* 266(32):21839-45

Stephens J, Pekala P. (1991). Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. *J Biol Chem.* 266(32):21839-45

Stephens J, Pekala P. (1992). Transcriptional repression of the C/EBP-alpha and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. Regulation is coordinate and independent of protein synthesis. *J Biol Chem.* 267(19):13580-4

Schawarz EJ, Reginato MJ, Shao D, Krakow SL, Lazar MA.(1997). Retinoic acid blocks adipogenesis by inhibiting C/EBPbeta-mediated transcription. *Mol Cell Biol.* 17:1552-61

Student A, Hsu R, Lane M. (1980).Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem.* 255(10):4745-50

Sorisky A, Pardasani D, Lin Y. (1996). The 3-phosphorylated phosphoinositide response of 3T3-L1 preadipose cells exposed to insulin, insulin-like growth factor-1, or platelet-derived growth factor. *Obesity Res.* 4(1):9-19

Suryawan A, Swanson L, Hu C. (1997). Insulin and hydrocortisone, but not triiodothyronine, are required for the differentiation of pig preadipocytes in primary culture. *J Anim Sci.* 75:105-11

Tan D, Khor H, Low W, Ali A, Gapor A. (1991). Effect of a palm-oil vitamin E concentrate on the serum and lipoprotein lipids in humans. *Am J Clin Nutr*. 53(4): 10275-305

Tanaka T, Yoshida N, Kishimoto T, Akira S. (1997). Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *EMBO J.* 16(24):7432-43

Tang Q, Lane M. (2012). Adipogenesis: from stem cell to adipocyte. *Annu Rev Biochem.* 81:715-36

Tang Q, Otto T, Lane M. (2003). Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci.* USA. 100(1):44-9

Tang Q, Grønborg M, Lane M, et al. (2005). Sequential phosphorylation of CCAAT enhancer-binding protein beta by MAPK and glycogen synthase kinase 3beta is required for adipogenesis. *Proc Natl Acad Sci.* USA. 102(28):9766-71

Tengku-Muhammad T, Hughes T, Cryer A, Ramji D. (1996). Differential regulation of lipoprotein lipase in the macrophage J774.2 cell line by cytokines. *Cytokine.* 8:525-33

Thong F, Bilan P, Klip A. (2007). The Rab GTPase-activating protein AS160 integrates Akt, protein kinase C, and AMP-activated protein kinase signals regulating GLUT4 traffic. *Diabetes.* 56(2):414-23

Togashi N, Ura N, Higashiura K, Murakami H, Shimamoto K. (2002).Effect of TNF-alpha--converting enzyme inhibitor on insulin resistance in fructose-fed rats. *Hypertens.* 39(2 Pt 2):578-80

Tomiyama K, Nakata H, Sasa H, Arimura S, Nishio E, Watanabe Y. (1995). Wortmannin, a specific phosphatidylinositol 3-kinase inhibitor, inhibits adipocytic differentiation of 3T3-L1 cells. *Biochem Biophys Res Commun*. 212(1):263-69

Tomiyama K, Nishio E, Watanabe Y. (1999). Both wortmannin and simvastatin inhibit the adipogenesis in 3T3-L1 cells during the late phase of differentiation. *Jpn J Pharmacol.* 80(4):375-8

Tontonoz P, Hu E, Graves R, Budavari A, Spiegelman B. (1994).mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* 8(10):1224-34

Tontonoz P, Hu E, Devine J, Beale E, Spiegelman B. (1995). PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol.* 15:351-7

Tontonoz P, Kim JB, Graves RA, Spigelman BM. (1993). ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol Cell Biol.* 13:4753-59

Tontonoz P, Hu E, Graves R, Budavari A, Spiegelman B. (1994). mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* 8(10):1224-34

Tontonoz P, Spiegelman B. (2008). Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem.* 77:289-312

Torti F, Dieckmann B, Beutler B, Cerami A, Ringold G. (1985). A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science.* 229(4716):867-9

Traber M. (2007). Vitamin E regulatory mechanisms. *Annu Rev Nutr.* 27:347-62

Tzameli I, Fang H, Flier J, et al. (2004). Regulated production of a peroxisome proliferator-activated receptor-gamma ligand during an early phase of adipocyte differentiation in 3T3-L1 adipocytes. *J Biol Chem.* 279(34):36093-102

Umek R, Friedman A, McKnight S. (1991). CCAAT-enhancer binding protein: a component of a differentiation switch. *Science.* 251(4991):288-92

Unger R, Clark G, Scherer P, Orci L. (2010). Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta.* 1801(3):209-14

Unger R. (1995). Lipotoxicity in the pathogenesis of obesity-dependent NIDDM: genetic and clinical implications. *Diabetes.* 44(8):863-8

Unger R, Zhou Y. (2001) Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes.* 50 Suppl 1:S118-S121

Unger R,Orci L. (2001) Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J.* 15(2): 312-21

Unger R. (2003) The physiology of cellular liporegulation. *Annu Rev Physiol.* 65:333-47

Unger R, Scherer P. (2010) Gluttony, sloth and the metabolic syndrome: a roadmap to lipotoxicity. *Trends Endocrinol Metab. TEM.* 21(6):345-52

Uto-Kondo H, Ohmori R, Kiyose C, Kishimoto Y, Saito H, Igarashi O, Kondo K. (2009). Tocotrienol suppresses adipocyte differentiation and Akt phosphorylation in 3T3-L1 preadipocytes. *J Nutr.*139(1):51-7

