IN VITRO ANALYSIS OF *PPAR-γ* AND *FABP4* EXPRESSION IN 3T3-L1 CELLS DURING ADIPOCYTE DIFFERENTIATION

Dissertation submitted to the University of Kerala in partial fulfilment of the requirement for the award of the degree of

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CERTIFICATE

This is to certify that the dissertation entitled **'In Vitro Analysis of *PPAR-γ* and *FABP4* Expression in 3T3-L1 Cells During Adipocyte Differentiation'** submitted to the University of Kerala in partial fulfilment of the requirements for the award of the degree of Master of Science in Biochemistry, is a record of original research work carried out by **GOPIKA LEKSHMI N. (Candidate Code: 63824142005)** of the Department of Biochemistry, T.K.M. College of Arts and Science, Kollam under the guidance of **Prof. (Dr.) R. Rajalakshmi** and **Dr. Lekshmi N.,** Inter University Centre for Genomics and Gene Technology (IU-CGGT), University of Kerala, Kariavattom. It is also certified that no part of this work has been presented for the award of any other degree, diploma, or similar title at any other university.

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Declaration

I hereby declare that the thesis titled **'In Vitro Analysis of *PPAR-γ* and *FABP4* Expression in 3T3-L1 Cells During Adipocyte Differentiation'** is based on original work carried out by me under the guidance and supervision of **Prof. (Dr.) R. Rajalakshmi** and **Dr. Lekshmi N.,** in the Inter University Centre for Genomics and Gene Technology (IU-CGGT), University of Kerala. I further declare that no part of this work has been presented for the award of any other degree, diploma, or similar title from any other university.

GOPIKA LEKSHMI N.

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Abbreviations

aP2	Adipocyte Protein 2
BAT	Brown Adipose tissue
WAT	White Adipose tissue
cAMP	Cyclic Adenosine Monophosphate
C/EBP	*CCAAT*/ Enhancer binding Protein
CRE	Cyclic AMP Response Element
CREB	Cyclic AMP Response Element Binding Protein
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EtBr	Ethidium Bromide
FABP4	Fatty Acid Binding Protein 4
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT1	Glucose transporter-1
GLUT4	Glucose transporter-4
KLF	Kruppel Like Factor
MAPK	Mitogen-Activated Protein Kinase
$NaHCO_3$	Sodium Bicarbonate
PBS	Phosphate Buffered Saline
PKA	Protein Kinase A
PPAR-γ	Peroxisome Proliferator-ActivatedReceptor Gamma
Pref-1	Preadipocyte Factor 1
RXR	Retinoid X Receptor
PFA	Paraformaldehyde
RT-qPCR	Quantitative Real Time PCR
TAE	Tris-Acetate-EDTA buffer

Abstract

Adipogenesis is a highly regulated process through which precursor stem cells differentiate into lipid laden adipocytes. Dysregulation of adipogenesis damages the healthy adipose tissue expansion characterized by increased adipocyte size and number, contributing to the development of obesity and related metabolic disorders. The present study aimed to evaluate the temporal expressional patterns of Peroxisome Proliferator-Activated Receptor Gamma (*PPAR-γ*) and Fatty Acid Binding Protein 4 (*FABP4*) using mouse 3T3-L1 preadipocyte cells. Cells were induced using a standard differentiation cocktail containing IBMX, Dexamethasone, and Insulin (MDI) and were monitored microscopically for any morphological changes including lipid droplet accumulation throughout the stages of differentiation.Oil Red O staining of lipid droplets on Day 12 further validated the efficient maturation of adipocytes. The relative gene expression analysis of *PPAR-γ* and *FABP4* demonstrated a progressive upregulation across the differentiation timeline confirming their role in the maturation of adipocytes. However, further studies are necessary to understand the molecular mechanisms of *PPAR-γ* and *FABP4* gene regulation to develop therapeutic strategies for addressing obesity and related metabolic disorders.

Chapter 1

Introduction

The occurrence of obesity has increased at an unprecedented rate worldwide between 2020 and 2026, which has become a major global public health issue. It is presently the most prevalent causes of death rates worldwide, continuing to increase across all population groups and geographical areas (Kushwah *et al*., 2026). Obese patients are increasing day by day, which is characterized by an increase in adipose tissue. Excessive adipocyte hypertrophy and hyperplasia in obesity increase the expandability of adipose tissue, resulting in fibrosis, hypoxia, and altered adipokine secretion (Sun *et al*., 2011). It increases the risk of cancer, coronary heart disease, and stroke, is a major pathophysiological cause of metabolic syndrome, which is characterized by insulin resistance, hypertension, dyslipidaemia, and hyperglycaemia. Estimates indicate that the number of obese patients will continue to increase, reaching over 1.13 billion individuals by 2030 (Kushwah *et al*., 2026).

Adipose tissue is an extremely active endocrine and metabolic organ. In addition to mediating local autocrine and paracrine actions that affect food intake, energy expenditure, and the metabolism of carbohydrates and fats, adipocytes secrete a variety of substances that circulate blood and operate on distant tissues (Havel, 2002). Hormonal signalling, lipid metabolism, and energy storage are all regulated by adipose tissue, as an active endocrine organ. Insulin signalling pathways in peripheral tissues are disrupted by long-term exposure to excess free fatty acids, inflammatory mediators, and oxidative stress, which reduces glucose absorption and increases hepatic gluconeogenesis. Eventually, high insulin levels stimulate more adipogenesis, further increasing the risk of type 2 diabetes and continued weight gain (Czech, 2017). Excessive accumulation of fat in our body is a major reason for the development of adiposity or obesity. Therefore, studying the molecular mechanisms involved in adipocyte differentiation is important to evaluate the extent of adiposity and related diseases. Obesity can cause damage to DNA and disrupt the normal functioning of DNA repair mechanisms, which may result in higher rate of mutations and the conversion of normal tissues into cancerous ones (Wlodarczyk and Nowicka, 2019).

Adipogenesis is the process by which the fibroblast-like mesenchymal stem cells differentiate into round shaped lipid laden adipocytes and are involved in regulating energy metabolism

(Rosen and MacDougald, 2006). It involves mainly two stages: Commitment (determination) phase in which mesenchymal stem cells committed to adipocyte lineage forming preadipocytes and the termination differentiation phase in which preadipocytes differentiate in mature fat storing adipocytes (Ghaben and Scherer, 2019). The process involves mitotic clonal expansion in which preadipocytes undergoes several rounds of cell division, enabling the activation of the adipogenic specific genes necessary for the maturation of adipocytes and lipid accumulation. Mature adipocytes have many roles, involving lipid production, transporting fatty acids across their membranes, responding to insulin signals, and releasing adipokines (Lee and Fried, 2014).

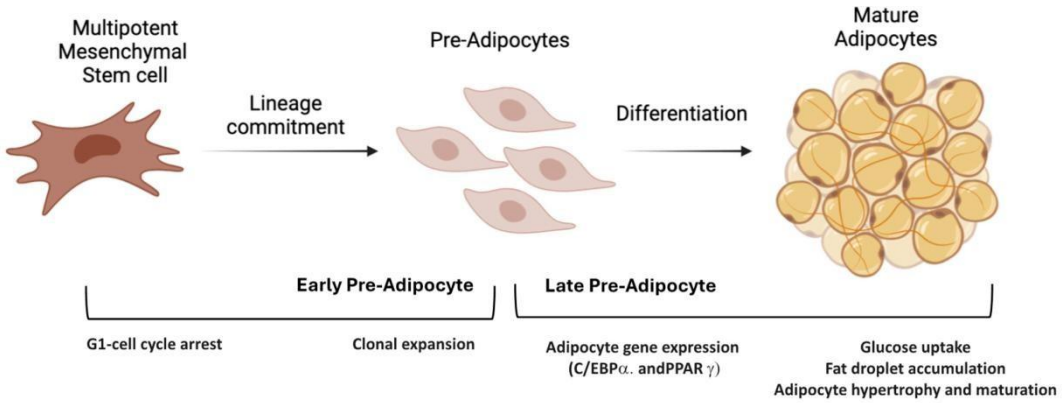


Figure 1: Stages of Adipogenesis (Giammona *et al*., 2024)

Hypertrophy and Hyperplasia of adipose tissue promote cAMP upregulation,where cAMP regulated activation of PKA result in the phosphorylation of CREB. The regulation of adipogenesis also appears to be a potential function of cyclic AMP response element-binding protein (CREB). By the activation of CREB it activates CRE of target genes like *C/EBP-β* and *C/EBP-δ* necessary for the terminal differentiation. *C/EBP-β* and *C/EBP-δ* are the early transcription factors that are activated when preadipocytes are differentiated into mature adipocytes. *C/EBPβ* might have a broader role in the development of adipocytes by helping pluripotent cells shift towards the adipocyte lineage also it has a role in mitotic clonal expansion. Disruption of the gene inhibits mitotic clonal proliferation, which is necessary for differentiation and supports the dependency of adipogenesis on *C/EBPβ* (Tang and Lane, 2012). Terminal adipocyte differentiation involves a sequence of transcriptional events. Both *C/EBP-β* and *C/EBP-δ* directly promote the expression of *PPAR-γ* and *C/EBP-α*, which are essential transcriptional regulators in adipocyte differentiation (Tang *et al*., 2005). *PPAR-γ* and *C/EBP-α* initiate a positive feedback loop that enhances their own expression and also activate numerous downstream target genes.

PPAR-γ is considered as a master regulator of adipocyte differentiation and maturation. It has three isoforms *PPAR-γ1*, *PPAR-γ2*, *PPAR-γ3*, *PPAR-γ2* is the main isoform specifically in adipocytes. Each isoform contains domain responsible for transactivation, DNA binding and dimerization. PPARs create heterodimers with the retinoid X receptors for binding to the peroxisome proliferator response elements in the target genes. The ectopic expression of *PPARγ2* in naive preadipocytes and non-adipogenic fibroblasts lead to the activation of genes associated with adipocytes and promotes their differentiation (Ghaben and Scherer, 2019)

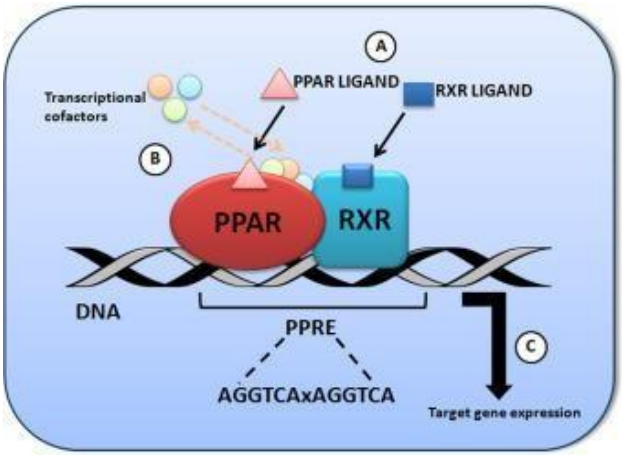


Figure 2: Molecular mechanism of *PPAR-γ* Mediated Transcriptional Activation (Rigano *et al*., 2017)

PPAR-γ2 isoform inhibits lipotoxicity by increasing the lipid buffering capacity of peripheral organs. Based on the actions of *PPAR-γ* on glucose and lipid metabolism they are suggested for the treatment of hyperglycaemia, dyslipidaemia etc. It plays a crucial role in the transcriptional activation of genes associated with adipogenesis including Fatty acid binding protein 4 *(FABP4)*, which help in the transport of fatty acids and increases insulin sensitivity by activating adipocyte-derived hormones such as adiponectin, regulate glucose metabolism by the translocation of *GLUT-4* which is a transporter protein helps in the transport of glucose across adipocytes. By the activation of various genes, it mainly regulates the intracellular lipid transport, glucose uptake and insulin sensitivity and facilitates lipid accumulation in fat cells. A balanced and sufficient production of adipocytokines (adiponectin and leptin), which are mediators of insulin action in peripheral tissues, is ensured by activated *PPAR-γ* in adipocytes, as a result, the body's overall insulin sensitivity is preserved (Kintscher and Law, 2005). Dysregulation of PPAR-Gamma (*PPAR-γ*) is associated with the development of obesity, type 2 diabetes, atherosclerosis, and other related diseases. Increasing *PPAR-γ* activity can improve metabolic parameters in type 2 diabetes and other chronic illnesses, whereas reduced activity of *PPAR-γ* has been found to have anti-obesity effects.

