EVALUATION OF *KLF4* AND *ADIPOQ* EXPRESSION DURING ADIPOCYTE DIFFERENTIATION

Dissertation submitted to the University of Kerala in partial fulfillment of the requirements for the award of the Degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled **'Evaluation of *KLF4* and *Adipoq* Expression During Adipocyte Differentiation'**, submitted to the University of Kerala in partial fulfillment 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 **THASLEEMA MUHAMMED (Candidate code: 63824142012)** 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**, in the 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 "**Evaluation of *KLF4* and *Adipoq* Expression during Adipocyte Differentiation"** is based on original research work carried out by me under the 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, Kariavattom . 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.

THASLEEMA MUHAMMED

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Abbreviations

ACC	:Acetyl-CoA Carboxylase
Akt	:Protein Kinase B
AMPK	: AMP Activated Protein Kinase
ATP	:Adenosine Triphosphate
C/EBPα	: CCAAT/Enhancer-Binding Protein Alpha
C/EBPβ	:CCAAT/Enhancer-Binding Protein Beta
C/EBPδ	: CCAAT/Enhancer-Binding Protein Delta
cAMP	: Cyclic Adenosine Monophosphate
cDNA	: Complementary DNA
CO_2	:Carbon Dioxide
Ct	:Cycle Threshold
DEX	:Dexamethasone
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	:Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
EDTA	:Ethylenediaminetetraacetic Acid
FABP4	:Fatty Acid-Binding Protein 4
FAS	:Fatty Acid Synthase
FBS	:Fetal Bovine Serum
GAPDH	: Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT4	: Glucose Transporter Type 4
IBMX	:3-Isobutyl-1-Methylxanthine
KLF4	: Krüppel-Like Factor 4
MDI	: 3-Isobutyl-1-Methylxanthine, Dexamethasone, and Insulin

PBS : Phosphate-Buffered Saline

PI3K : Phosphoinositide 3-Kinase

PPARγ : Peroxisome Proliferator-Activated Receptor Gamma

qRT-PCR : Quantitative Real-Time Polymerase Chain Reaction

SREBP-1c : Sterol Regulatory Element-Binding Protein-1c

SYBR Green : Syto®/SYBR Green Fluorescent DNA-Binding Dye

Abstract

Adipocyte differentiation is a complex biological process regulated by several transcription factors and adipocyte-specific genes that play important roles in lipid metabolism and insulin sensitivity. Any dysregulation in these processes can contribute to obesity associated diseases and other metabolic disorders. The present study evaluated the gene expression patterns of*KLF4* and *Adipoq* during the differentiation of the 3T3-L1 preadipocyte cell line, an established *in vitro* model for adipogenesis. Upon chemical induction,3T3-L1 preadipocyte cells exhibited distinct morphological changes accompanied by intracellular lipid accumulation, confirming successful adipogenesis. The increase in number of lipid droplet formation and adipocyte maturation was validated microscopically by Oil Red O staining at distinct stages of differentiation.Gene expression analysis by qRT-PCRdemonstrated that *KLF4* expression was elevated during the early stages of differentiation**,** indicating its role in the initiation of adipogenesis, whereas *Adipoq* expression increased progressively during later stages, suggesting its involvement in adipocyte maturation and metabolic regulation. The differential expression patterns of these genes highlight their stage specific role in adipocyte development. The findings of this study provide a valuable platform for further investigation into the molecular mechanisms of adipogenesis,contributing to a better understanding of obesity, insulin resistance, and other metabolic disorders.

Chapter 1

Introduction

Adipose tissue is a highly specialized connective tissue that serves as the primary site for energy storage and also functions as an active endocrine organ. Adipocytes, commonly known as fat cells, arise from mesenchymal stem cells through a complex and tightly regulated process known as adipocyte differentiation or adipogenesis. During adipogenesis, fibroblast-like preadipocytes undergo morphological and biochemical changes to become mature adipocytes capable of storing triglycerides and secreting various adipokines.

This differentiation process occurs in distinct stages involving growth arrest, mitotic clonal expansion, and terminal differentiation. Early adipogenic transcription factors initiate the differentiation program, followed by activation of master regulators and adipocyte-specific genes.

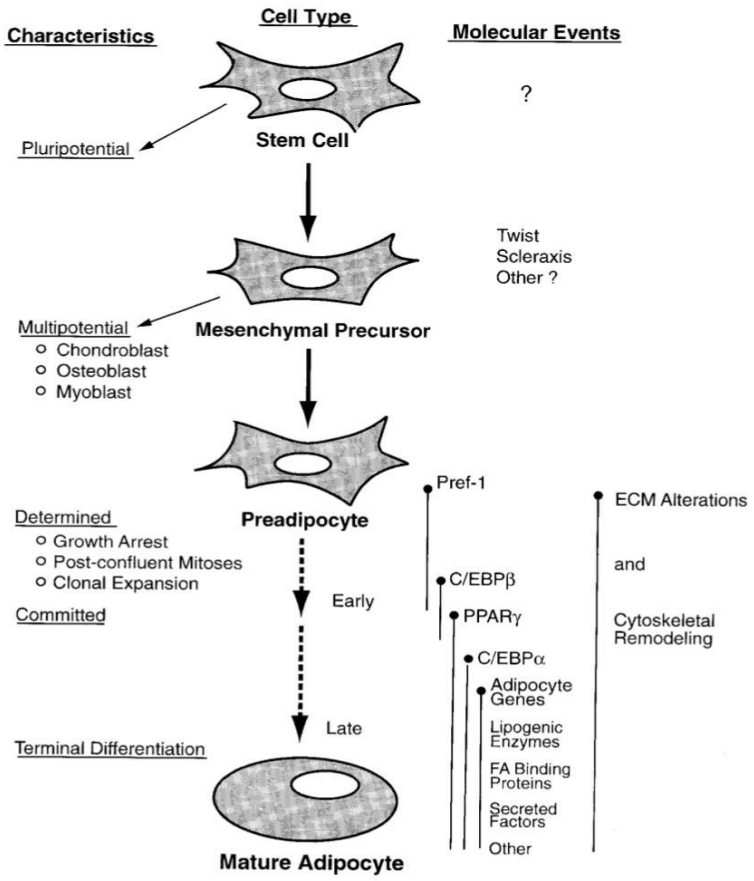


Figure 1: Schematic representation of adipocyte differentiation from pluripotent stem cells to mature adipocytes (Gregoire *et al.*, 1998)

Mature adipocytes are characterized by lipid droplet accumulation and expression of genes responsible for glucose and lipid metabolism. Besides their role in energy homeostasis, adipocytes secrete biologically active molecules that regulate insulin sensitivity, inflammation, appetite, and overall metabolic balance. Therefore, adipocyte differentiation is a fundamental biological process that plays a critical role in maintaining metabolic health.

The increasing prevalence of obesity and metabolic disorders has made adipocyte biology an important field of research. Studying adipocyte differentiation is therefore essential for regulating lipid storage, maintaining energy balance, and controlling glucose metabolism. Impaired adipogenesis contributes to obesity, insulin resistance, type 2 diabetes mellitus, cardiovascular diseases, and metabolic syndrome. Understanding the molecular mechanisms underlying adipocyte differentiation thus provides valuable insights into the development and progression of these disorders. Furthermore, studies on adipogenesis help identify potential therapeutic targets for obesity and diabetes and aid in the development of novel drugs that regulate adipose tissue function. Since adipose tissue acts as an endocrine organ that secretes various adipokines involved in metabolic homeostasis, studying adipocyte differentiation is important for understanding the relationship between obesity and associated diseases. Therefore, investigations into adipogenesis have significant implications for both basic biological research and clinical medicine.