Uysal K, Wiesbrock S, Marino M, Hotamisligil G. (1997). Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature.* 389(6651):610-4

Van der Horst G, Farith-Sips H, Lowik CWGM, Karperien M. (2003).Hedgehog stimulates only osteoblastic differentiation of undifferentiated KS483 cells. *Bone.* 33:899-910

Vats V, Grover J, Rathi S.(2002). Evaluation of anti-hyperglycemic and hypoglycemic effect of Trigonella foenum-graecum Linn, Ocimum sanctum Linn and Pterocarpus marsupium Linn in normal and alloxanized diabetic rats. *J Endocrinol.* 79(1):95-100

Vertino AM, Taylor-Jones JM, Longo KA, Bearden ED, Lane TF, et al. (2005). Wnt10b deficiency promotes coexpression of myogenic and adipogenic programs in myoblasts. *Mol Biol Cell.* 16:2039-48

Wada T, Sasaoka T, Kobayashi M, et al. (2001). Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes via its 5'-phosphatase catalytic activity. *Mol Cell Biol*. 21(5):1633-46

Wang Q, Somwar R, Klip A, et al. (1999). Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol.* 19(6):4008-18

Wang Z, Chen H, Roa W, Finlay W. (2003). Farnesol for aerosol inhalation: Nebulization and activity against human lung cancer cells. *J Pharm Pharm Sci*. 6(1), 95-100

Watson R, Khan A, Pessin J, et al. (2004). Entry of newly synthesized GLUT4 into the insulin-responsive storage compartment is GGA dependent. *EMBO J.* 23(10):2059-70

Weiner F, Shah A, Smith P, Rubin C, Zern M. (1989). Regulation of collagen gene expression in 3T3-L1 cells.Effects of adipocyte differentiation and tumor necrosis factor alpha. *Biochemistry.* 28:4094-99

Williams S, Cantwell C, Johnson P. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev.* 5:1553-67

Willumsen B, Christensen A, Hubbert N, Papageorge A, Lowy D. (1984). The p21 ras C-terminus is required for transformation and membrane association. *Nature.* 310(5978):583-6

Wu S, Huang G, Ng L. (2013). γ-Tocotrienol induced cell cycle arrest and apoptosis via activating the Bax-mediated mitochondrial and AMPK signaling pathways in 3T3-L1 adipocytes. *Food Chem Toxicol.* 59:501-13

Wu Z, Bucher N, Farmer S. (1996). Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Mol Cell Biol.* 16:4128-36

Wu Z, Xie Y, Bucher N, Farmer S. (1995). Conditional ectopic expression of C/EBP beta in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. *Genes Dev.* 9:2350-63

Xu J, Liao K. (2004). Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J Biol Chem.* 279(34):35914-22

Xu H, Uysal K, Becherer J, Arner P, Hotamisligil G. (2002). Altered tumor necrosis factor-alpha (TNF-alpha) processing in adipocytes and increased expression of transmembrane TNF-alpha in obesity. *Diabetes.* 51(6):1876-83

Yamauchi T, Kamon J, Kadowaki T, et al. (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine.*7(8):941-6

Yamauchi T, Kamon J, Kadowaki T, et al. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med.* 8(11):1288-95

Yang T, Espenshade P, Brown M, et al. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell.*110(4):489-500

Yano M, Matsumura T, Araki E, et al. (2007). Statins activate peroxisome proliferator-activated receptor gamma through extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase-dependent cyclooxygenase-2 expression in macrophages. *Circ Res.* 100(10):1442-51

Ye J, Dzamko N, Kraegen E, et al. (2004). Direct demonstration of lipid sequestration as a mechanism by which rosiglitazone prevents fatty-acid-induced insulin resistance in the rat: comparison with metformin. *Diabetologia*. 47(7):1306-13

Yellaturu C, Deng X, Park E, Raghow R, Elam M. (2009).Insulin enhances the biogenesis of nuclear sterol regulatory element-binding protein (SREBP)-1c by posttranscriptional down-regulation of Insig-2A and its dissociation from SREBP cleavage-activating protein (SCAP).SREBP-1c complex. *J Biol Chem.* 284(46):31726-34

Yu J, Javorschi S, Olefsky J, et al. (2002). The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes.* 51(10):2968-74

Zahorska-Markiewicz B, Janowska J, Olszanecka-Glinianowicz M, Zurakowski A. (2000). Serum concentrations of TNF-alpha and soluble TNF-alpha receptors in obesity. *Int J Obes Relat Metab Disord.* 24(11):1392-5

Zaiden N, Yap WN, Ong S, et al. (2010). Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion. *J Atheroscler Thromb*. 17(10): 1019-32

Zehentner BK, Leser U, Burtscher H. (2000). BMP-2 and sonic hedgehog have contrary effects on adipocyte-like differentiation of C3H10T1/2 cells. *DNA Cell Biol.* 19:275-81

Zhang Y, Li X, Tang Q, et al. (2011). Transcriptional activation of histone H4 by C/EBPβ during the mitotic clonal expansion of 3T3-L1 adipocyte differentiation. *Mol Biol Cell.* 22(13):2165-74

Zhang F, Casey P. (1996). Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem.* 65: p. 241-69.

Zhou Y, Shimabukuro M, Unger R, et al. (1998). Enhanced de novo lipogenesis in the leptin-unresponsive pancreatic islets of prediabetic Zucker diabetic fatty rats: role in the pathogenesis of lipotoxic diabetes. *Diabetes.* 47(12):1904-8.