Fatty Acid Binding Protein 4 (*FABP4*), commonly referred to as adipocyte P2 (*aP2*), is a type of hormone-like substance secreted by mature adipocytes. Its main role is to help transport fatty acids through the membranes of cells. It plays a key role in maintaining the balance and metabolic functions of adipocytes. It achieves this by interacting with the peroxisome proliferator-activated receptor-γ (*PPAR-γ)*. *FABP4* primarily functions as a transporter within the cell (Schwenk *et al.,* 2010), by attaching to fatty acids (FAs) that enter the cell membrane. This process helps in transporting FAs inside the cell and contributes to the regulation of both glucose and lipid metabolism by increasing the levels of FAs. The primary role of *FABP4* is to control the metabolic processes of fatty acids, which can also serve as signalling molecules to influence the expression of FABP4 itself. In individuals who are obese, the levels of FABP4 in the blood are notably higher, likely due to increased production from larger fat tissues(Liu *et al*., 2022). FABP4 is recognized as a common risk factor for coronary heart disease and type 2 diabetes (T2D) (Zhao *et al.,* 2017). Experimental studies have demonstrated that a lack of FABP4 provides a significant protection against atherosclerosis (Erbay *et al.,* 2009). In addition, obese patients diagnosed with breast cancer exhibit significantly elevated levels of *FABP4* in their serum, which are linked to larger tumour sizes and a less favourable prognosis. *FABP4* levels are greater in metastatic ovarian tumors compared to the primary tumors, and it has been considered as a possible indicator of cancer spread.

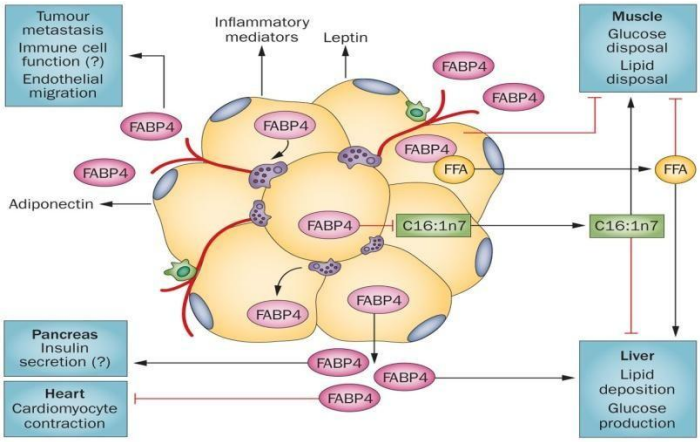


Figure 3: Metabolic functions of FABP4 (Hotamisligil and Bernlohr, 2015)

In vivo study of preadipocyte differentiation process in living organisms is very complex due to the various developmental processes. Because of this difficulty, in vitro cell models are used to understand the molecular mechanisms of adipogenesis. 3T3-L1 is one of the most frequently used preadipocyte clonal cell lines from mice. These cells are derived from disaggregated Swiss 3T3 mouse embryos that are approximately 17 to 19 days old, and they initially have a

fibroblast like appearance (Green and Meuth, 1974). A specific combination of adipogenic induction agents, such as Insulin (maturation promoter), Dexamethasone (activates glucocorticoid receptor), and 3-Isobutyl-1-Methylxanthine (IBMX) (inhibits phosphodiesterase and increases cyclic AMP levels), induce differentiation and results in the development of adipocyte-like characteristics in cell cultures. For these reasons, 3T3-L1 cell line has been widely accepted as a standard in vitro model for research on adipogenesis.

Accumulating evidence highlights that *PPAR-γ* and *FABP4* plays important role in lipid accumulation, fatty acid transport and adipocyte maturation. However, the precise temporal expression profiles of these genes in adipogenic differentiation remain to be fully characterized. Although *PPAR-γ* is the key regulator of adipogenesis that directly regulates the expression of *FABP4* which promote fatty acid transport and lipid accumulation, they are also interconnected through a regulatory loop; *FABP4* provides the ligands required for the *PPAR-γ* activity and subsequent lipid accumulation. Therefore, the present study aims to analyse the expression levels of *PPAR-γ* and *FABP4* during adipogenic differentiation using 3T3-L1 to evaluate their sequential relationship in promoting the differentiation of mature adipocyte phenotype. The evaluation of expression patterns in these genes will provide valuable implications in obesity-related diseases, Type II diabetes mellitus, cardiovascular diseases, metabolic syndromes and may contribute to the identification and development of potential drugs for the treatment of these diseases.

Chapter 2

Review of Literature

2.1 Adipogenesis

Adipogenesis is the process by which cells, fibroblasts or mesenchymal stem cells differentiate into mature fat cells, known as adipocytes capable of lipid storage and regulation of energy metabolism (Farmer, 2006; Song *et al.*, 2019). Studies (Tang and Lane, 2012) report that this process helps in the expansion of adipose tissue, which occurs through two main ways: an increase in the number of cells (hyperplasia) and an increase in the size of existing cells (hypertrophy) and this process includes several stages: the commitment of preadipocytes, cell growth arrest, mitotic clonal expansion, terminal differentiation, and developing into mature adipocytes. Mature adipocytes have many roles, involving producing lipids, transporting fatty acids across their membranes, responding to insulin signals, and releasing adipokines (Lee and Fried, 2014). Understanding the processes that drive adipogenesis and the factors that affect it is crucial for developing new treatments for obesity and related metabolic conditions (Abbas, 2017).

2.2 Adipose Tissue

Romacho *et al*. (2014) described that adipose tissue plays a crucial role in maintaining energy balance by actively storing and managing lipids, as well as producing and releasing a variety of hormone-like substances into the bloodstream, which affect metabolism throughout the body. Individuals with greater amounts of adipose tissue are more likely to develop metabolic disorders (Despres and Lemieux, 2006). White adipose tissue (WAT) and brown adipose tissue (BAT) are the two categories of adipose tissue, which makes up less than 5 % of body weight in some people and more than 60 % in others, is a highly malleable and dynamic tissue (Droyvold *et al*., 2006; Guo *et al*., 1999). The most prevalent kind of adipose tissue is white adipose tissue, which is made up of adipocytes with huge unilocular lipid droplets that are primarily used for energy storage and also helps to maintain energy homeostasis, and secretes adipokines like leptin and adiponectin (Sanchez-Gurmaches *et al*., 2016). WAT is a vital location for energy balance, insulin signalling, and endocrine function, and it makes up the bulk of the fat seen in adult humans (Farmer, 2008).

Brown adipose tissue (BAT) stores triglycerides in multilocular adipocytes, serving as readily accessible fuel for thermogenesis via the mitochondrial uncoupling of oxidative

phosphorylation of free fatty acids (FFA) (Cannon and Nedergaard, 2004; Kajimura *et al*., 2010; Betz and Enerback, 2015). Within WAT, there is a third type of adipose tissue called Brite/beige, which is caused by cold stress and contains adipocytes with BAT-like characteristics (Cousin *et al*., 1992). Brite adipocytes improve the control of glucose levels throughout the body and could be useful in the management of type 2 diabetes (Sanchez-Gurmaches *et al*., 2016).

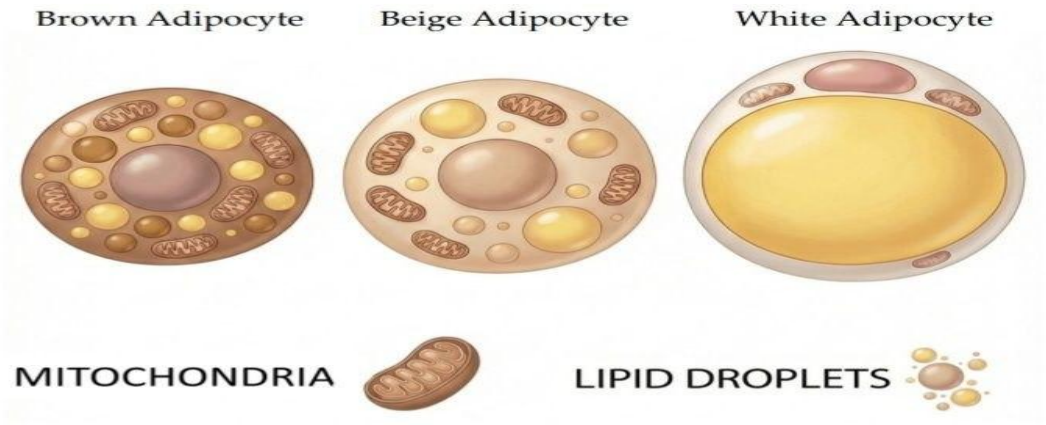


Figure 4: Types of Adipocytes (Sebaa, 2026)

2.3 Pathophysiology of Obesity

Excessive accumulation of body fat leads to various metabolic issues and diseases, including insulin resistance, atherogenic dyslipidaemia (characterized by high plasma triglycerides and low HDL cholesterol levels), Non-Alcoholic Fatty Liver Disease (NAFLD), beta-cell dysfunction, prediabetes, and type 2 diabetes. Generally, an increasing body mass index, which reflects the amount of body fat, is linked to a greater risk of developing type 2 diabetes (Colditz *et al*., 1995). However, the distribution of fat and triglycerides can influence the risk of metabolic complications caused by excessive body fat (Klein *et al*., 2004).

Triglycerides are stored in a compact form as an oil within adipocytes and yield about 9.3 kcal per gram when broken down, while glycogen is stored as a gel inside cells, with approximately 2 grams of water bound to each gram of glycogen, resulting in only 4.1 kcal per gram when metabolized (Klein *et al*., 2004). Men who are lean typically survive about 60 days of starvation when they lose more than 35 % of their body weight (Leiter and Marliss, 1982), whereas individuals with extreme obesity can endure extended periods of fasting; the longest recorded fast was by a man with severe obesity who fasted for 382 days, consuming only calorie-free

fluids, vitamins, and minerals, which led to a 60 % reduction in body weight without harmful effects (Stewart and Fleming, 1973).

Manolopoulos *et al*. (2010) studied that individuals who are obese with a higher concentration of fat in the upper body, including subcutaneous and visceral abdominal fat, intrahepatic triglycerides, intramyocellular lipids, and pancreatic fat, are at a greater risk of developing type 2 diabetes compared to those with a lower body fat pattern, primarily in the gluteofemoral region. In fact, greater gluteofemoral fat mass is associated with lower plasma triglyceride levels, increased HDL cholesterol, reduced fasting blood glucose and insulin levels, improved oral glucose tolerance and insulin sensitivity, and a lower risk of type 2 diabetes, regardless of whether a person is lean, overweight, or obese (Manolopoulos *et al*., 2010).

Fuster *et al*. (2017) reported that the body mass index of ≥30 kg/m² or higher is classified as obesity, which is now widely recognized as a major global health concern that has probably reached epidemic levels and condition is largely caused by changes in diet and worsened by a lack of physical activity. It also raises the risk of various health problems, including heart diseases, diabetes, and certain types of cancer, and is connected with a shorter lifespan. (Leung *et al*., 2017; Ligibel *et al*., 2014; Scherer and Hill, 2016). Obesity causes the fat tissue to grow larger, become dysfunctional, and inflamed, which in turn alters the structure and function of the cardiovascular system (Zhai and Haddad, 2017). This includes changes such as left ventricular remodelling, increased left ventricular mass, enlargement of the left atrium, and higher stroke volume (Cuspidi *et al*., 2014).

Koliaki *et al*. (2019) found that an increase in total and central blood volume, while reducing systemic vascular resistance, cardiac output, left ventricular filling pressure, and pulmonary arterial pressure. Additionally, when fat deposits occur in other organs outside of adipose tissue, it results in abnormal fat accumulation around the heart, which has been consistently linked to an increased risk of cardiovascular disease (Shimabukuro *et al*., 2013). It was recognized as the primary risk factor for type 2 diabetes mellitus (T2DM), and about one-third of individuals who are obese eventually develop T2DM (Bjerregaard *et al*., 2018; Chukir *et al*., 2018). Adults with a body mass index (BMI) higher than 35 kg/m² are 20 times more likely to develop T2DM compared to those with a BMI within the normal range of 18.5 to 24.9 kg/m² (Magkos *et al*., 2020; Malone and Hansen, 2019). It is widely thought that the main cause of T2DM involves obesity-induced insulin resistance in non-fat tissues, along with an inadequate ability of pancreatic beta cells to produce enough insulin to counteract this resistance (Rohm *et al*., 2022).