Several researchers have contributed significantly to understand the mechanisms of adipocyte differentiation. Rosen and Spiegelman (2000) demonstrated Peroxisome Proliferator-Activated Receptor gamma (*PPARγ*) as the master regulator of adipogenesis and is indispensable for the formation of mature adipocytes. Farmer (2006) further emphasized the coordinated action of transcription factors such as *CCAAT*/enhancer-binding proteins and *PPARγ* in controlling adipocyte differentiation and lipid metabolism. Another study by Birsoy *et al.*, (2008) reports Krüppel-like factor 4 (*KLF4*) as an early adipogenic transcription factor that stimulates the expression of *C/EBPβ a*nd initiates the adipogenic transcriptional cascade. Adiponectin (*Adipoq*) discovered by Scherer *et al.*, (1995) is an adipocyte-specific secretory protein that enhances insulin sensitivity and regulates glucose metabolism. Cristancho and Lazar (2011) studied the transcriptional and epigenetic mechanisms involved in adipocyte differentiation and highlighted the complex network of genes and signaling pathways that regulate adipogenesis. These studies collectively established adipogenesis as a highly coordinated process involving multiple genes and regulatory mechanisms.

Various experimental approaches are employed to investigate adipocyte differentiation and gene expression patterns. The mouse 3T3-L1 preadipocyte cell line is widely used as an in vitro model system for studying adipogenesis. Adipocyte differentiation is regulated by a cascade of transcription factors and adipocyte-specific genes that act sequentially to control the maturation process. During the early phase of adipogenesis, transcription factors such as Krüppel-like factor 4 (*KLF4*), *CCAAT*/enhancer-binding protein beta (*C/EBPβ*), and *CCAAT*/enhancer-binding protein delta (*C/EBPδ*) are rapidly induced and initiate the adipogenic program.

These early factors activate the expression of master regulators including Peroxisome Proliferator-Activated Receptor Gamma (*PPARγ*) and *CCAAT*/enhancer-binding protein alpha*(C/EBPα*), which are essential for terminal differentiation and maintenance of the mature adipocyte phenotype. In the later stages, adipocyte-specific genes such as adiponectin, leptin, Fatty Acid-Binding Protein 4 (*FABP4*), and Glucose Transporter Type 4 (*GLUT4*) are expressed. These genes are involved in lipid metabolism, glucose uptake, fatty acid transport, and endocrine regulation. The coordinated expression of these genes ensures the successful conversion of preadipocytes into fully differentiated and metabolically active adipocytes.

Krüppel-like factor 4 (*KLF4*) is an important zinc finger transcription factor that functions during the early stage of adipocyte differentiation. It is rapidly induced following adipogenic stimulation and activates the expression of *C/EBPβ*, thereby initiating the transcriptional cascade required for adipogenesis. *KLF4* directly binds to the *C/EBPβ* promoter and acts as an immediate early regulator of adipogenesis. Experimental studies have demonstrated that knockdown of *KLF4* significantly impairs adipocyte differentiation and reduces the expression of adipogenic markers, indicating its essential role in adipocyte development (Birsoy *et al*., 2008). In addition, *KLF4* contributes to the regulation of cell cycle progression during adipogenesis, facilitating the transition of preadipocytes towards terminal differentiation. Reviews have further highlighted *KLF4* as one of the earliest transcription factors that triggers the adipogenic regulatory cascade by activating *C/EBPβ* before the induction of *PPARγ* and *C/EBPα* (Wu & Wang, 2013).

In contrast, adiponectin is a hormone secreted predominantly by mature adipocytes and serves as a marker of terminal adipocyte differentiation. Adiponectin enhances insulin sensitivity, promotes fatty acid oxidation, regulates glucose metabolism, and exhibits anti-inflammatory and anti-atherogenic properties (Scherer *et al*., 1995; Yamauch*i et al*., 2001).

High levels of adiponectin expression are associated with improved metabolic health and a reduced risk of obesity, insulin resistance, and type 2 diabetes mellitus (Kadowaki & Yamauchi, 2005). Therefore, while *KLF4* is involved in initiating adipogenesis, adiponectin reflects successful maturation and the metabolic functionality of adipocytes (Scherer *et al*., 1995; Kadowaki & Yamauchi, 2005).

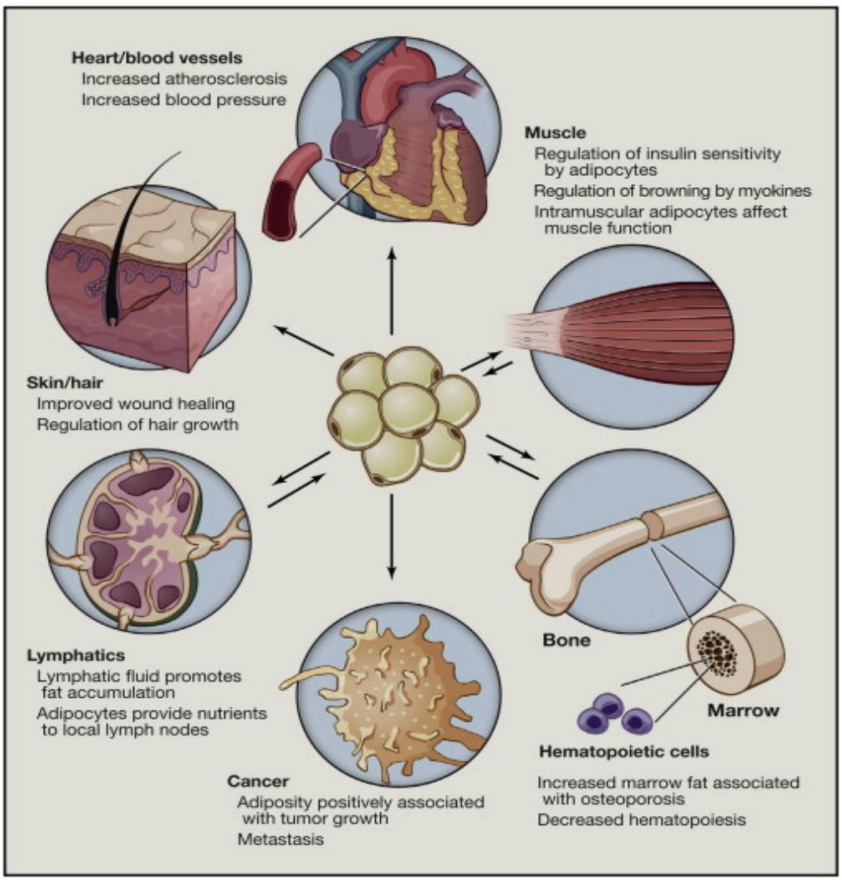


Figure 2: Adipose crosstalk with other tissues (Rosen, E. D., *et al*., 2014)

Research on adipocyte differentiation has gained considerable importance because of the increasing burden of obesity and metabolic diseases worldwide. Future studies on adipogenesis will contribute to identifying novel genes, transcription factors, and signaling pathways involved in fat cell development. Advances in molecular biology, genomics, transcriptomics, and gene-editing technologies are expected to provide deeper insights into the mechanisms regulating adipogenesis. Understanding these mechanisms may lead to the development of targeted therapies for obesity, diabetes, cardiovascular diseases, and metabolic syndrome. Adipocyte differentiation studies also have applications in regenerative medicine, stem cell biology, and tissue engineering. Furthermore, identifying biomarkers

associated with adipogenesis could improve disease diagnosis and facilitate the development of personalized therapeutic strategies. Therefore, continued research in this field holds great promise for improving human health and combating metabolic disorders.

In conclusion, the gene expression analysis of *KLF4* and *Adipoq* revealed their significant roles during different phases of adipocyte differentiation. *KLF4* functions as an early regulator that initiates the adipogenic transcriptional cascade, whereas *Adipoq* serves as a marker of terminal differentiation and metabolic functionality of mature adipocytes. The observed expression patterns confirm the successful progression of adipogenesis and emphasize the coordinated regulation of adipocyte-specific genes. Understanding the molecular functions of *KLF4* and *Adipoq* contributes to a better understanding of adipose tissue biology and provides a foundation for future studies aimed at developing novel therapeutic approaches for obesity, insulin resistance, and associated metabolic diseases.

Chapter 2

Review of Literature

2.1 Overview of Adipogenesis

Adipocyte differentiation, often known as adipogenesis, represents a complex, multistep biological process wherein undifferentiated precursor cells transform into mature adipocytes that are specialized for lipid storage and endocrine functions (Rosen *et al.*, 2000). This process is crucial for sustaining whole-body energy homeostasis, regulating lipid metabolism, and managing insulin sensitivity (Rosen *et al.*, (2000), Kershaw *et al.*, (2004) indicating that adipose tissue is no longer viewed solely as a fat storage organ; it is now acknowledged as an active endocrine organ that secretes various adipokines that affect systemic metabolism.