2.4 Obesity and Associated Diseases

The International Agency for Research on Cancer has identified several types of cancer linked to overweight and obesity, such as postmenopausal breast cancer, endometrial cancer, renal cell carcinoma, oesophageal adenocarcinoma, pancreatic cancer, colorectal cancer, and liver cancer (Wlodarczyk and Nowicka, 2019). Approximately 55 % of all cancers diagnosed in women and 24 % of those diagnosed in men are connected to overweight and obesity (Arnold *et al*., 2015). Breast cancer is the second most common cause of death from cancer in women globally, and fat cells are the most common type of cells found in the surrounding tissue of human breast tumors (De Santis *et al*., 2017)

A meta-analysis of 82 studies on breast cancer, including 213,075 breast cancer survivors with 23,182 deaths, showed that the relative risk of mortality was 1.75 for premenopausal and 1.34 for postmenopausal breast cancer in obese women and an increase in BMI by 5 kg/m^2 before and after a cancer diagnosis was associated with an 18 % and 29 % higher risk of breast cancer mortality, respectively (Wlodarczyk and Nowicka, 2019). In this context, obesity was linked to poorer survival outcomes for breast cancer patients, regardless of when BMI was measured (Chan *et al*., 2014).

Obesity can cause damage to DNA and disrupt the normal functioning of DNA repair mechanisms, which may result in a higher rate of mutations and the conversion of normal tissues into cancerous ones and also leads to chronic inflammation, insulin resistance, and changes in gene expression, all of which can promote cancer cell proliferation, migration, resistance to apoptosis, and tumor angiogenesis (Wlodarczyk and Nowicka, 2019). Cozzo *et al*. (2018) and Strong *et al*. (2015) highlighted that fat cells work as hormone-producing cells by releasing hormones, growth factors, cytokines, and other substances, which can influence the environment around tumors. In patients with ovarian cancer, Zhang *et al*. (2018) found that fat around the kidneys is especially prone to this browning, and a thicker layer of this fat is linked to worse outcomes in terms of how long cancer remains under control.

Studies by Sundararajan *et al*. (2018), Higuchi *et al*. (2018), and Lin *et al*. (2018) together showed that exosomes from cancer cells help in sending signals between cells and are vital for the growth of tumors. In the case of fat tissue, Wang *et al*. (2018) found that exosomes released by fat cells can make atherosclerosis worse by encouraging the formation of new blood vessels. Yu *et al*. (2018) also showed that these exosomes can cause insulin resistance in muscle cells

by reducing the activity of a protein called *PPAR-γ*. Fat cells, which are good at storing and releasing free fatty acids (FFAs), can change the metabolism of cancer cells through small vesicles, making it easier for cancer cells to use FFAs (Lazar *et al*., 2022).

2.5 Cell Culture Models for Adipogenesis Research

Green and Kehinde (1974) reported that the most popular culture models are the 3T3-L1 and 3T3-F422A culture lines, which are generated from disaggregated Swiss 3T3 mouse embryos. The pluripotent fibroblasts (10T1/2, Balb/c 3T3, 1246, RCJ3.1, and CHEF/18 fibroblasts) may differentiate into many cell types and 5-azacytidine, an inhibitor of DNA methylation, may transform 10T1/2 fibroblasts, which were generated from C3H mouse embryos (Reznikoff *et al*., 1973), into pre-adipose, pre-muscle, and pre-cartilage tissue (Taylor and Jones, 1979). These multipotent fibroblasts serve as useful models for comprehending the processes that determine the individual cell fates. Additionally, TA1 (Chapman *et al*., 1984) and 30A5 preadipocytes (Konieczny and Emerson, 1984) derived from 10T1/2 fibroblasts treated with 5azacytidine have been used. Aspects of differentiation arising from hereditary obesity are studied using Ob1771 preadipocytes derived from genetically fat adult mice (Negrel *et al*., 1978). The effects of fatty acids on adipocyte development have been investigated using the 1256 cell line (Serrero *et al*., 1992).

2.6 Adipocyte Differentiation and the Molecular Mechanisms Involved

Pluripotent mesenchymal stem cells, which may differentiate into adipocytes, myocytes, chondrocytes, and osteocytes, are the source of adipocytes (Covas *et al*., 2008; Lin *et al*., 2010). During late embryonic development, preadipocytes that are present in humans start to differentiate into adipose tissue; most of this differentiation takes place soon after birth (Burdi *et al*., 1985). A "cocktail" of agents is used to induce differentiation, which starts a series of synchronized phases in the differentiation process. Treatment with insulin, a glucocorticoid, a substance that raises intracellular cAMP levels, and fetal bovine serum results in maximal differentiation (Student *et al*., 1980). In the adipogenic cocktail, IGF-1 can replace insulin (Smith *et al*., 1988). Traditionally, the glucocorticoid receptor pathway has been stimulated by the synthetic glucocorticoid agonist dexamethasone (DEX). Traditionally, the cAMPdependent protein kinase pathway is stimulated by the cAMP-phosphodiesterase methylisobutylxanthine (IBMX) inhibitor. This adipogenic mixture, which includes insulin, DEX, and IBMX, is frequently shortened to MDI (Ntambi and Kim, 2000). In vivo insulin resistance is known to be caused by glucocorticoids, and a prior work employing 3T3-L1 adipocytes discovered that

long-term dexamethasone administration reduced insulin-induced glucose absorption (Sakoda *et al*., 2000; Bazuine *et al*., 2004). Primary preadipocytes from young animals often only need high concentrations of insulin (1-10 μM) in serum-free media or low concentrations (1-10 nM) in the presence of fetal calf serum (Deslex *et al*., 1987; Gregoire *et al*., 1992; Hauner *et al*., 1989; Suryavan *et al*., 1997).

Different preadipocytes have a post confluent mitosis and subsequent growth halt about 24 hours after MDI induction (Bern Lohr *et al*., 1985). The cells divide and replicate their DNA at least once. The cells finish post confluent mitosis by day two of differentiation, at which point they go into an uncommon growth arrest known as GD (Scott *et al*., 1982). It is thought that mitosis is required to unravel DNA so that transcription factors can access regulatory response elements found in genes that control the phenotype of mature adipocytes (Cornelius *et al*., 1994). Cells are committed to forming adipocytes following the growth halt. For further differentiation, the growth arrest is necessary at day three, growth-arrested cells start to show late hallmarks of differentiation (Ntambi and Kim, 2000). By days five to seven, the cells had rounded up, accumulated fat droplets, resulting into terminally differentiated adipocytes (Ntambi and Kim, 2000).

Preadipocytes undergo many rounds of mitosis, known as mitotic clonal expansion and synchronously rejoin the cell cycle (Student *et al*., 1980). After leaving the cell cycle, the cells acquire the appearance and metabolic characteristics of adipocytes, lose their fibroblastic shape, and accumulate cytoplasmic triglycerides (Green and Kehinde, 1974; Student *et al*., 1980). An elevated rate of de novo lipogenesis and a coordinated increase in the expression of the fatty acid and triacylglycerol biosynthesis enzymes are strongly associated with triglyceride accumulation (MacDougald and Lane, 1995; Student *et al*., 1980; Coleman *et al*., 1978). The rapid change in cell shape from fibroblastic to spherical is the first characteristic of the adipogenesis process and the changes in the amount and kind of extracellular matrix (ECM) components as well as the quantity of cytoskeletal components coincide with these morphological changes (Gregoire *et al*., 1998). Cell shape change, adipocyte-specific gene expression, and lipid accumulation all depend on the plasminogen cascade mediating the proteolytic destruction of the stromal extracellular matrix of preadipocytes (Selvarajan *et al*., 2001).

2.7 Transcription Factors in Adipogenesis

2.7.1 *C/EBP-β* and *C/EBP-δ*

The initial stage of adipogenesis is marked by a temporary and significant increase in the levels of *C/EBP-β* and *C/EBP-δ,* which are triggered in laboratory settings by a hormonal differentiation cocktail (Ramji and Foka, 2002). These genes begin to accumulate within 24 hours after the start of adipogenesis and the cells re-enter the cell cycle, synchronizing their progression through the mitotic cell cycle (Tang *et al*., 2003). As the cells move from the G1 phase to the S phase, *C/EBP-β* is phosphorylated and activated in a sequence involving glycogen synthase kinase-3 β and mitogen-activated protein kinase (MAPK). Following this, both *C/EBP-β* and *C/EBP-δ* directly promote the expression of *PPAR-γ* and *C/EBP-α,* which are essential transcriptional regulators in adipocyte differentiation (Tang *et al*., 2005). *PPAR-γ* and *C/EBP-α* initiate a positive feedback loop that enhances their own expression and also activate numerous downstream target genes, whose expression is crucial for the development of adipocytes. By the second day of the differentiation process, *C/EBP-α* starts to accumulate and is then phosphorylated by cyclin D3, which inhibits cell proliferation and allows the cells to enter the final phase of adipogenesis (Wang *et al*., 2013). Both in vivo and in vitro studies have demonstrated that *C/EBPα*, *C/EBPβ*, and *C/EBPδ* are involved in the process of adipocyte differentiation. McKnight and his team were the first to show that these proteins are induced in a coordinated manner during adipogenesis (Cao *et al*., 1991). *C/EBPβ* is mainly activated by methylisobutylxanthine, while *C/EBPδ* is strongly induced by dexamethasone in the early stages of adipogenesis. *C/EBP-β* and *C/EBP-δ* work together to activate *C/EBPα* (Cao *et al*., 1991). Experiments that involved reducing the function of these proteins in 3T3-L1 and NIH3T3 cell lines confirmed their importance, as blocking either *C/EBP-β* or *C/EBP-δ* reduced the formation of fat cells (Yeh *et al*., 1995). Interestingly, studies where *C/EBP-α* was overexpressed showed that it alone could induce adipogenesis in cells that are capable of becoming fat cells, as well as in non-fat cell fibroblasts (Freytag *et al*., 1994; Yeh *et al*., 1995). In animal studies, mice that lacked *C/EBP-β* or *C/EBP-δ* showed problems in the development of fat tissue (Tanaka *et al*., 1997). Mice lacking *C/EBP-α* had significantly lower levels of fat accumulation (Wang *et al*., 1996; Flodby *et al*., 1996).

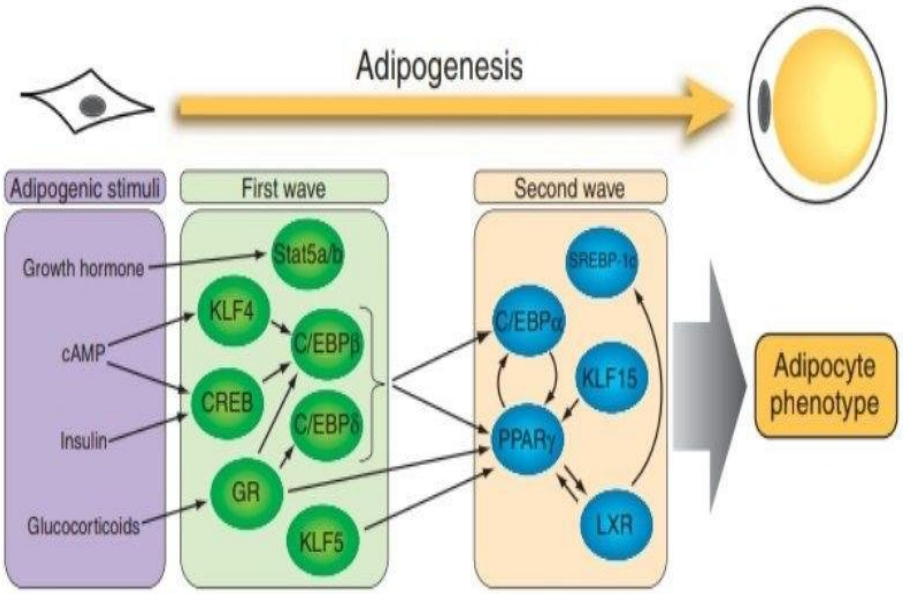


Figure 5: Transcriptional cascade of Adipocyte Differentiation (Siersbæk and Mandrup, 2011)

2.7.2 *PPAR-γ*

PPAR-γ is most expressed in adipocytes and is essential for controlling lipid production, energy balance, and adipogenesis (Kliewer *et al*., 1997; Sheu *et al*., 2005; Lehrke and Lazar, 2005; Medina-Gomez *et al*., 2007). All *PPAR-γ* isoforms are crucial for glucose metabolism and adipocyte development, *PPAR-γ2* is mostly expressed in adipose tissue, *PPAR -γ1* is found in almost all cells (Feige *et al*., 2006). On the other hand, *PPAR-γ2* is the isoform that is controlled in response to obesity and nutritional consumption (Werman *et al*., 1997) using *PPAR-γ*2 from genetically obese POKO mice reduces fat storage in adipocytes when compared to normal obese mice on the same diet, according to one study based on an animal model. Medina-Gomez *et al*. (2007) demonstrated that the *PPAR-γ2* isoform inhibits lipotoxicity through a variety of mechanisms, such as stimulating the growth of adipose tissue, increasing the lipid-buffering capacity of peripheral organs (liver, muscle, and pancreatic beta cells), and stimulating the proliferative response of b-cells to insulin resistance. Adipose *PPAR-γ* preserves normal organ function (liver and skeletal muscle) and shields non-adipose tissues from severe lipid accumulation (Janani and Kumari, 2015). A balanced and sufficient production of adipocytokines (adiponectin and leptin), which are mediators of insulin action in peripheral tissues, is ensured by activated *PPAR-γ* in adipocytes, as a result, the body's overall insulin sensitivity is preserved (Kintscher and Law, 2005). In addition to its adipogenetic action, *PPAR-γ* plays a significant role in lipid metabolism by controlling genes involved in the release,

transport, and storage of fatty acids, including lipoprotein lipase (LPL) and the fatty acid transporter CD36 (Lehrke and Lazar, 2005; Medina-Gomez *et al*., 2007; Ren *et al*., 2002)

2.7.3 *KLF*s

KLF5 expression is activated during the early phases of adipocyte differentiation and has been demonstrated to be essential for adipocyte differentiation both in laboratory settings and within living organisms and also *KLF5* small interfering RNA (siRNA) is introduced into 3T3-L1 preadipocytes, it leads to reduced differentiation of adipocytes and a significant decline in lipid accumulation (Oishi *et al*., 2005). *KLF15* is significantly upregulated during the differentiation of 3T3-L1 cells, and mutations that produce dominant-negative effects or RNA interference techniques that reduce its expression led to reduced adipocyte differentiation (Mori *et al*., 2005). These studies also revealed that when *KLF15* is artificially expressed, it can promote adipogenesis by enhancing the activation of *PPAR* in NIH-3T3 cells. *KLF15* also plays a critical role in the physiological function of adipocytes, as research has shown that *KLF15* interacts with the GLUT4 promoter, suggesting a connection between adipocyte insulin sensitivity and *KLF15* (Gray *et al*., 2002). Further examination indicated that the presence of KLF4 in adipose tissue is due to its high levels in preadipocytes within the stromal vascular fraction of white adipose tissue (WAT), as reported by Soukas *et al*. (2001). This led to the idea that *KLF4* might play a significant role in the process of adipogenesis. In 3T3-L1 cells, *KLF4* mRNA is expressed early during differentiation, and when its expression is reduced using siRNA, there is a significant decrease in lipid accumulation, as shown by Birsoy *et al*. (2008). Additionally, *KLF6* has been found to support adipogenesis. *KLF6* is activated during the differentiation of 3T3-L1 cells (Inuzuka *et al*., 1999), and research suggests that it helps in the development of adipocytes by reducing the expression of the delta-like protein-1 (DLK-1) gene in 3T3-L1 preadipocytes (Li *et al*., 2005). *KLF2* is artificially expressed in 3T3-L1 preadipocytes, it significantly interferes with lipid accumulation by reducing the expression of the *PPAR-γ2* gene (Banerjee *et al*., 2003), and also partially restores the expression of Pref-1 (Wu *et al*., 2005). The role of *KLF2* in adipocyte development is further supported by studies on mouse embryonic fibroblasts derived from *KLF2* knockout mice, which show increased lipid accumulation (Wu *et al*., 2005).

2.8 Adipocyte Marker Genes

2.8.1 GLUT1 and GLUT4

Adipocytes predominantly use two glucose transporter isoforms, GLUT1 and GLUT4, to move glucose. GLUT4 is an insulin-sensitive glucose transporter, whereas GLUT1 is a constitutive one. Both proteins are integral membrane proteins with like structural characteristics and under appropriate culture conditions, 3T3-L1 cells, which come from mouse embryonic tissue, differentiate from fibroblast-like preadipocytes into mature adipocytes (Green and Meuth, 1974; Green and Kehinde, 1976). In differentiation, GLUT1 is expressed in both fibroblastic and adipocytic types; GLUT4 expression is only seen in mature adipocytes (Kaestner *et al*., 1989; de Herreros and Birnbaum, 1989). GLUT1 is found throughout the plasma membrane and intracellular vesicular compartments in mature adipocytes, whereas GLUT4 is mostly found intracellularly under basal conditions and translocate to the plasma membrane following a strong insulin stimulus (Gould *et al*., 1989; Gibbs *et al*., 1990; Piper *et al*., 1992; Blok *et al*., 1988). It has been shown that long-term exposure to high insulin levels decreases GLUT4 expression at the mRNA and protein levels by reducing transcription and increasing mRNA turnover (Flores-Riveros *et al.*, 1993). Earlier studies reveal that roughly 23 nM insulin is necessary to produce a 50 % decrease in GLUT4 mRNA expression; this is significantly higher than the physiological level of insulin in human blood (Olefsky and Nolan, 1995).

The insulin-responsive glucose transporter 4 (GLUT4) is acknowledged as a key regulator of glucose uptake in tissues sensitive to insulin, including skeletal muscle and adipose tissue (Huang and Czech, 2007). GLUT4 is essential for maintaining glucose homeostasis by enabling insulin-stimulated glucose transport through cell membranes. GLUT4 recruitment to the cell surfaces of muscle and adipose cells is immediately stimulated by both insulin and exercise, independent of transcription or translation (Herman and Kahn, 2006; Rose and Richter, 2005). However, these two physiological stressors trigger different signalling pathways that result in increased glucose absorption and GLUT4 translocation. Recent studies have covered the insulin signalling route to GLUT4 in great depth (Bryant *et al*., 2002; Watson and Pessin, 2007).

More investigation showed that regular exposure of 3T3-L1 adipocytes to physiological insulin levels results in insulin resistance, which is characterized by lowered GLUT4 expression and glucose transport activity desensitization. About 100 pM insulin reduced glucose transport activity by 50 %, but 10 nM insulin resulted in the highest desensitization in 8 hours. Furthermore, it was found that glucose or glucosamine were crucial for the emergence of insulin

resistance (Garvey *et al*., 1987; Traxinger and Marshall, 1989; Traxinger and Marshall, 1991). Post-translational loss of GLUT4 is a major contributor to the start of insulin resistance in adipocytes, as seen by the link between decreased glucose transport activity and decreased GLUT4 protein expression and compromised GLUT4 translocation.

2.8.2 *FABP*4

Fatty acid-binding protein 4 (*FABP4*), a small protein that carries long-chain fatty acids and other hydrophobic substances, is involved in the exchange of metabolic signals between fat cells and cancer cells (Nieman *et al*., 2011). Nieman and colleagues (2011) suggested that higher levels of *FABP4* in metastatic ovarian cancer cells near fat cells help maintain this exchange by allowing fatty acids to move from fat cells into cancer cells. Studies have found that *FABP4* levels are greater in metastatic ovarian tumors compared to the original tumors, and it has been considered as a possible indicator of cancer spread. Guaita-Esteruelas *et al*. (2018) showed that the production of *FABP4* by fat cells is a key part of the process that allows fatty acids to reach cancer cells. In clinical observations, Tucker *et al*. (2014) noted that higher FABP4 levels in the tissue of primary high-grade serous carcinoma were linked to the presence of remaining cancer cells after initial surgery.

2.9 Adipokines

2.9.1 Leptin

Zhang *et al.* (1994) identified and sequenced the mouse obese (ob) gene using positional cloning, which led to the discovery of leptin as a satiety factor secreted by white adipose tissue (WAT) in genetically obese (ob/ob) mice demonstrated that this finding established WAT as an active endocrine organ. Although leptin is predominantly expressed and secreted by adipocytes within WAT (Zhang *et al*., 1994). Leptin functions as a satiety signal within a feedback loop that regulates WAT mass (Zhang *et al*., 1994). In the fed state, circulating leptin concentrations are directly correlated with body fat mass (Considine *et al*., 1996). Ahima *et al*. (1996) demonstrated that the onset of starvation induces a disproportionate decrease in leptin levels relative to changes in adipose tissue mass. A mutation in the leptin gene or receptor is linked to obesity in both humans and rodents, and leptin administration, either centrally or peripherally, reduces food intake and body weight (Friedman and Halaas, 1998). Leptin affects energy expenditure in addition to suppressing food intake. Leptin treatment raised body temperature and physical activity in ob/ob mice, according to earlier research. Pelley mounter *et al*. (1995).

Chen *et al*. (2013) and Modzelewska *et al*. (2019) found that activating LEPR increases the production of proteins such as cyclin D1 and Mcl-1, which prevent cells from dying and encourage their growth in ovarian cancer cells. Storti *et al.* (2021) noted that the amount of leptin in the blood is closely linked to the total body fat. Uddin *et al*. (2009) showed that high levels of leptin are connected with worse outcomes in women from the Middle East who have epithelial ovarian cancer. Wei *et al*. (2017) demonstrated in an animal model that blocking the leptin receptor can stop the spread of ovarian cancer cells in the abdominal cavity and limit tumor growth, suggesting that leptin could be a target for treatment.

2.9.2 Adiponectin

Adipose tissue is an extremely active endocrine and metabolic organ. In addition to mediating local autocrine and paracrine actions that affect food intake, energy expenditure, and the metabolism of carbohydrates and fats, adipocytes secrete a variety of substances that circulate blood and operate on distant tissues (Havel, 2002). Adipocytes release adiponectin, which has been epidemiologically linked to obesity, insulin resistance, type 2 diabetes, and cardiovascular diseaseillness (Fu *et al*., 2005). Reduced expression of the adiponectin gene in adipose tissue is associated with low plasma adiponectin levels in various illness conditions (Hotta *et al*., 2001; Weyer *et al*., 2001).

Adiponectin gene polymorphisms have also been linked to insulin resistance, type 2 diabetes, and hypoadiponectinemia (Waki *et al*., 2003). Consequently, obesity and insulin resistance have been linked to low adiponectin levels and variations in the adiponectin gene (Fu *et al*., 2005). It is involved in regulating a variety of metabolic processes and possesses antiatherogenic (Ouchi *et al*., 1999; Ouchi *et al*., 2000) and anti-inflammatory (Yokota *et al*., 2000) properties. gene expression and protein secretion of this protein is reduced in obesity (Arita *et al*., 1999), CVD severity (Matsuzawa *et al*., 2004), insulin resistance and T2DM (Hotta *et al*., 2001), and dyslipidaemia (Matsubara *et al*., 2002) also it decreases the TG content of adipose tissue by increasing FA oxidation, thus making it an insulin sensitiser (Yamauchi *et al*., 2001). As insulin sensitivity increases, as in weight loss or insulin-sensitising drug treatment, adiponectin concentrations rise (Yang *et al*., 2001; Matsazawa *et al*., 2004), which has led to the proposal that adiponectin contributes to the development of the metabolic syndrome (Matsuzawa *et al*., 2004).

2.10 Role of microRNAs in Adipogenesis

Several microRNAs that seem to be involved in adipogenesis have been found. While some of these microRNAs appear to adversely control adipogenesis (Kim *et al*., 2010; Sun *et al*., 2009), others appear to accelerate it (Kennel *et al*., 2008; Wang *et al*., 2008). By blocking Wnt signalling, mammalian homologs of miR-8 stimulate adipogenesis (Kennel *et al*., 2008). Let7 introduced spontaneously decreased both terminal cell differentiation and clonal growth in 3T3-L1 cells (Sun *et al*., 2009).

2.11 Future Perspectives

Adipocytes have remarkable adaptability, exhibiting an exceptional ability to increase in size when faced with excessive nutrition (Markussen and Mandrup, 2023). Obesity is a common public health issue in industrialized countries (Kuczmarski *et al*., 1994 and Wolf and Colditz, 1998). Studying how fat cells develop and identifying genes that are altered in various monogenic mouse models of obesity has helped us gain insight into the cellular and molecular mechanisms underlying the growth of fat tissue in both normal and disease conditions. A thorough comprehension of the molecular components involved in adipogenesis and their connection to the function of adipose tissue may lead to innovative therapeutic strategies for tackling obesity while preserving the normal function of adipose tissue (Ambele *et al*., 2020)

Chapter 3

Aim and Objectives

Aim

The present study aims to evaluate the role of *PPAR-γ* and *FABP4* during adipocyte differentiation by analysing their temporal expression patterns in 3T3-L1 cells.

Objectives

1) To induce adipocyte differentiation in 3T3-L1 cells.
2) To assess adipocyte differentiation through morphological observations.
3) To isolate total RNA from cells and synthesis cDNA.
4) To analyse the temporal expression patterns of *PPAR-γ* and *FABP4* by RT-qPCR.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Laboratory Materials

T25 culture flasks, 6-well culture plates, Cryovials, Sterile centrifuge tubes, Eppendorf tubes, Micropipette tips, Haemocytometer (Neubauer chamber), Coverslips, Gel casting tray and comb, Erlenmeyer flask, PCR tubes, Aluminium foil, Syringe filter unit, Disposable gloves and laboratory consumables.