2.2 Adipose Tissue as an Endocrine Organ and Types of Adipocytes

Adipose tissue consists of various types of adipocytes that vary in their morphology, function, and metabolic activity, all of which are essential for maintaining energy balance and systemic metabolism (Rosen *et al.*, 2014). White adipocytes represent the predominant type and are mainly adapted for energy storage, accumulating triglycerides in a single large lipid droplet (Kershaw *et al.*, 2004). Additionally, these cells act as endocrine entities by releasing adipokines such as leptin and adiponectin, which play a role in regulating appetite, insulin sensitivity, and inflammatory responses (Rosen *et al.*, 2014). The excessive buildup of white adipose tissue has been closely linked to obesity, insulin resistance, and metabolic syndrome (Kershaw *et al.*, 2004). Brown adipocytes, on the other hand, are specialized for energy expenditure via non-shivering thermogenesis and are distinguished by the presence of numerous small lipid droplets and a high density of mitochondria (Cannon *et al.*, 2004).

2.3 3T3-L1 Preadipocyte Cell Line as an In Vitro Model

The murine 3T3-L1 preadipocyte cell line has been widely employed as a model system for investigating adipogenesis due to its capacity to consistently differentiate into adipocytes under controlled in vitro conditions (Green *et al.*, 1975). This model closely resembles the physiological process of adipocyte development, rendering it highly valuable for exploring molecular mechanisms, gene expression patterns, and signaling pathways associated with adipogenesis.

Student *et al*., (1980) noted that 3T3-L1 cell line is derived from mouse embryonic fibroblasts and has become one of the most widely accepted in vitro models for examining adipocyte biology and also these cells display a fibroblast-like morphology in their undifferentiated state but adopt a rounded shape with intracellular lipid droplets upon differentiation. Under suitable hormonal stimulation, 3T3-L1 cells experience a synchronized differentiation process that encompasses growth arrest, mitotic clonal expansion, early differentiation, and terminal maturation (Tang *et al*., 2003). The reproducibility and clearly defined stages of differentiation render this cell line particularly appropriate for mechanistic studies (Tang *et al*., 2003).

Adipogenesis in 3T3-L1 cells occurs through several distinct phases. Initially, the cells achieve confluence and experience growth arrest, entering the G0 phase of the cell cycle. Following induction, the cells re-enter the cell cycle and undergo mitotic clonal expansion, which is crucial for subsequent differentiation (Tang *et al*., 2003). Reports suggests that after clonal expansion, the cells commence early differentiation, which is characterized by the activation of transcription factors and metabolic enzymes. Ultimately, terminal differentiation takes place, during which the cells accumulate lipids and express genes specific to adipocytes (Tang *et al*., 2003).

Lane *et al*., (1999) identified the differentiation of 3T3-L1 cells is typically induced by a hormonal cocktail that includes Insulin, Dexamethasone, and IBMX. Each of these components serves a specific function in initiating adipogenesis. Insulin activates the PI3K/Akt signaling pathway, facilitating glucose uptake and lipid synthesis. Dexamethasone, a glucocorticoid, promotes the transcription of early adipogenic genes (Zhang *et al.,* 2004). IBMX functions as a phosphodiesterase inhibitor, elevating intracellular cAMP levels and activating protein kinase A (PKA), which subsequently activates CREB mediated transcription (Zhang *et al.,* 2004).

2.4 Transcriptional Regulation of Adipogenesis

Rosen *et al*., (2002) suggested that the differentiation process is governed by a hierarchical cascade of transcription factors. The early phase is characterized by the induction of *C/EBPβ* and *C/EBPδ*, which activate downstream adipogenic genes. Peroxisome proliferator-activated receptor gamma (*PPARγ*) is recognized as the principal regulator of adipogenesis. Its activation is crucial for both the initiation and maintenance of the adipocyte phenotype

(Tontonoz *et al.*, 1994). *PPARγ* collaborates with *C/EBPα* to modulate genes that are integral to lipid metabolism and insulin sensitivity (Farmer *et al*., 2006).

Adipogenesis is governed by various signaling pathways. The PI3K/Akt pathway is vital for insulin-mediated adipogenesis, as it facilitates glucose uptake and lipogenesis (Salties *et al*., 2001). Conversely, the Wnt/β-catenin signaling pathway acts to inhibit adipogenesis by down regulating *PPARγ* expression. The activation of Wnt signaling keeps cells in an undifferentiated state (Ross *et al.*, 2000). AMP-activated protein kinase (AMPK) serves as a metabolic regulator, inhibiting adipogenesis by decreasing lipid accumulation (Hardie *et al*., 2012).

2.5 Molecular Markers of Adipogenesis

Spiegelman *et al*., (2001) conducted a study for the synchronized upregulation of *FABP4*, *FAS*, *Adipoq*, and Leptin during the differentiation of 3T3-L1 cells which signifies the transition of preadipocytes into metabolically active and functionally mature adipocytes. Each of these genes plays a distinct role in lipid metabolism, energy regulation, and endocrine signaling, thereby validating the successful adipogenesis and functional maturation of adipose cells.

PPARγ is acknowledged as the key regulator of adipocyte differentiation and is vital for the initiation of the adipogenic program (Rosen *et al*., 1999; Moseti *et al*., 2016). It is a member of the nuclear receptor superfamily and operates as a ligand-activated transcription factor that governs genes associated with lipid metabolism, glucose homeostasis, and adipocyte maturation (Moseti *et al*., 2016). During the process of adipogenesis, *PPARγ* forms a heterodimer with retinoid X receptor (RXR) and binds to specific DNA response elements to activate adipocyte-specific genes such as *FABP4*, *Adipoq*, and Leptin (Reactome, 2023). The expression of *PPARγ* is stimulated following the activation of early transcription factors like C/EBP (Reactome, 2023). The expression of *PPARγ* is upregulated following the activation of early transcription factors, including *C/EBPβ* and *C/EBPδ* (Rosen *et al.*, 1999). Moreover, PPARγ creates a positive feedback mechanism with *C/EBPα*, which helps to stabilize and sustain the differentiated adipocyte phenotype (Tang *et al*., 2003). The absence of *PPARγ* function leads to disrupted adipogenesis, thereby affirming its pivotal role in the development of adipocytes (Moseti *et al*., 2016).

Krüppel-like factor 4 (*KLF4*) is a zinc finger-containing transcription factor that belongs to the Krüppel-like factor family of DNA-binding proteins. It plays a crucial role in regulating

cellular processes such as proliferation, differentiation, apoptosis, and maintenance of stem cell pluripotency. *KLF4* was initially identified in epithelial tissues of the gastrointestinal tract but is now recognized as an important regulator in various biological processes (Dang *et al*., 2000).

The *CCAAT*/enhancer-binding proteins (*C/EBPs*) constitute a family of transcription factors that oversee various stages of adipocyte differentiation (Rosen *et al*., 1999). *C/EBPβ* and *C/EBPδ* are swiftly activated during the initial phase of adipogenesis in response to hormonal stimulation with MDI (Reactome, 2023). *C/EBPβ* and *C/EBPδ* are swiftly activated during the initial phase of adipogenesis in response to hormonal stimulation with MDI (Reactome, 2023). These transcription factors are triggered by signaling pathways such as cAMP/PKA and glucocorticoid signaling (Tang et al., 2003). They are essential for initiating adipogenesis by promoting the expression of *PPARγ* and *C/EBPα* (Rosen *et al*., 1999; Moseti *et al.*, 2016). Additionally, *C/EBPβ* and *C/EBPδ* govern mitotic clonal expansion, which is necessary for the advancement of differentiation (Tang *et al*., 2003).

FABP4 is a cytoplasmic fatty acid-binding protein that is predominantly expressed in mature adipocytes and acts as a significant marker of adipocyte differentiation (Kershaw *et al*., 2004). Its expression markedly increases during the final stages of adipogenesis (Moseti *et al.*, 2016). Gan *et al.*, (2015) reported that *FABP4* binds to long-chain fatty acids and aids in their intracellular transport to organelles such as mitochondria, endoplasmic reticulum, and lipid droplets, and this role is crucial for the coordination of lipid metabolism, encompassing fatty acid storage and oxidation. In addition to its function in lipid transport, *FABP4* also modulates metabolic signaling pathways and engages with transcription factors such as *PPARγ* (Gan *et al.*, 2015; Moseti *et al*., 2016). An increase in *FABP4* expression signifies heightened lipid accumulation and suggests successful maturation of adipocytes (Kershaw *et al.*, 2004). Moreover, *FABP4* has been associated with metabolic disorders including obesity and insulin resistance (Gan *et al*., 2015).