4.1.2 Reagents for Cell Culture

- Dulbecco's Modified Eagle Medium (DMEM, high glucose) (Gibco).
- 10 % Fetal Bovine Serum (FBS) (Sigma, USA)
- 1 % Penicillin–Streptomycin (Sigma, USA)
- Phosphate Buffered Saline (PBS) (Sigma, USA)
- 0.25 % Trypsin–EDTA solution
- Dimethyl sulfoxide (DMSO)
- Sodium bicarbonate ($NaHCO_3$)

4.1.3 Reagents for Cryopreservation

- Freezing medium (DMEM supplemented with 10 % FBS + 20 % DMSO)

4.1.4 Reagents for Cell Counting

- 0.4 % Trypan blue solution.

4.1.5 Reagents for Adipocyte Differentiation Induction Cocktail

- 0.5 mM 3-Isobutyl-1-Methylxanthine (IBMX)
- 1 μM Dexamethasone (DEX)
- 10 μg/ml Insulin

4.1.6 Reagents for Oil Red O Staining

- Oil Red O
- Isopropanol
- 4 % Paraformaldehyde (PFA)
- Sodium hydroxide (NaOH)
- Hydrochloric acid (HCl)

4.1.7 Reagents for RNA Isolation

- TRIzol™ reagent (Invitrogen)
- Chloroform
- Isopropanol
- 75 % Ethanol
- DEPC-treated water
- RNaseZap solution (Ambion)

4.1.8 Reagents for Agarose Gel Electrophoresis

- Agarose (1.2 % w/v of 50 mL)
- 1X TAE buffer (40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA) - pH 8.3
- Ethidium bromide (EtBr)
- 6X loading dye (Bromophenol Blue, Xylene Cyanol and glycerol)
- RNA molecular weight marker.

4.1.9 Reagents for cDNA Synthesis and RT-qPCR

- iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA)
- SYBR Green Master mix (Bio-Rad Laboratories, USA)
- Forward and reverse primers

4.1.10 Instruments

- Class II HEPA filtered Biosafety Cabinet (ESCO Lifesciences, Singapore)
- CO_2 Incubator (Eppendorf, Germany)
- Inverted phase-contrast microscope (Leica microsystems, Germany)
- Refrigerated centrifuge (Eppendorf, Germany)
- Centrifuge (Eppendorf, Germany)
- Micropipettes (Thermo Scientific, USA)
- Vortex mixer (Shimadzu, Japan)
- pH meter (Thermo Scientific, USA)
- Vacuum filtration system (0.22 µm)
- Analytical balance (Shimadzu, Japan)
- Magnetic stirrer / hot plate
- Microwave oven
- Gel electrophoresis apparatus
- Gel documentation system (Bio-Rad, USA)
- Thermal cycler (Thermo Fisher Scientific, USA)
- Refrigerator and deep freezer (-20 °C and -80 °C)
- UV visible Spectrophotometer (Thermo Scientific, USA)
- Biophotometer (Eppendorf, Germany)
- CFX96 Real-Time PCR System (Bio-Rad, USA)

4.2 Methodology

4.2.1 Cell Culture

The 3T3-L1 cell line used in this study was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in high glucose DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % Penicillin-Streptomycin in a humidified environment of 5 % CO_2 at 37 ℃. Cell morphology and confluency were monitored regularly using an inverted

phase-contrast microscope. Cells were passaged at 70-80 % confluency to prevent contact inhibition and cryopreservation of cells with extremely low temperatures was used to maintain their structural integrity with the use of a cryoprotectant (20 % DMSO).

4.2.2 Preparation of DMEM

3T3-L1 cells maintained in Dulbecco's Modified Eagle Medium (DMEM) was prepared according to the manufacturer's instructions. The powdered DMEM was dissolved in 900 mL of high-purity sterile distilled water with continuous stirring until completely dissolved. Sodium bicarbonate (3.7 g) was added and mixed until the solution became clear. The final volume was made up to 1000 mL using sterile distilled water. The prepared basal medium was sterilized using a 0.22 µm vacuum filtration system and collected in a sterile storage bottle. Under aseptic conditions in a laminar airflow hood, 10 % Fetal Bovine Serum (FBS) and 1 % Penicillin-Streptomycin were added to prepare complete DMEM medium.

4.2.3 Subculture of 3T3-L1 cells

To ensure optimal differentiation of 3T3-L1 preadipocytes and to prevent contact inhibition, cells were sub cultured upon reaching 70-80 % confluency and the passage number (typically 3-15) was maintained. The culture medium was first aspirated from the T25 flasks and discarded. The remaining cell monolayer was washed twice with 1 mL of phosphate-buffered saline (PBS) to remove residual serum. To detach the cells, 400 µL of 0.25 % trypsin-EDTA solution was added, ensuring the entire cell monolayer was coated and incubated at 37 ℃ for 1-2 min. Cell detachment was monitored using an inverted phase-contrast microscope and observed about 70-80 % of the cells were rounded and detached. The enzymatic activity of the trypsin was neutralized by adding 1 mL DMEM supplemented with 10 % FBS. To obtain a homogenous, single-cell suspension, it was gently resuspended along the sides of the culture flask. Finally, 500 µL cell suspension was added to two new T25 flasks containing 5 mL of DMEM supplemented with 10 % FBS and incubated in a humidified environment of 5 % CO_2 at 37 ℃.

4.2.4 Cell Seeding

Prior to differentiation, 3T3-L1 preadipocytes maintained in a T25 culture flask were seeded. Adherent cells cultured in a T25 flask were harvested by first aspirating the culture medium and washing the cell monolayer twice with 1 mL of Phosphate Buffered Saline (PBS) to remove residual serum. To detach the cells, 400 µL of 0.25 % Trypsin-EDTA solution was added,

ensuring the entire cell monolayer was coated and incubated at 37 ℃ for 1-2 min. Cell detachment was monitored using an inverted phase-contrast microscope and observed about 70-80 % of the cells were rounded and detached. The enzymatic activity of the trypsin was neutralized by adding 1 mL DMEM supplemented with 10 % FBS. To obtain a homogeneous, single-cell suspension, it was gently resuspended along the sides of the culture flask. Cell concentration and viability were determined using the Neubauer chamber via the trypan-blue dye exclusion method. Briefly, the single-cell suspension was mixed with 0.4 % trypan blue dye to achieve a 1:1 (v/v) ratio and 10 µL of this mixture was loaded onto the Neubauer chamber. Cell counting was performed according to the L rule, where cells touching the top and left borders were counted, while those touching the bottom and right borders were excluded. The number of viable cells was determined using the following formulae.

$$\text{Total cells/ml} = \frac{(\text{Total cells counted x Dilution factor x } 10^4 \text{ cells/ml})}{\text{Number of squares counted}}$$

The cell concentration was calculated as 140 x 4 x 10^4 cells / mL. The cell suspension was then appropriately diluted with fresh complete medium, and the 3T3-L1 preadipocytes were seeded into 6-well culture plates at a density of 10000-30000 cells/well.

4.2.5 3T3-L1 Differentiation

To differentiate 3T3-L1 pre-adipocytes into adipocytes, cells were cultured in complete (DMEM supplemented with 10 % FBS) medium for 24 hours. Differentiation of 3T3-L1 cells is induced by an adipogenic cocktail containing Insulin, Dexamethasone (DEX), and 3-Isobutyl-1-Methylxanthine (IBMX).

4.2.5.1 MDI Induction

The induction medium was formulated by DMEM supplemented with 10 % FBS and 100 µL of 0.5 mM 3-Isobutyl-1-Methylxanthine (IBMX), 4 µL of 1 µM dexamethasone, and 20 µL of 10 µg/mL Insulin stock solution was prepared by dissolving in dimethyl sulfoxide (DMSO) in the laminar air flow hood under aseptic conditions and the final volume was made up to 20 mL. Six well plate was observed under an inverted microscope and was observed 70-80 % confluency. Culture media was aspirated and discarded from each well in the laminar air flow hood under aseptic conditions. 3 mL of freshly prepared MDI induction medium was added to each well to initiate adipogenic differentiation except control wells. The plate was then incubated in a humidified environment of 5 % CO_2 at 37 ℃ for 4 days.

4.2.5.2 Insulin Treatment

After 48 hours of MDI induction, six well plate was taken from the incubator and observed under an inverted microscope to monitor cell morphology and differentiation. Then Induction medium was aspirated from each well and discarded under aseptic conditions. The insulin medium was freshly prepared by supplementing DMEM containing 10 % FBS. Specifically, 20 µL of 10 µg/mL insulin was added to 20 mL of DMEM supplemented with 10% FBS. Subsequently, 3 mL of insulin medium was added to each well except control well (undifferentiated) and cell collected wells. The plate was then incubated in a humidified environment of 5 % CO_2 at 37 ℃ for 24 hours.

4.2.5.3 Post Induction Maintenance

Following insulin induction, six well plate was taken from the incubator and observed under an inverted microscope to monitor cell morphology and differentiation. Insulin containing medium was aspirated from each well and discarded. 3 mL of DMEM supplemented with 10 % FBS was added under aseptic conditions in a laminar air flow hood. Maintenance media was replaced after every 24 hrs. for the adipocyte maturation and incubated in a humidified environment of 5 % CO_2 at 37 ℃.

Table 1: Work flow of 3T3-L1 differentiation

Day	Activity
Day 0	DMEM + 10 % FBS with Induction (MDI) medium (100 µL of 5 mM 3-Isobutyl-1-Methylxanthine (IBMX), 4 µL of 1 µM Dexamethasone, and 20 µL of 10 µg/mL Insulin (3 mL MDI medium for each well).
Day 2	Cell collection for RNA isolation
Day 4	MDI medium replaced with Insulin medium (DMEM + 10 % FBS with 20 µL of 10 µg/mL insulin) (3 mL for each well)
Day 6	Cell collection for RNA isolation and Insulin media changed to DMEM + 10 % FBS
Day 8 and Day 10	Media renewal (DMEM +10 % FBS)
Day 12	Cell collection for RNA isolation and Oil Red O staining

4.2.6 Oil Red O Staining

The differentiation of 3T3-L1 preadipocytes to mature adipocytes is characterized by the intracellular lipid droplet accumulation. Oil Red O staining is used to stain lipid droplets in (mature) 3T3-L1 adipocytes as described by Pittenger *et al*. (1999).

4.2.6.1 Preparation of Oil Red O Staining Reagents

The Oil Red O working solution was prepared immediately prior to use. Oil Red O stock solution (0.5 % w/v in 100 % isopropanol) diluted to 3:2 using deionized H2O to make Oil Red O working solution. Syringe filter unit can be used in place of a Whatman paper/ funnel system to filter the Oil Red O working solution. 4 % paraformaldehyde (PFA) in PBS was prepared by heating of 10 ml of 1X PBS on a hot plate at 60 °C. Then, added 0.4 g of PFA powder while stirring and added 1 N NaOH drop wise until the solution clears, adjusted the pH 7.2 – 7.4 using 1N HCL. A 60 % (v/v) isopropanol solution was prepared by diluting 6 mL of 100 % isopropanol and made up to 10 mL using distilled water.

4.2.6.2 Procedure for Oil Red O Staining

For fixation of cells, the culture media was completely aspirated from the wells and discarded, followed by a wash with 1 mL Phosphate Buffered Saline (PBS) to remove any residual serum. 2 mL of 4 % paraformaldehyde (PFA) was carefully added along the sides of each well and the culture plate was then covered with aluminium foil to protect the light-sensitive PFA and incubated for 30 minutes at room temperature. Following incubation, added 4 % PFA was aspirated and discarded, and the fixed cells were washed with 1 mL of 60 % isopropanol for 5 minutes at room temperature. After the isopropanol treatment, the solution was aspirated and stain with 2 mL of freshly prepared Oil Red O working solution. The plate was gently rotated to achieve uniform stain distribution and incubated for 15 minutes at room temperature. Following staining, the Oil Red O was removed, and 1 mL of isopropanol was briefly added and then aspirated, the wells were rinsed gently with distilled water until the wash solution ran clear. The plates were maintained in a hydrated state with distilled water until imaging. Stained lipid droplets were visualized and imaged using a phase-contrast microscope.

4.2.7 RNA Isolation

The total RNA isolation using TRIzol™ reagent was performed according to the manufacturer's instructions (Invitrogen), and the extracted RNA was stored at -80 ℃ in a freezer (Thermo Scientific, USA).

4.2.7.1 Cell Collection

Cells were collected for RNA isolation on 2^{nd}, 6^{th} and 12^{th} day using 1 mL of TRIzol™ (Invitrogen) reagent under aseptic conditions in a laminar air flow hood. Culture media was aspirated and discarded from the well. 1 mL of TRIzol™ reagent was added directly to the well to lyse the cells. Following lysis, the lysate was transferred to Eppendorf tube and stored at -80 ℃ (Thermo scientific, USA).