*Fatty Acid Synthase (*FAS) is a crucial enzyme that plays a significant role in de novo lipogenesis and is markedly upregulated during the differentiation of adipocytes (Moseti *et al*., 2016). It facilitates the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA, resulting in the formation of palmitate (Gan *et al*., 2015). These fatty acids are then esterified into triglycerides and stored in lipid droplets, which are typical of mature adipocytes (Gan *et al., 2015).*

Adiponectin is a hormone specific to adipocytes that is secreted in large quantities by mature adipocytes and plays a vital role in metabolic regulation (Kershaw *et al.*, 2004). Its expression significantly rises during the later stages of adipocyte differentiation (Moseti *et al*., 2016). Adiponectin enhances insulin sensitivity by facilitating glucose uptake in skeletal muscle and decreasing hepatic glucose production. It also promotes fatty acid oxidation and diminishes lipid accumulation in peripheral tissues (Hotamisligil *et al*., 2006). Therefore, its increased expression during adipogenesis indicates the acquisition of endocrine function by mature adipocytes (Moseti *et al.*, 2016).

Leptin is a hormone produced by adipocytes that is essential for the regulation of energy homeostasis and appetite (Kershaw *et al.*, 2004). Its levels are closely linked to adipocyte differentiation and rise in response to lipid accumulation (Gan *et al*., 2015). Leptin influences the hypothalamus to reduce appetite and boost energy expenditure. Additionally, it plays a role in the regulation of glucose metabolism and fatty acid oxidation in peripheral tissues (Hotamisligil *et al.*, 2006). During the process of adipogenesis, the expression of leptin is increased in conjunction with *PPARγ*, signifying its function as a marker for mature adipocytes (Moseti *et al*., 2016). However, in cases of obesity, heightened levels of leptin can result in leptin resistance, which hinders its regulatory capabilities (Hotamisligil *et al*., 2006). Therefore, leptin functions as both a biologically active hormone and a marker of adipocyte maturation (Kershaw *et al*., 2004).

2.6 Role of Insulin in Adipogenesis

Insulin plays a crucial role in both the initiation and progression of adipocyte differentiation by activating key signaling pathways that regulate glucose uptake, lipid synthesis, and gene expression (Saltiel *et al*., 2001; Ros*en et* al., 2000). In the 3T3-L1 model, insulin is an essential component of the MDI cocktail and acts primarily during the early and intermediate stages of adipogenesis (La*ne et al.*, 1999).

At the molecular level, insulin binds to the insulin receptor (IR), leading to its autophosphorylation and activation of downstream signaling cascades such as the phosphoinositide 3-kinase (PI3K)/Akt pathway (Saltiel *et al.*, 2001). Activation of the PI3K/Akt pathway promotes glucose uptake and enhances lipogenesis, which are critical for adipocyte maturation (Kohn *et al.*, 1996). Akt signalling also stimulates the expression of adipogenic transcription factors, including *PPARγ* and *C/EBPα*, thereby promoting differentiation (Rosen *et al.*, 2000).

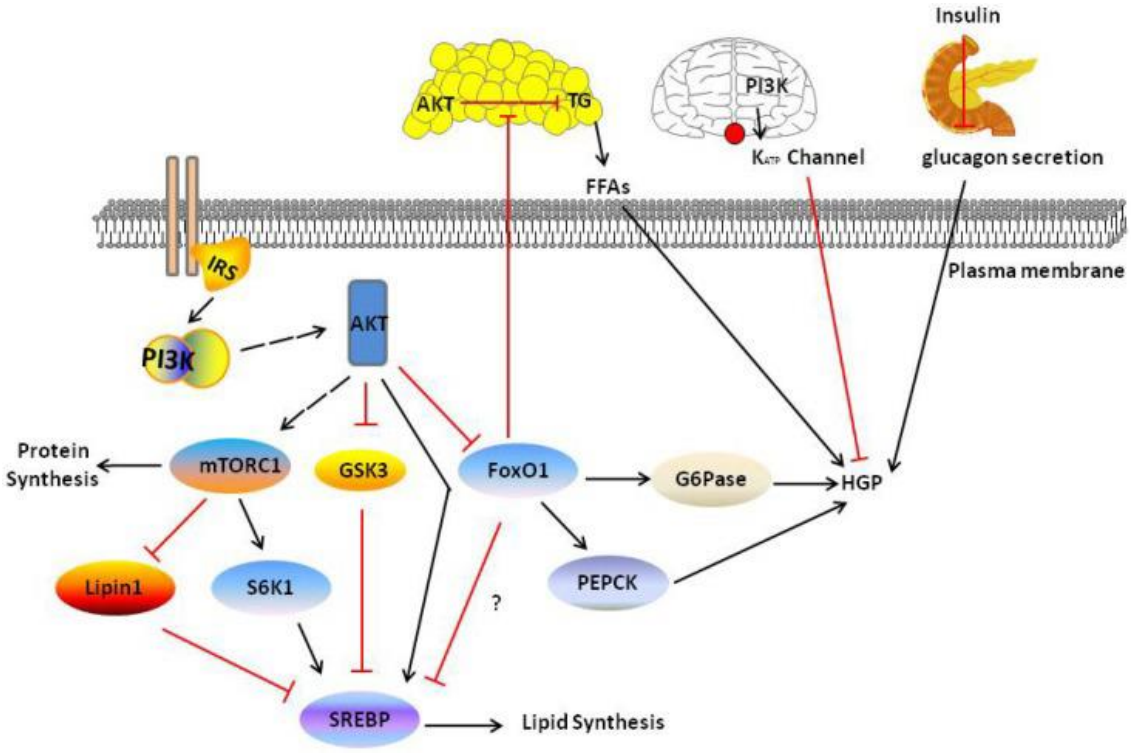


Figure 3: Role of insulin in molecular signaling pathways regulating adipocyte differentiation (Huang, X *et al*., 2018)

Insulin further promotes adipogenesis by enhancing the expression and translocation of Glucose Transporter Type 4 (*GLUT4*) to the plasma membrane, thereby increasing glucose uptake into the cell (Shepherd *et al*., 1999). The elevated intracellular glucose is then utilized for the synthesis of glycerol and fatty acids, which are crucial for the formation of triglycerides and the accumulation of lipid droplets (Saltiel *et al*., 2001). In addition to its role in metabolic regulation, insulin also affects gene expression by modulating transcriptional activity. It boosts the expression of lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), which are essential for fatty acid synthesis during the differentiation of adipocytes (Rosen *et al.*, 2000). Insulin signaling also aids in maintaining the differentiated state by supporting *PPARγ* activity (Farmer *et al*., 2006).

Furthermore, insulin is involved in the mitotic clonal expansion phase of adipogenesis, a period during which preadipocytes re-enter the cell cycle prior to terminal differentiation (Tang *et al.*, 2003). In the absence of insulin signaling, this phase is compromised, resulting in diminished adipocyte formation. Additionally, insulin regulates the secretion of adipokines such as adiponectin and leptin, which further enhance the endocrine functions of mature adipocytes (Kershaw *et al.*, 2004). However, prolonged exposure to elevated insulin levels

can result in insulin resistance, disrupting adipocyte function and contributing to metabolic disorders such as obesity and type 2 diabetes (Hotamisligil *et al.,* 2006).

2.7 Lipid Accumulation During Adipocyte Maturation

During the final phases of adipocyte differentiation, 3T3-L1 cells experience significant metabolic alterations marked by the buildup of intracellular lipid droplets and the activation of lipid metabolic pathways and also this phenomenon entails the synchronized upregulation of lipogenic enzymes, including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and glycerol-3-phosphate acyltransferase, which together promote the synthesis and storage of triglycerides (Rosen *et al.,* 2014; Moseti *et al.,* 2016).