4.2.7.2 Procedure for RNA Isolation

The workbench for RNA isolation, pipettes, all plasticwares, and centrifuge were wiped twice with RNaseZap (Ambion) to remove RNase present on the surface. Solutions for RNA isolations were treated with 0.1 % diethylpyrocarbonate (DEPC, Sigma) and then incubated overnight at room temperature. The treated solutions were autoclaved for 20 minutes to degrade the traces of DEPC. All of the reagents used in the protocols were prepared using the DEPC-treated water to inactivate RNases.

To initiate RNA isolation, the lysed sample was vortexed and incubated at room temperature for 5 minutes. 200 µL chloroform per 1mL of TRIzol™ reagent was added and shaked vigorously by hand for 15s and incubated at room temperature for 3 min. For phase separation, the samples were centrifuged at 12000 rpm for 15 min at 4 ◦C. The resulting upper aqueous phase was carefully transferred to 1.5 ml Eppendorf tubes. RNA precipitation was done by mixing with 0.5 mL (500 µL) isopropanol per 1 mLTRIzol™ reagent and incubated at 10 minutes at room temperature. Then centrifuged at 12000 rpm for 10 min at 4 ℃. Following centrifugation, the supernatant was completely removed, and the RNA pellet was washed with 1mL 75 % ethanol (diluted with DEPC treated water) and the samples were vortexed once and centrifuged at 2000 rpm for 4 min at 4 ℃. The supernatant was discarded and the RNA pellet was allowed to air-dry at room temperature for 5 minutes. Finally, the pellet was dissolved in 50 µL of DEPC-treated water and aliquoted into separate 0.5 mL Eppendorf tubes and stored at - 20 ℃.

4.2.8 RNA Quality Assessment

4.2.8.1 Purity

The purity of the isolated RNA was assessed by measuring the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} measured using a biophotometer. An A_{260}/A_{280} ratio and A_{260}/A_{230} ratio of approximately 2.0 indicated high-quality RNA with minimal protein or phenol contamination. RNA samples exhibiting A_{260}/A_{280} and A_{260}/A_{230} ratios approximately 2.0 were considered as pure and used for downstream applications such as cDNA synthesis and quantitative real-time PCR (RT-qPCR).

4.2.8.2 Integrity

RNA quality and integrity have been assessed by agarose gel electrophoresis of total RNA, before downstream applications such as cDNA synthesis and RT-qPCR. Electrophoresis gel tank, gel comb, and gel tray were soaked in buffer (0.1 M NaOH and 100 mM ethylenediaminetetraacetic acid) for 2 h and rinsed well with DEPC-treated water. Weighed out 0.6 g (1.2 % w/v of 50 mL) agarose and added it to the Erlenmeyer Flask. Followed by added 50 mL of 1x TAE buffer (40 mM Tris base (pH 7.6), 20 mM acetic acid, 1 mM EDTA) with final pH 8.3 prepared with DEPC water. Dissolved the agarose completely by placing it in a microwave. It was then removed and allowed to cool on the lab bench until the heat became bearable for handling (approximately 60 ℃). Then added 2 µl ETBR (Ethidium Bromide) in to the flask and swirled to mix. The prepared agarose solution was then poured into the casting tray with the inserted comb in place. The gel was left undisturbed at room temperature for 30 minutes to allow complete solidification before downstream electrophoresis. Mix 5 µl RNA sample and 1 µl of 6X loading dye. The solidified gel was placed in an electrophoresis tank containing 1X TAE running buffer prepared with DEPC-treated water, ensuring that the gel is fully submerged in the buffer. The RNA samples and an RNA molecular weight ladder were loaded into the wells. Electrophoresis was performed at 80 V for 45–60 minutes until the tracking dye had migrated approximately 75 % of the gel length. The gel was visualized using a gel documentation system (Bio-Rad Laboratories, USA).

4.2.9 cDNA Synthesis

iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) is used for two-step reverse transcription quantitative PCR (RT-qPCR).

Table 2: Reaction mixture used for cDNA synthesis

Components	Volume per reaction (µL)
5xiScript Reaction Mix	4 µL
iScript Reverse Transcriptase	1 µL
RNA template	5 µL
Nuclease- free water	10 µL
Total Volume	20 µL

Incubate the complete reaction mix in a thermal cycler using the following.

Table 3: Thermal cycling conditions for cDNA synthesis

Reaction	Temperature	Time
Priming	25 ℃	5 min
Reverse transcription	46 ℃	20 min
RT inactivation	95 ℃	1 min
Optional Step	Hold at 4 ℃	

4.2.10 Primer Dilution

The lyophilized forward and reverse primers were procured commercially and diluted using nuclease free water. The lyophilized primer was briefly centrifuged to collect the contents of the bottom of the tube. Nuclease free water added according to manufacturer's data sheet to obtain a final concentration of 100 µM (100 p mol/µL) stock solution. Vortex and briefly centrifuged. A 10 µM (10 p mol/L) working was prepared from the stock solution by diluting 10 µL of stock solution with 90 µL nuclease- free water. Vortex and stored at -20 ℃ until further use for qPCR analysis.

Table 4: Primer sequence used for RT-qPCR

SL No	Gene symbol	Gene Name	Primer sequence	Reference
1	*PPAR-γ*	Peroxisome proliferatoractivated receptor gamma	Forward: - 5'GAGTTCATGCTTGTCAAGGATGC-3' Reverse: - 5'- CGATATCACTGGAGATCTCGCC -3'	La Camera *et al.* (2021)
2	*FABP4*	Fatty acidbinding protein 4	Forward: - 5'- ATGTGTGATGCCTTTGT - 3' Reverse: - 5'- TTTCCATCCCACTTCTG - 3'	La Camera *et al.* (2021)
3	*GAPDH*	Glyceraldehyde 3-phosphate dehydrogenase	Forward: 5'-ACTCCACTCACGGCAAATTC - 3' Reverse: 5'- TCTCCATGGTGGTGAAGACA -3'	Xie *et al.* (2025)

4.2.11 Quantitative Real-Time PCR (RT-qPCR)

Gene expression analysis was performed using SYBR® Green master mix (Bio-Rad Laboratories, USA) on a CFX96 Real-Time system (Bio-Rad Laboratories, USA). Thawed universal SYBR® Green master mix and other frozen reaction components at room temperature. Mixed thoroughly and centrifuged briefly for collecting solutions at the bottom of tubes, and then stored on ice protected from light. Prepared (on ice or at room temperature) enough reaction setup for all qPCR reactions by adding all required components except the template according to the following protocol.

Table 5: Reaction mixture used for RT-qPCR

Components	Volume per reaction, µL
SYBR® Green master mix (2x)	5 µL
Forward primer	1 µL
Reverse primer	1 µL
Nuclease- free water	2 µL
cDNA template	1 µL
Total Volume	10 µL

Mixed the assay master mix thoroughly to ensure homogeneity and dispense equal aliquots into each PCR tube or into the wells of a PCR plate. Then added the samples (and nuclease-free H2O if needed) to the PCR tubes containing the reaction mixture. Sealed tubes with flat caps and vortexed 30 sec to ensure thorough mixing of the reaction components. Spin the tubes to remove any air bubbles and collected the reaction mixture in the vessel bottom.

Programmed thermal cycling protocol on the real-time PCR instrument. Load the PCR tubes onto the real-time PCR instrument and runs the PCR. Performed data analysis according to the instrument-specific instructions.

Table 6: Thermal cycling conditions for RT-qPCR (40 cycles)

Reaction	Temperature	Time
Initial denaturation	95 ℃	2-3 min
Denaturation	98 ℃	15 sec
Annealing/Extension+ Plate	60 ℃	30 sec

Melt Curve Analysis was conducted at the end of each run to verify the specificity of amplification.

4.2.12 Gene Expression Analysis

The relative gene expression levels of target genes were calculated using $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001). GAPDH was used as the endogenous reference gene (housekeeping gene) for normalization. All reactions were performed as triplicates and mean Ct values were used for further analysis. Data were expressed as Mean ± Standard Error. Fold changes in gene expression were calculated by comparing the Ct values of 2nd, 6th and 12th day samples with control sample.

Relative fold change was calculated by the equation: -

$$\Delta Ct = Ct\ (Target\ gene) - Ct\ (Reference\ Gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (Test) - \Delta Ct\ (control)$$

$$Fold\ change = 2^{(-\Delta\Delta Ct)}$$

Chapter 5

Results

5.1 Morphological Assessment

5.1.1 Morphological Observation of Undifferentiated 3T3-L1 cells

The undifferentiated 3T3-L1 cells observed under an inverted phase contrast microscope exhibited fibroblast like appearance (spindle-shaped, elongated cells) using prior to induction. Because of the adherent characteristics they formed a uniform homogenous monolayer (Fig 6).

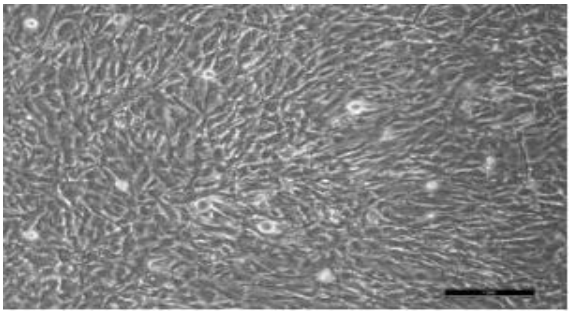
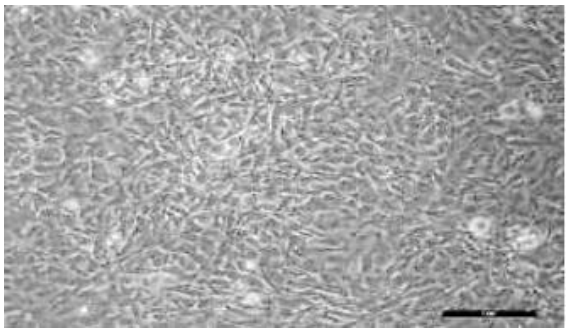


Figure 6: Microscopic view of undifferentiated 3T3-L1 cells (10x magnification)

5.1.2 Morphological Changes and Lipid Droplet Accumulation During Adipocyte Differentiation

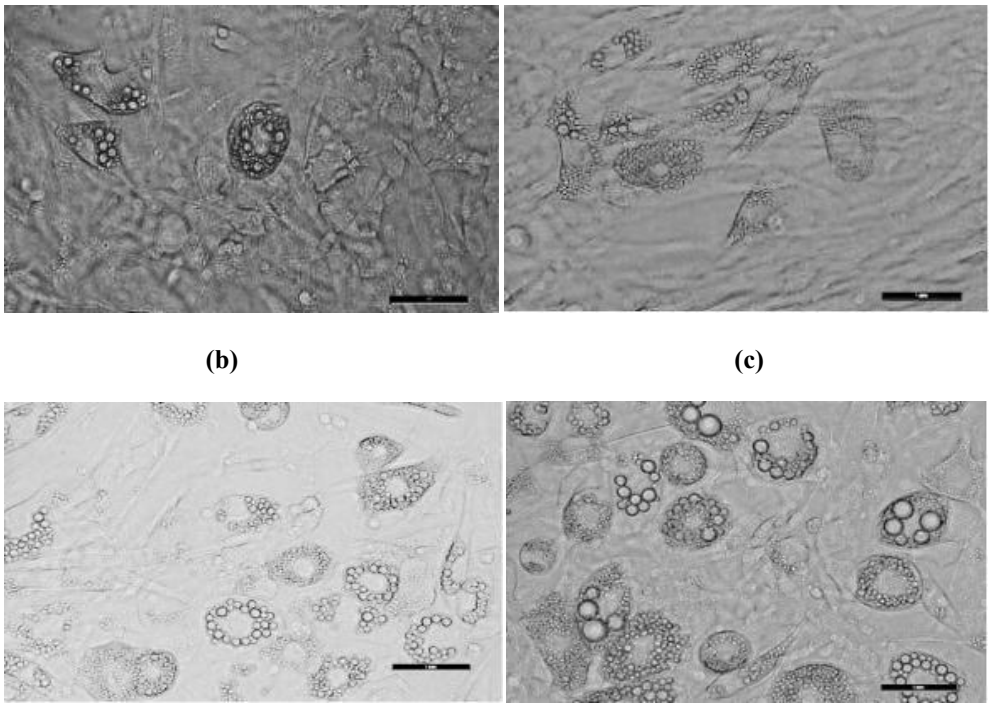
Following induction with MDI (IBMX, Dexamethasone, Insulin), preadipocyte cells lose their fibroblast-like (spindle shaped elongated cell) morphology and gradually change day by day to rounded mature adipocytes along with the accumulation of intracellular lipid droplets (Fig 7).