The buildup of lipid droplets is a hallmark of mature adipocytes and indicates the functional shift of the cell towards energy storage (Farmer *et al., 2*006). Besides lipid synthesis, adipocytes also manage lipolysis via enzymes such as hormone-sensitive lipase (HSL), thus preserving a balance between lipid storage and mobilization also metabolic activities are meticulously regulated by transcription factors like *PPARγ* and SREBP-1c, which guarantee the appropriate expression of genes involved in lipid homeostasis (Farmer *et al.,* 2006).

Adipocyte differentiation is essential for sustaining metabolic health, and its disruption is closely linked to the onset of metabolic disorders such as obesity, insulin resistance, and type 2 diabetes (Kahn *et al.,* 2000). Adequate adipogenesis enables the safe storage of excess energy in adipose tissue; however, dysfunctional differentiation results in ectopic lipid accumulation in organs like the liver and muscle, leading to metabolic impairment (Rosen *et al.*, 2014).

2.8 Adipogenesis in Obesity and Metabolic Disorders

Excessive adipogenesis leads to the expansion of adipose tissue and the development of obesity, which is frequently associated with altered secretion of adipokines and chronic inflammation and disruption between adipocyte hypertrophy and hyperplasia further intensifies metabolic complications, underscoring the necessity for tightly regulated adipogenic processes (Kahn *et al*., 2000; Rosen *et al.,* 2014). Consequently, comprehending the molecular mechanisms that control adipocyte differentiation is vital for formulating therapeutic approaches to address metabolic diseases (Kahn *et al.,* 2000; Rosen *et al*., 2014).

Hotamisligil *et al.,* (2006) reported that adipocytes are not solely responsible for lipid storage; they also act as active endocrine cells that release a range of cytokines and

adipokines, which are essential in regulating inflammation and maintaining metabolic homeostasis. In the context of obesity, adipose tissue experiences chronic low-grade inflammation, marked by an increased release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). These cytokines disrupt insulin signalling pathways, resulting in insulin resistance and metabolic dysfunction (Lane *et al*., 1999). Moreover, the infiltration of immune cells, particularly macrophages, into adipose tissue further exacerbates the inflammatory response (Lane *et al.*, 1999).

Oxidative stress has a complex impact on adipocyte differentiation, influenced by the concentration of reactive oxygen species (ROS) within the cell also moderate ROS levels function as signaling molecules that enhance adipogenesis by activating transcription factors like *PPARγ* and *C/EBPs* (Tormos *et al.,* 2011). In contrast, excessive production of ROS causes oxidative damage, which hinders adipocyte differentiation and disrupts cellular function (Saltie*l et al*., 2001). Additionally, oxidative stress plays a role in inflammation and insulin resistance, thereby connecting it to various metabolic disorders (Saltiel *et al*., 2001). Epigenetic mechanisms, such as DNA methylation, histone modification, and chromatin remodeling, are crucial in the regulation of adipocyte differentiation and also these modifications affect gene expression without changing the DNA sequence, thereby allowing for a dynamic control of adipogenic genes (Musri *et al*., 2010). During the process of adipogenesis, there is an increase in histone acetylation at the promoters of essential genes like *PPARγ* and *C/EBPα*, which facilitates their transcription. Additionally, alterations in DNA methylation patterns enable the activation of genes specific to adipocytes (Musri *et al*., 2010). These epigenetic modifications are vital for ensuring the correct progression of differentiation and the preservation of the adipocyte phenotype (Musri *et al*., 2010).

MicroRNAs (miRNAs) are small non-coding RNAs that modulate gene expression at the post transcriptional level and are significant in the process of adipogenesis (Kajimoto *et al.*, 2006). Certain miRNAs encourage adipocyte differentiation by inhibiting specific pathways, while others hinder adipogenesis by targeting critical transcription factors such as *PPARγ*. The dysregulation of miRNA expression has been associated with metabolic disorders, underscoring their significance in adipocyte biology (Kajimoto *et al.,* 2006).

2.9 Therapeutic Implications of Adipogenesis

Farmer *et al.,* (2006) reported that understanding the molecular mechanisms involved in adipocyte differentiation carries substantial therapeutic significance for addressing metabolic

diseases and also focusing on pivotal transcription factors like *PPARγ* and signaling pathways such as PI3K/Akt and AMPK presents potential approaches for influencing adipogenesis. The investigations by Rayalam *et al*., (2008) are underway into pharmacological agents that improve insulin sensitivity or curb excessive adipogenesis for the management of obesity and type 2 diabetes. Furthermore, natural substances like polyphenols have demonstrated encouraging effects in modulating adipocyte differentiation and lipid metabolism.

The differentiation of adipocytes in 3T3-L1 cells is a meticulously coordinated process that encompasses transcriptional regulation, signaling pathways, epigenetic modifications, and metabolic alterations (Rosen *et al.*, 2014). Adequate regulation of adipogenesis is crucial for sustaining metabolic homeostasis, whereas its disruption is linked to the onset of metabolic disorders such as obesity and diabetes (Rosen *et al.*, 2014). 3T3-L1 cell model continues to yield significant insights into adipocyte biology and acts as a robust tool for investigating the mechanisms that underlie adipogenesis and its related diseases (Green *et al.*, 1975; Tang *et al*., 2003).

2.10 Summary of Adipocyte Differentiation and 3T3-L1 Model

Krüppel-like factor 4 (*KLF4*) is an early adipogenic transcription factor that plays a pivotal role in initiating adipocyte differentiation by regulating the expression of key transcription factors, including *C/EBPβ*, *C/EBPδ*, *PPARγ*, and *C/EBPα* (Birsoy *et al*., 2008). Among these, *Adipoq* is a major adipokine secreted by mature adipocytes that enhances insulin sensitivity, promotes fatty acid oxidation, and regulates glucose homeostasis (Kadowaki *et al*., 2008). Furthermore, dysregulation of *KLF4* or *Adipoq* expression has been associated with obesity, insulin resistance, and type 2 diabetes, highlighting their potential as therapeutic targets for the prevention and treatment of metabolic disorders (Muhi *et al*., 2025).

Chapter 3

Aim and Objectives

Aim

To investigate the expression of *KLF4* and *Adipoq* genes in 3T3-L1 cells during different stages of adipogenesis.

Objectives

- To induce adipocyte differentiation in 3T3-L1 cells.
- To confirm adipogenesis by morphological observation.
- To isolate total RNA and synthesise cDNA.
- To determine the expression levels of *KLF4* and *Adipoq* using qRT-PCR.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Consumables: T-25 tissue culture flasks, six-well culture plates, cryovials, sterile centrifuge tubes, 0.5 mL and 1.5 mL micro centrifuge tubes, sterile micropipette tips, Neubauer haemocytometer with coverslips, gel casting tray and comb, Erlenmeyer flask, PCR tubes and PCR plates, aluminium foil, syringe filter units, Whatman filter paper, disposable gloves, and other sterile laboratory consumables.

4.1.2 Cell Culture Reagents: High-glucose Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin solution, 0.25 % Trypsin-EDTA, Dimethyl Sulfoxide (DMSO) are procured from Sigma-Aldrich, (USA). Fetal Bovine Serum (FBS) (Gibco), Phosphate-Buffered Saline (PBS), Sodium bicarbonate (3.7 g/L), and sterile distilled water.

4.1.3 Cryopreservation Reagents: Cryopreservation medium consisting of DMEM, Fetal Bovine Serum and DMSO was used for long-term storage of cells. 70 % ethanol was used for surface disinfection during cryopreservation and revival procedures.

4.1.4 Cell Counting Reagents: A 0.4 % Trypan blue solution was used to determine cell viability and perform viable cell counting using a Neubauer haemocytometer.

4.1.5 Adipocyte Differentiation Reagents: Adipogenic differentiation cocktail consisted of 3-Isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX), and Insulin. These reagents were used to induce differentiation of 3T3-L1 preadipocytes into mature adipocytes.

4.1.6 Oil Red O Staining Reagents: Oil Red O powder, isopropanol, paraformaldehyde (4 %), sodium hydroxide, hydrochloric acid, and deionised water were used for visualisation of intracellular lipid droplets in differentiated adipocytes.