(1)



(a)

(2)



(b) **(c)**

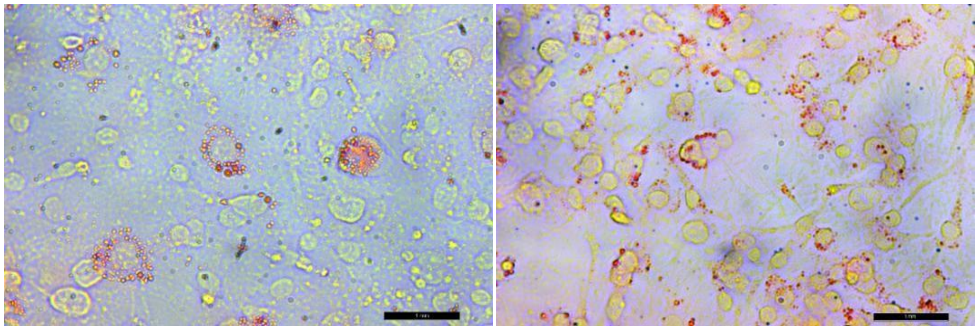
(d) **(e)**

Figure 7: Microscopic view of 3T3-L1 during course of differentiation: (1) Early differentiation phase showing transition from spindle shaped-preadipocytes to a rounded cell shape: (a) Day 2 (2) Progressive intracellular lipid droplet accumulation: (b) Day 4, (c) Day 6, (d) Day 8, (e) Day 12

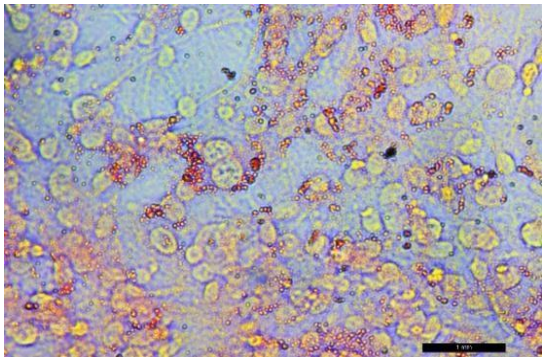
5.1.3 Morphological Changes and Lipid Droplet Accumulation During Adipocyte differentiation visualized by Oil Red O Staining

Oil-Red-O staining validated the maturation of adipocytes with extensive red stained intracellular lipid droplets within the cytoplasm and confirmed successful time dependent adipogenesis (Fig 8(1) & Fig 8(2)).

(1)

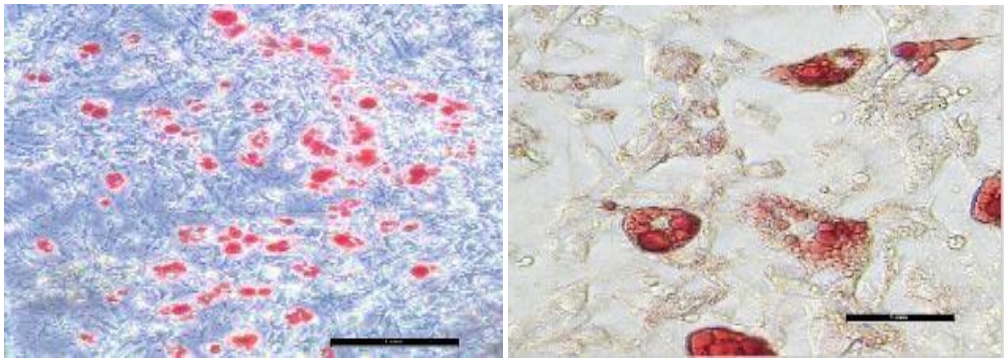


(a) **(b)**



(c)

(2)



(d) **(e)**

Figure 8: (1) Oil Red O staining of 3T3-L1 cells during differentiation showing intracellular lipid droplet accumulation (a) Day 4, (b) Day 8, (c) Day 12) (20x magnification).(2) Representative images of Oil Red O staining of differentiated 3T3-L1 cells (d) 10x magnification, (e) 40x magnification

5.2 Analysis of RNA Purity and Integrity

5.2.1 Purity

The purity and quantity of RNA was assessed by spectrophotometric analysis. The A_{260}/A_{280} and A_{260}/A_{230} absorbance ratio of all samples was ~ 2 (Table 7), indicating good quality RNA with minimal contamination.

Table 7: Spectrophotometric analysis of RNA quantity and purity

Sample	Concentration (ng /µl)	A_{260}/A_{280}	A_{260}/A_{230}
Pre-adipocyte	273.24	1.95	1.88
2	623.84	1.99	1.89
6	251.10	1.98	1.91
12	321.64	2.00	1.92

5.2.2 Integrity

RNA integrity was assessed by 1.2 % agarose gel electrophoresis. Distinct 28S and 18S rRNA bands with no smearing were observed in all samples, indicating good quality, intact RNA (Fig 9).

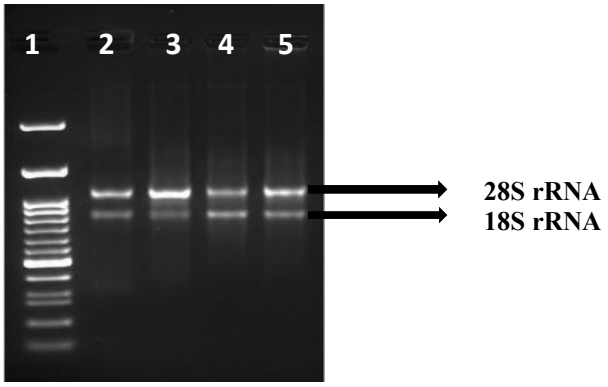


Figure 9: Total RNA isolated from cells at various stages of differentiation.
Lane 1: 100 bp DNA ladder; Lane 2: RNA from preadipocytes (3T3L-1); Lane 3: RNA from the cells at 2nd day of differentiation; Lane 4: RNA from the cells at 6th day of differentiation; Lane 5: RNA from the cells at 12th day of differentiation.

5.3 Quality Assessment of cDNA

Quantitative real-time PCR (RT-qPCR) analysis was performed using cDNA synthesized from the isolated total RNA. The housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as the endogenous control to assess cDNA quality, normalize target gene expression and synthesis efficiency. Amplification of GAPDH yielded highly reproducible results with low quantification cycle values, indicating substantial and consistent template abundance across samples, confirming its suitability for downstream gene expression analysis (Fig 10).

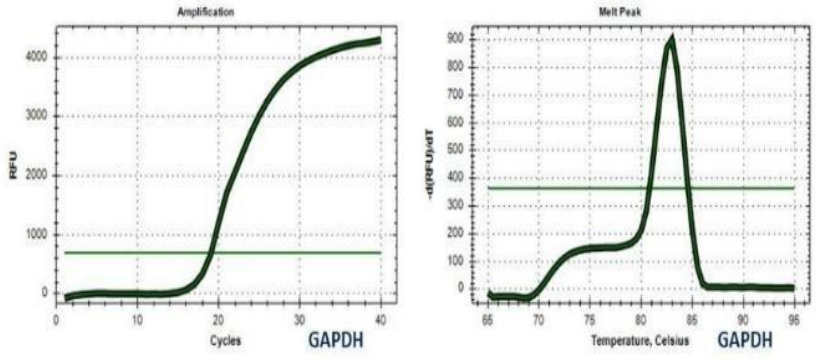


Figure 10: Representative RT-qPCR amplification and melt curve analysis of the GAPDH reference gene used for cDNA quality assessment.

5.4 RT-qPCR Analysis

5.4.1Amplification Curve Analysis

The RT-qPCR amplification plot exhibited a characteristic sigmoidal curve (Fig 11), consisting of a baseline phase, an exponential phase with rapid amplification of the target sequence, a linear phase, and a plateau phase, indicating efficient amplification of target genes.

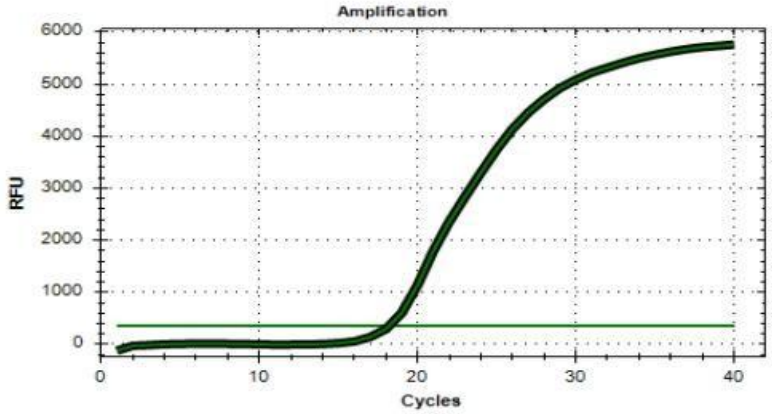
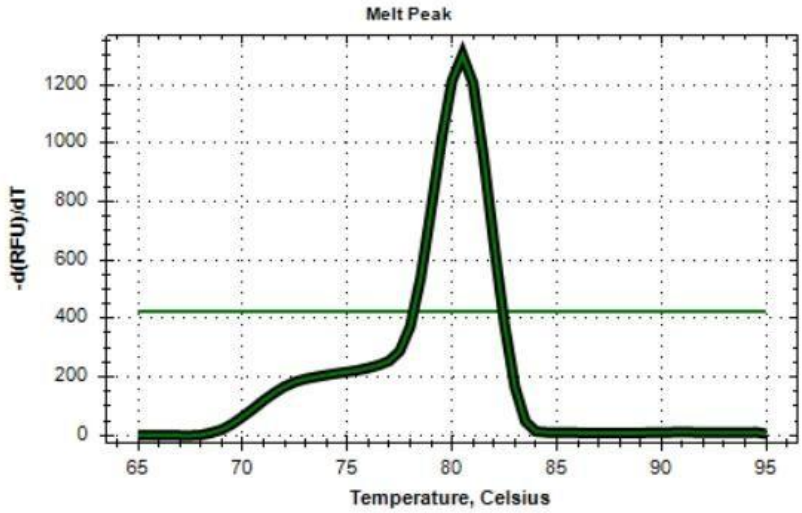


Figure 11: Representative amplification curve demonstrating efficient RT-qPCR amplification, characterized by an early quantification followed by a strong exponential phase

5.4.2 Melt Curve Analysis

Melt curve analysis confirmed the high specificity of the reaction, characterized by a single sharp melt peak indicating specific amplification of *PPAR-γ* (Fig 12(A)), *FABP4* (Fig 12(B)) with no significant primer-dimer formation or non-specific PCR products.

(A)



(B)

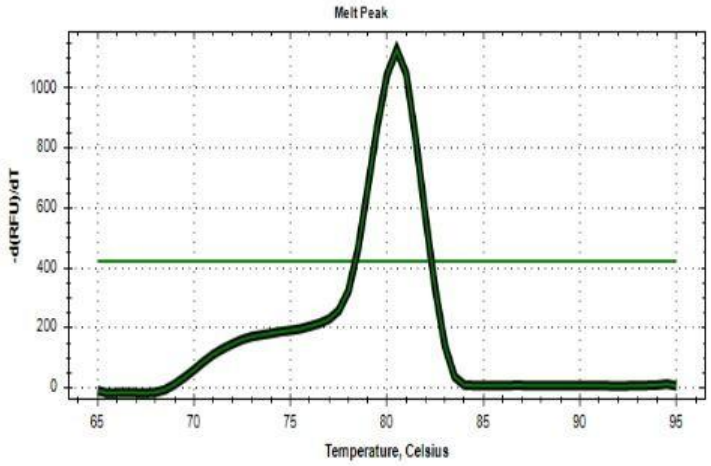


Figure 12: Melt curve analysis: Single, sharp melt peak showing accurate amplification (A) *PPAR-γ* (B) *FABP4*

5.4.3 Relative Gene Expression Analysis by Quantitative Real Time PCR

5.4.3.1 Relative Expression Analysis of *PPAR-γ*

Quantitative real-time PCR was conducted to evaluate the relative expression levels of *PPAR-γ* during different stages of adipocyte differentiation (Day 2, Day 6 and Day 12) and the relative fold change of *PPAR- γ* were calculated using $2^{-(\Delta\Delta Ct)}$ method by comparing the undifferentiated cells (control) which was normalized to 1. The results are presented in Table 8 and figure 13.

Table 8: Relative fold change in *PPAR-γ* gene expression during adipocyte differentiation, determined by RT-qPCR using the $2^{-(\Delta\Delta Ct)}$ method

Sample	Fold change
Day 2	0.05
Day 6	0.25
Day 12	1.89

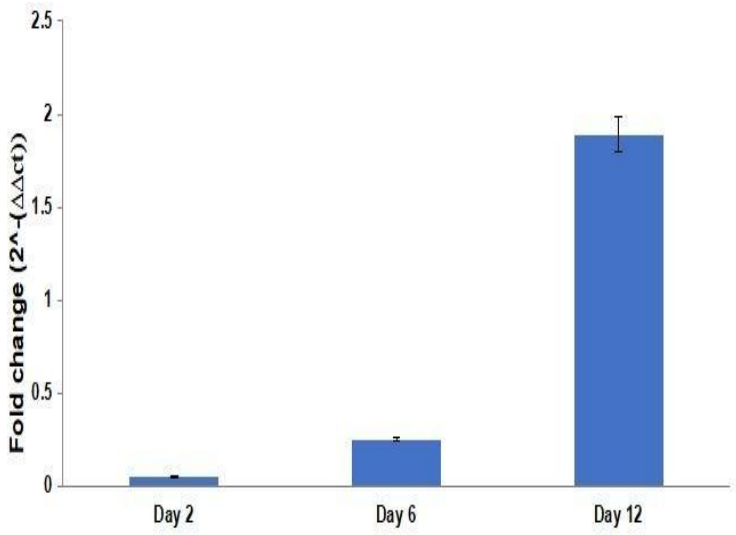


Figure 13: Relative fold change in *PPAR-γ* gene expression during adipocyte differentiation, determined by RT-qPCR using the $2^{(-\Delta\Delta Ct)}$ method at Day 2, Day 6, and Day 12

PPAR-γ expression showed a low expression in the early phase (day-2), a gradual increase during the mid-differentiation stage (day-6) and a marked upregulation at the terminal stage (day-12) compared to the undifferentiated cells (control), which was normalized to 1.

5.4.3.2 Relative Expression Analysis of FABP4

Quantitative real-time PCR was conducted to evaluate the relative expression levels of *FABP4* during different stages of adipocyte differentiation (Day 2, Day 6 and Day 12) and the relative fold change of *FABP4* were calculated using $2^{-(\Delta\Delta Ct)}$ method by comparing the undifferentiated cells (control) which was normalized to 1. The results are presented in Table 9 and figure 14.

Table 9: Relative fold change in *FABP4* gene expression during adipocyte differentiation, determined by RT-qPCR using the $2^{-(\Delta\Delta Ct)}$ method

Sample	Fold change
Day 2	0.4954
Day 6	42.81
Day 12	435.54

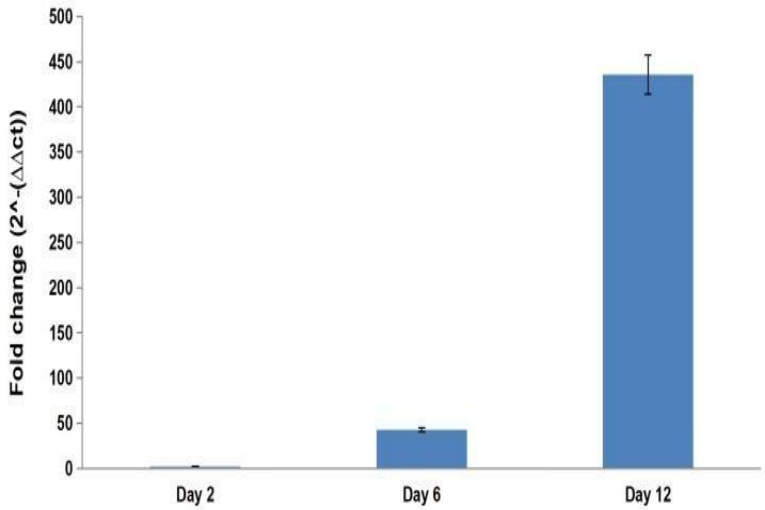


Figure 14: Relative fold change in *FABP4* gene expression during adipocyte differentiation, determined by RT-qPCR using the $2^{(-\Delta\Delta Ct)}$ method at Day 2, Day 6, and Day 12

FABP4 expression showed a progressive increase during adipocyte differentiation, with the highest expression observed at day 12 indicating its role in terminal differentiation compared to the undifferentiated cells (control), which was normalized to 1.

Chapter 6

Discussion

The biological process of adipogenesis is characterized by various structural morphological and molecular events that drive the differentiation of preadipocytes into mature lipidaccumulating adipocytes. In this study,we designed the experiments to elucidate the morphological changes and the expression patterns of *PPAR-γ* and *FABP4* in 3T3-L1 preadipocytes and differentiated adipocyte cells.In addition, RNA quality, purity assessment and cDNA synthesis was conducted to evaluate the reliability of gene expression analysis. Excessive adipocyte differentiation and adipose tissue expansion can lead to dysregulations in the molecular events related obesity and associated diseases like Type II diabetes mellitus, cardiovascular diseases,dyslipidaemia, hypertension, metabolic syndromes, certain types of cancers etc. By examining, how *PPAR-γ* and *FABP4* are expressed will provide a detailed understanding of these genes in the process of adipocyte development and their relevance in disease associated with obesity. The results of this study indicate successful adipogenic differentiation characterized by a progressive increase of *PPAR-γ* and *FABP4* throughout the differentiation process.

3T3-L1 cells show typical fibroblast like morphology that display the elongated, spindleshaped structure, under microscopic observation which indicates the maintained undifferentiated preadipocyte state necessary for the adipogenic induction. Preadipocyte cells were differentiated into mature lipid-accumulating adipocyte cells by inducing with differentiation cocktail (IBMX, Dexamethasone and Insulin, often called MDI). The conversion of the preadipocyte phenotype of 3T3-L1 to mature adipocyte is as established in previous studies (Green and Kehinde, 1975; Rubin *et al*., 1978; Russell and Ho, 1976). Preadipocytes have a post confluent mitosis and subsequent growth arrest at about 24 hours after MDI induction (Scott *et al*., 1982; Bernlohr *et al*., 1985). After 4^{th} day of induction, the lipid droplets accumulated within the cytoplasm of cells which indicated the early initiation of adipogenic differentiation. A progressive increase in cell number and size, along with increase in accumulation of lipid droplets over culture time particularly at 6^{th} day and 8^{th} day associated with a transition of rounded morphology of cells indicated the successful adipogenic differentiation. These findings support the previous reports by Gregoire *et al*. (1998) described as the rapid change in cell shape from fibroblastic to rounded morphology is a characteristic feature of the adipogenesis process and these morphological changes is also associated with the changes in the amount and kind of extracellular matrix (ECM) components as well as the

quantity of cytoskeletal components. Furthermore, the observed morphological changes are associated with the terminal differentiation as stated by Ntambi and Kim (2000).

Oil red O staining was used to visualize the intracellular lipid droplets in the cytosol at 12th day of differentiation. Differentiated cells with intracellular lipid droplets stains red with Oil Red O and the cells show negligible staining and remain colourless. According to the observations of Green and Kehinde (1974) and Ramirez-Zacarias *et al*. (1992), Oil Red O is a widely accepted method for confirming adipocyte maturation and intracellular lipid accumulation thereby indicated the successful adipogenic differentiation.

Analysis of RNA quality, purity and integrity plays a pivotal role in downstream applications such as cDNA synthesis, gene expression analysis. RNA can be easily degraded due to the ubiquitous presence of RNases enzymes in the environment. Therefore, the assessment of RNA is crucial. In this study, the purity of RNA isolated from different stages of adipocyte differentiation was evaluated by spectrophotometric analysis, where the $A_{260}/_{280}$ ratio and A_{260}/A_{230} around ~2.0 indicate RNA samples with minimal protein and phenol contamination. Based on the absorbance values, it was confirmed that the RNA isolated from different stages of adipocyte differentiation was intact and of good purity. Furthermore, the RNA quality and integrity were assessed by agarose gel electrophoresis. Distinct bands corresponding to the 28 S and 18 S rRNA were observed in all samples. The absence of smearing indicated that the isolated RNA was of good quality and with minimal degradation. In summary, the RNA isolated from different stages of differentiation was of good quality, purity and integrity suitable for subsequent cDNA synthesis and RT-qPCR analysis.

Evaluation of the gene expression patterns of *PPAR-γ* and *FABP4* indicated a progressive increase at various stages of adipocyte differentiation. The increased expression levels of *PPAR-γ* from early to late stage indicates its steady involvement in the regulation of adipogenesis supporting its role as the master regulator that initiates the activation of various adipogenic genes. *PPAR-γ* mRNA is induced by several transcription factors, mainly the *C/EBPβ*, *C/EBPδ* (Wu *et al*., 1995)

The peroxisome proliferator-activated receptor gamma (*PPAR-γ*) functions as a key regulator of adipogenesis by activating the expression of adipocyte-specific genes as reported by Schoonjans *et al*. (1996). *PPAR-γ* belongs to a large group of nuclear receptors that are activated by hormones crucial for the adipocyte development (Tontonoz *et al*., 1994; Hu *et al*., 1995; Rosen *et al*., 1999). A balanced and sufficient production of adipocytokines (adiponectin and

leptin), which are mediators of insulin action in peripheral tissues, is ensured by activated *PPAR-γ* in adipocytes, as a result, the body's overall insulin sensitivity is preserved (Kintscher and Law, 2005). *PPAR-γ* is also involved in the insulin stimulated glucose uptake by the activation of GLUT4 (Wu *et al*., 1998). In particular, the ubiquitin-proteasome system has been identified as a significant regulator of *PPAR-γ*, as demonstrated by studies from Hauser *et al*. (2000). Dennis *et al*. (2001) and Floyd and Stephens (2002). Another study (Lehmann *et al*., 1995) demonstrated that the antidiabetic thiazolidinedione drugs had a high affinity for *PPARγ*, this research was the first to show that *PPAR-γ* serves as a target for this class of antidiabetic medications.

FABP4 is a downstream target of *PPAR-γ* and the expression levels exhibited a progressive increase during the late stages of adipocyte differentiation involving the adipocyte maturation and terminal differentiation which indicates its role in adipocyte maturation and lipid handling. *FABP4* primarily functions as a transporter of fatty acids within the cell (Schwenket *et al*., 2010). Experimental studies have demonstrated that a lack of FABP4 provides a significant protection against atherosclerosis (Erbay *et al*., 2009). In addition, obese patients diagnosed with breast cancer exhibit significantly elevated levels of *FABP4* in their serum, which are linked to larger tumour sizes and a less favourable prognosis. *FABP4* also associated with the increased triglycerides and reduced high-density lipoprotein evidenced by the findings of Kaess *et al*. (2012). Although, *PPAR-γ* and *FABP4* interconnected through a regulatory loop the ligands for the *PPAR-γ* activity are provided by the *FABP4*.

Overall, the successful differentiation was confirmed by assessing morphology and analysis of intracellular lipid droplets by Oil Red O staining. RNA quality, purity and integrity was verified using spectrophotometric analysis and electrophoretic technique and cDNA synthesis provide the high-quality template for the RT-qPCR based gene expression analysis and also the increased progression of the adipogenic key regulators, *PPAR-γ* and *FABP4* through different stages of adipocyte differentiation indicates their crucial role in the adipocyte maturation and differentiation. The upregulation of *PPAR-γ* supports its function as a master regulator and high expression levels of *FABP4* in terminal differentiation indicates its role in adipocyte maturation and lipid accumulation. Studying how fat cells develop and identifying genes provide a detailed understanding of the cellular and molecular mechanisms of adipose tissue expansion. Despite these findings, the present study has certain limitations and in-depth investigations are therefore essential to understand the molecular mechanisms of *PPAR-γ* and *FABP4* gene regulation,

which is critical for developing novel therapeutic strategies against weight gain, obesity and associated metabolic diseases.

Summary and Conclusion

The current study successfully demonstrated the differentiation of 3T3-L1 preadipocytes into mature adipocytes, as confirmed by morphological assessment and the accumulation of intracellular lipid droplets, as validated by Oil Red O staining. The expression analysis revealed a progressive increase in the expression of *PPAR-γ* and *FABP4* during different stages of adipocyte differentiation. The increased expression levels of *PPAR-γ* throughout the course of differentiation confirm its role as a master regulator in adipogenesis. Furthermore, the upregulated expression levels of *FABP4* during the terminal stages of differentiation indicates its involvement in adipocyte maturation and lipid accumulation in the cells. Based on the findings of this study, it can be concluded that the temporal expression patterns of *PPAR-γ* and *FABP4* plays an important role in adipose tissue development and lipid metabolism. Understanding the expression dynamics of these genes may contribute to the identification of potential therapeutic targets for the management of obesity and related metabolic diseases.

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