4.1.7 RNA Isolation Reagents: Total RNA was isolated using TRIzol™ reagent (Invitrogen,USA). Chloroform, 75 % ethanol, DEPC-treated water, and RNaseZap solution were used during RNA extraction and purification to ensure RNase-free conditions.

4.1.8 Agarose Gel Electrophoresis Reagents: Agarose, 1× TAE buffer, ethidium bromide, 6× loading dye, and RNA molecular weight marker were used for evaluation of RNA integrity by agarose gel electrophoresis.

4.1.9 cDNA Synthesis and Quantitative Real-Time PCR Reagents: First-strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Gene expression analysis was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA) together with gene-specific forward and reverse primers.

4.1.10 Instruments : The major instruments used during the study included a 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),Thermal cycler (Thermo Fisher Scientific, USA), CFX96 Real-Time PCR System (Bio-Rad Laboratories, USA), Electrophoresis apparatus (Bio-Rad, USA), Gel documentation system (Bio-Rad, USA), Vortex mixer, Analytical balance (Shimadzu, Japan), pH meter (Thermo Scientific, USA), Biophotometer (Eppendorf, Germany), UV-Visible Spectrophotometer (Thermo Scientific, USA), Vacuum filtration system, Refrigerator, Deep freezer (-20°C and -80°C), Magnetic stirrer, Microwave oven, and standard laboratory micropipettes.

4.2 Methodology

4.2.1 Cell Culture

In the present study, 3T3-L1cell line was procured from National Centre for Cell Science (NCCS), Pune, India, and maintained according to standard cell culture procedures under aseptic conditions. Cells were maintained in high-glucose DMEM supplemented with 10 % fetal bovine serum containing 1 % penicillin-streptomycin and incubated at 37 °C in a humidified incubator containing 5 % CO_2. Cell morphology, growth characteristics, and confluency were monitored daily using an inverted phase-contrast microscope. The culture medium was replaced every two days to ensure an adequate supply of nutrients and to remove accumulated metabolic waste products. Cells were sub cultured once they reached approximately 70–80 % confluency.

4.2.2 Preparation of Dulbecco's Modified Eagle Medium (DMEM)

Complete Dulbecco's Modified Eagle Medium (DMEM) was prepared according to the manufacturer's instructions under sterile conditions. Powdered high-glucose DMEM (Gibco) was dissolved in approximately 900 mL of sterile distilled water with continuous stirring using a magnetic stirrer until the medium was completely dissolved. Sodium bicarbonate (3.7 g/L) was then added gradually, and the solution was stirred until it became completely clear and the final volume was made up to 1000 mL with sterile distilled water. The prepared basal medium was sterilized through a 0.22 μm membrane vacuum filtration unit under aseptic conditions and collected in a sterile storage bottle. Subsequently, within a Class II biosafety cabinet, fetal bovine serum (10 %) and 1 % penicillin-streptomycin solution were aseptically added to prepare complete DMEM. The prepared complete medium was stored at 4°C until further use in cell culture experiments.

4.2.3 Subculturing procedure of 3T3-L1cells

Cells were sub cultured to prevent over-confluency and maintain healthy cell growth. Enzymatic dissociation using 0.25 % trypsin-EDTA was employed to detach adherent 3T3-L1 cells from the culture flask, followed by transfer into fresh culture flasks containing complete growth medium.

- The spent culture medium was carefully aspirated and discarded.
- The cell monolayer was washed twice with 1 mL sterile phosphate-buffered saline (PBS) to remove residual serum and dead cells.
- 400 µL of 0.25 % Trypsin-EDTA solution was added to completely cover the cell monolayer.
- The culture flask was incubated at 37°C for 1–2 minutes.
- Cell detachment was monitored under an inverted phase-contrast microscope until approximately 70–80 % of the cells became rounded and detached.
- The enzymatic activity of trypsin was neutralised by adding 1 mL of complete DMEM supplemented with 10 % FBS.
- The detached cells were gently pipetted several times along the sides of the flask to obtain a uniform single-cell suspension.
- Adequate of cell suspension was transferred into two T25 flask containing 5 ml medium.

- The cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 for further growth.
- Cell morphology and proliferation were monitored regularly using an inverted phase-contrast microscope.

4.2.4 Cell Counting Using Trypan Blue Exclusion Method

Cell counting was performed using the Trypan Blue exclusion assay**,** which differentiates viable cells from non-viable cells based on membrane integrity. Viable cells exclude the dye and appear transparent, whereas non-viable cells absorb the dye and appear blue under the microscope. The total viable cell count was determined using a Neubauer haemocytometer before cell seeding.

- The detached cell suspension was mixed thoroughly to obtain a uniform suspension.
- 10 µL of cell suspension was mixed with 10 µL of 0.4 % Trypan Blue solution in a sterile micro centrifuge tube.
- The mixture was gently mixed by pipetting and incubated at room temperature for less than 3 minutes.
- Approximately 10 µL of the stained cell suspension was loaded into a Neubauer haemocytometer fitted with a coverslip.
- The haemocytometer was placed under an inverted phase-contrast microscope.
- Viable (unstained) and non-viable (blue-stained) cells were observed.
- Viable cells in the four large corner squares of the haemocytometer were counted.
- The L-rule was followed during counting, where cells touching the top and left boundary lines were included, whereas those touching the bottom and right boundary lines were excluded.
- The total viable cell concentration was calculated using the following formula:
  Total cells/mL = (Total cells counted × Dilution factor × 10^4) / Number of squares counted.

4.2.5 Cell Seeding

- 1mL of the prepared cell suspension was diluted with complete DMEM to a final volume of 6 mL.
- The cell suspension was seeded into 6-well culture plate at a density of 10000-30000 cells/well.

- The volume of each well was adjusted to 3 mL with complete DMEM supplemented with 10 % FBS.
- The culture plate was gently swirled to ensure uniform distribution of cells.
- The seeded plates were incubated at 37°C in a humidified atmosphere containing 5 % CO_2 for 48 hours.
- Cell morphology and confluency were monitored regularly using an inverted phase-contrast microscope.

4.2.6 Adipogenic Differentiation of 3T3-L1 Cells

In the present study, differentiation was induced using a standard adipogenic cocktail consisting of3-Isobutyl -1-methylxanthine (IBMX), dexamethasone (DEX), and insulin.

Table 1: Differentiation Schedule of 3T3-L1 Cells

Day	Procedure
Day 0	Cells reached 70–80 % confluency and were induced with MDI medium (DMEM + 10 % FBS supplemented with 100 µL IBMX, 4 µL DEX, and 20 µL insulin).
Day 2	Cells were collected for RNA isolation.
Day 4	MDI medium was replaced with insulin medium (DMEM + 10 % FBS supplemented with insulin).
Day 6	Cells were collected for RNA isolation, and insulin medium was replaced with complete DMEM.
Day 8	Media renewal with DMEM (10 %).
Day 10	Media renewal with DMEM (10 %).
Day 12	Cells were collected for RNA isolation, and Oil Red O staining was performed to evaluate lipid accumulation.

4.2.7 Oil Red O Staining

Oil Red O staining is a histological technique used to detect and visualize neutral lipids accumulated in differentiated adipocytes (Pittenger *et al*. (1999)). Oil Red O is a fat-soluble

dye that selectively stains intracellular lipid droplets red, enabling qualitative assessment of adipogenic differentiation under an inverted phase-contrast microscope.

4.2.7.1 Staining Procedure

- The culture medium was aspirated from each well. Cells were washed once with 1 mL PBS.
- 2 mL of 4 % paraformaldehyde was added gently along the sides of each well.
- Plates were covered with aluminium foil and incubated for 30 minutes at room temperature.
- The fixative was discarded, and cells were washed with 60 % isopropanol for 5 minutes.
- The isopropanol was removed completely.
- 2 mL of freshly prepared Oil Red O working solution was added to each well.
- Plates were incubated for 15 minutes at room temperature. Wells were briefly rinsed with isopropanol and lipid droplets were observed using an inverted phase-contrast microscope.

4.2.8 RNA Isolation

Total RNA was isolated from differentiated and undifferentiated 3T3-L1 cells using the TRIzol™ reagent method. TRIzol lyses cells, inactivates RNases, and facilitates separation of RNA from DNA and proteins through phase separation using chloroform. The purified RNA was subsequently used for complementary DNA (cDNA) synthesis and quantitative gene expression analysis.

- Cell culture medium was carefully aspirated from each well.
- Cells were washed gently with 1 mL sterile phosphate-buffered saline (PBS).
- 1 mL of TRIzol™ reagent was added directly to each well.
- Cells were lysed by repeated pipetting until a homogeneous lysate was obtained.
- The lysate was transferred into a sterile RNase-free micro centrifuge tube.
- The tubes were incubated at room temperature for 5 minutes.
- 200 µL of chloroform was added to each tube.
- Tubes were tightly capped and shaken vigorously for 15 seconds.
- Samples were incubated at room temperature for 2–3 minutes.
- The lysates were centrifuged at 12,000 × g for 15 minutes at 4 °C.
- Following centrifugation, three layers were observed:

- Upper aqueous phase (RNA)
- Interphase (DNA)
- Lower organic phase (proteins)

- The clear upper aqueous phase was carefully transferred to a fresh RNase-free tube.
- An equal volume of isopropanol was added and mixed gently.
- Samples were incubated at room temperature for 10 minutes.
- Tubes were centrifuged at 12,000 × g for 10 minutes at 4 °C.
- The supernatant was discarded carefully without disturbing the RNA pellet.
- The pellet was washed with 1 mL of 75 % ethanol.
- Samples were centrifuged at 7,500 × g for 5 minutes at 4 °C.
- Ethanol was removed completely, and the RNA pellet was air-dried for 5–10 minutes.
- The RNA pellet was dissolved in RNase-free water.
- RNA samples were stored at −80 °C until further analysis.

4.2.9Assessment of RNA Quality

4.2.9.1 Purity

The quantification and purity check of the isolated RNA was done using a biophotometer by measuring the absorbance. A A260/A280 ratio and A260/A230 ratio of approximately 2.0 indicated high-quality RNA with minimal protein or phenol contamination. RNA samples exhibiting A260/A280 and A260/A230 ratios approximately 2.0 were considered as pure and used for downstream applications such as cDNA synthesis and quantitative real-time PCR (qRT-PCR).

4.2.9.2 Integrity

RNA integrity was assessed by agarose gel electrophoresis. Intact RNA produces distinct 28S and 18S ribosomal RNA bands**,** whereas degraded RNA appears as smeared bands. This analysis ensured that high-quality RNA was used for cDNA synthesis.

- A 1.2 % agarose gel was prepared using 1× TAE buffer.
- Agarose was completely dissolved by heating.
- After cooling, ethidium bromide was added carefully.
- The gel solution was poured into a casting tray fitted with a comb.
- The gel was allowed to solidify completely.

- RNA samples were mixed with 6× loading dye.
- Samples and an RNA ladder were loaded into individual wells.
- Electrophoresis was carried out in 1× TAE buffer at 80–100 V.
- Following electrophoresis, RNA bands were visualized using a gel documentation system.
- The presence of sharp 28S and 18S rRNA bands confirmed good RNA quality.

4.2.10 cDNA Synthesis

Complementary DNA (cDNA) was synthesized from purified RNA using the iScript™ cDNA Synthesis Kit. Reverse transcriptase converts RNA into cDNA, which serves as the template for quantitative real-time PCR.

Table 2: cDNA reaction mixture

Component	Volume (µL)
5× iScript Reaction Mix	4
iScript Reverse Transcriptase	1
RNA Template	1
Nuclease-free Water	14
Total Volume	20

Table 3: cDNA Thermal Cycling Condition

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

4.2.11 Primer Dilution

Lyophilized primers were briefly centrifuged, Nuclease-free water was added to prepare the stock solution according to the manufacturer's instructions. Working primer solutions were prepared by appropriate dilution. Forward and reverse primers were mixed separately. Primer solutions were stored at −20°C until use.

Table 4: Primer sequence used for qRT-PCR

Sl No	Gene Symbol	Gene Name	Primer Sequence	Reference
1	*KLF4*	Krüppel-like factor 4	Forward: 5′GCGGGAAGGGAGAAGACA 3’ Reverse: 5′GGCTCGGGCAGCTGCT 3’	Yamaguchi *et al*., (2017).
2	*Adipoq*	Adiponectin	Forward: 5′CCACTTTCTCCTCATTTCTG 3’ Reverse: 5′CTAGCTCTTCAGTTGTAGTAAC3’	Odeniyi *et al*., (2024).
3	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	Forward: 5′ACTCCACTCACGGCAAATTC 3’ Reverse: 5′TCTCCATGGTGGTGAAGACA 3’	Xie *et al*., (2025).

4.2.12 Quantitative Real-Time PCR

Quantitative real-time PCR was performed using SYBR Green chemistry to quantify the relative expression of *KLF4*and *Adipoq* genes. Gene expression levels were normalized to the housekeeping gene and analysed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method **(** Livak, K. J., & Schmittgen, T. D. (2001)).

- The qPCR master mix was prepared on ice.
- Appropriate volumes of SYBR Green Supermix, forward primer, reverse primer, cDNA template, and nuclease-free water were mixed thoroughly.
- 20 µL of reaction mixture was dispensed into each PCR well.
- Each sample was analysed in triplicate.
- The PCR plate was sealed, briefly centrifuged, and placed in the CFX96 Real-Time PCR System (Bio-Rad Laboratories, USA).

- Amplification was carried out using the optimized thermal cycling conditions, data were collected during each extension cycle.

Table 5: qRT-PCR Reaction Mixture

Component	Volume (µL)
SYBR Green Supermix	10
Forward Primer	1
Reverse Primer	1
cDNA Template	2
Nuclease-free Water	6
Total volume	20

Table 6 : qRT-PCR Thermal Cycling Conditions

Step	Temperature	Time	Cycles
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	10 sec	40
Annealing	60°C	30 sec	40
Extension	72°C	30 sec	40

4.2.13 Melt Curve Analysis

Melt curve analysis was performed immediately after PCR amplification to verify amplification specificity. A single sharp melting peak indicated amplification of a specific PCR product, whereas multiple peaks suggested non-specific amplification or primer-dimer formation.

- Melt curve analysis was initiated automatically after completion of PCR amplification.
- The temperature was gradually increased while continuously monitoring fluorescence.

- Melting peaks were analysed using Bio-Rad CFX Manager software.
- Samples showing a single sharp peak were considered to have specific amplification.

4.2.14 Analysis of Gene Expression

Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method **(**Livak,Schmittgen, T. D., *et al*., 2001), which compares Ct values of target genes after normalization with the housekeeping gene.

- Ct values for each sample were obtained from the Bio-Rad CFX Manager software.
- The average Ct value of triplicate reactions was calculated and data were expressed as mean ± standard error. ΔCt was calculated by subtracting the Ct value of the housekeeping gene from the Ct value of the target gene. ΔΔCt was calculated by comparing the ΔCt of treated samples with that of the control group.
- Relative fold change in gene expression was calculated using the following equation:

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

$$\Delta\Delta \text{ Ct} = \Delta \text{ Ct (Test)} - \Delta \text{ Ct (control)}$$

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

- Gene expression data were expressed as relative fold changes with respect to the control samples.

Chapter 5

Results

5.1 Assessment of Morphological Changes during Adipocyte Differentiation

The 3T3-L1 preadipocytes exhibited fibroblast-like morphology before induction (Fig 4). Upon treatment with differentiation medium containing IBMX, Dexamethasone, and Insulin, gradual morphological changes were observed. Cells lost their spindle-shaped appearance and became rounded. As differentiation progressed, intracellular lipid droplets accumulated (Fig 5 - Fig 8), indicating successful conversion of preadipocytes into mature adipocytes. Maximum lipid accumulation was observed on day 12 (Fig 9), confirming adipocyte maturation.

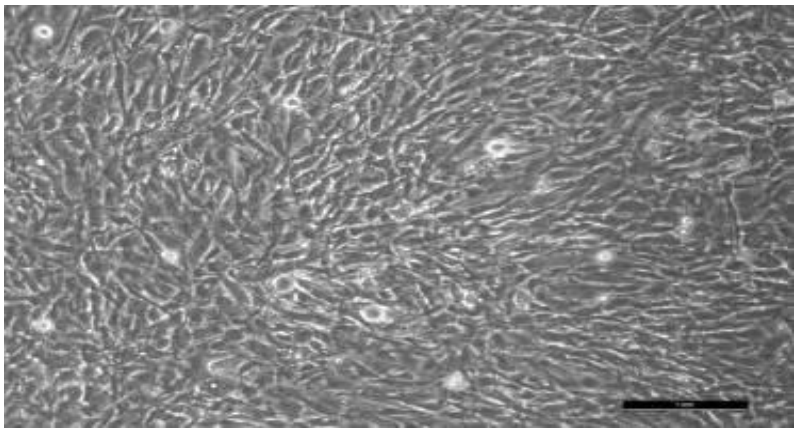


Figure 4: Microscopic Observation of 3T3-L1 cells

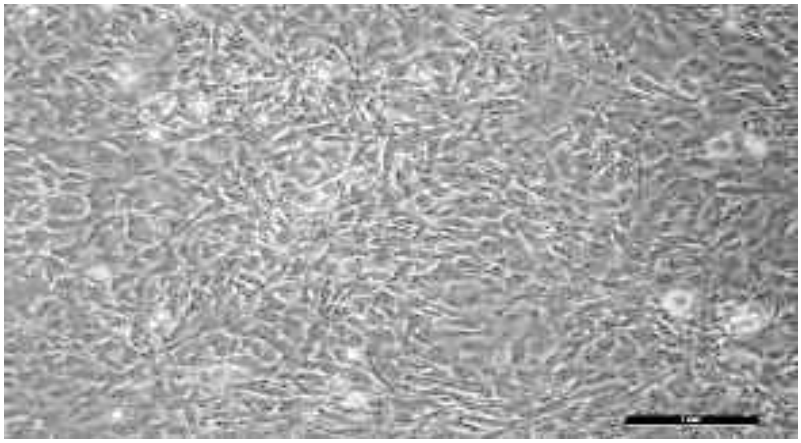


Figure 5: Microscopic Observation of 3T3-L1 cells at 2nd day of adipocyte differentiation.



Figure 6: Microscopic Observation of 3T3-L1 cells at 4th day of adipocyte differentiation.

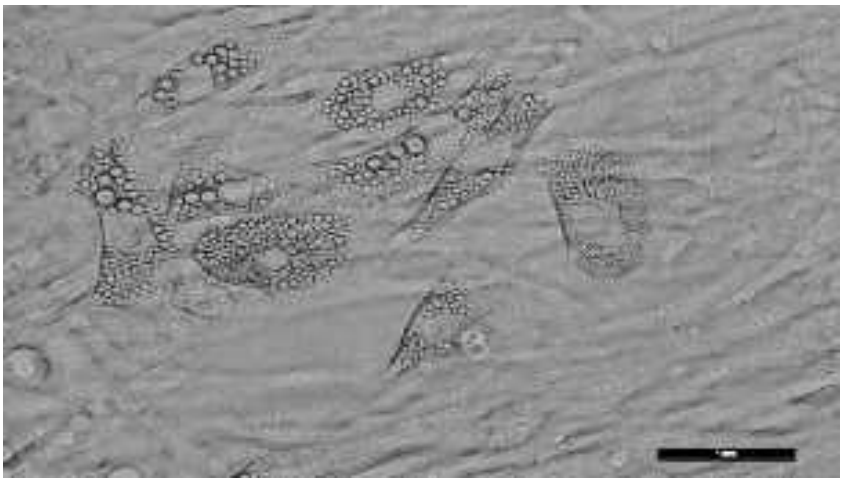


Figure 7: Microscopic Observation of 3T3-L1 cells at 6th day of adipocyte differentiation.

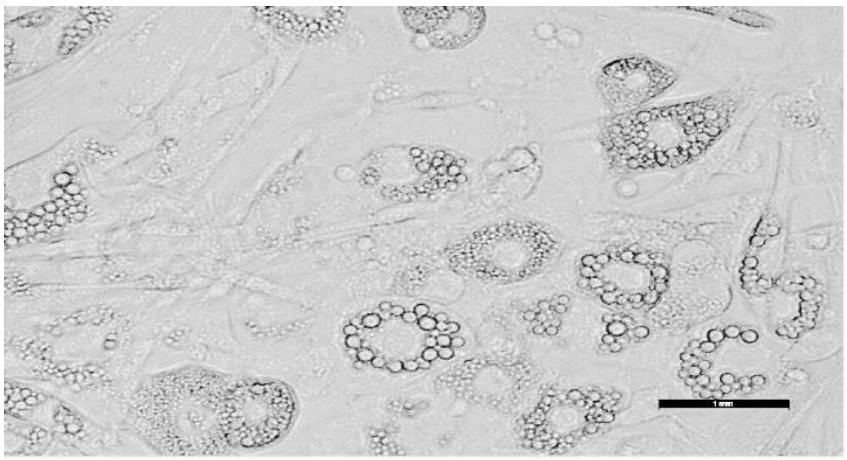


Figure 8: Microscopic Observation of 3T3-L1 cells at 8th day of adipocyte differentiation.

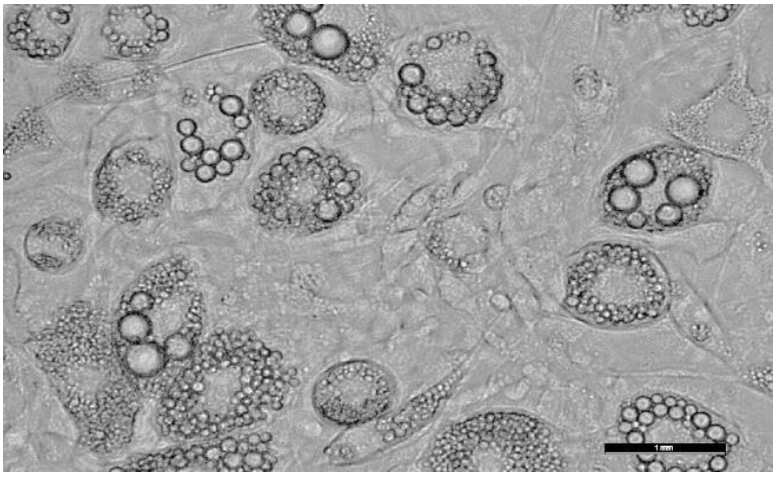
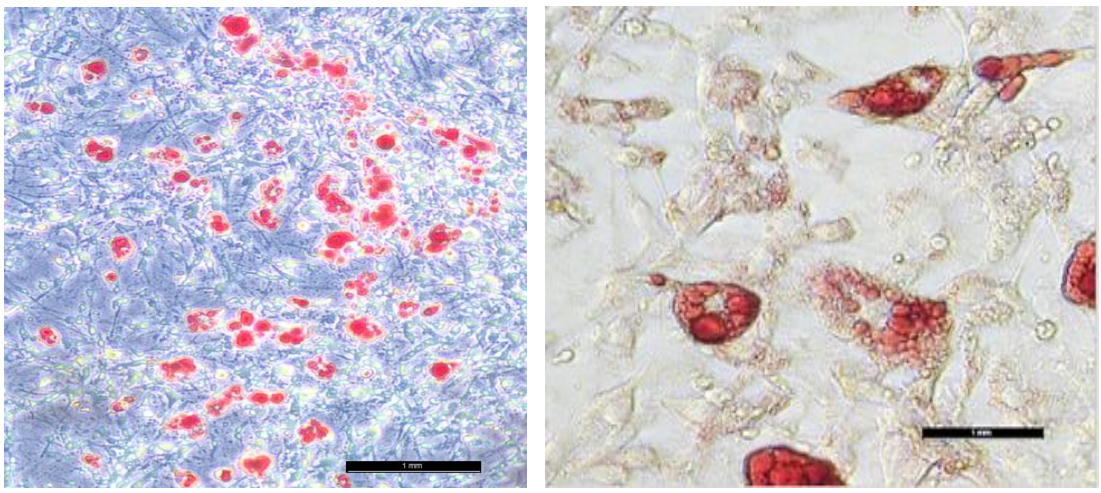


Figure 9: Microscopic Observation of 3T3-L1 cells at 12th day of adipocyte differentiation.

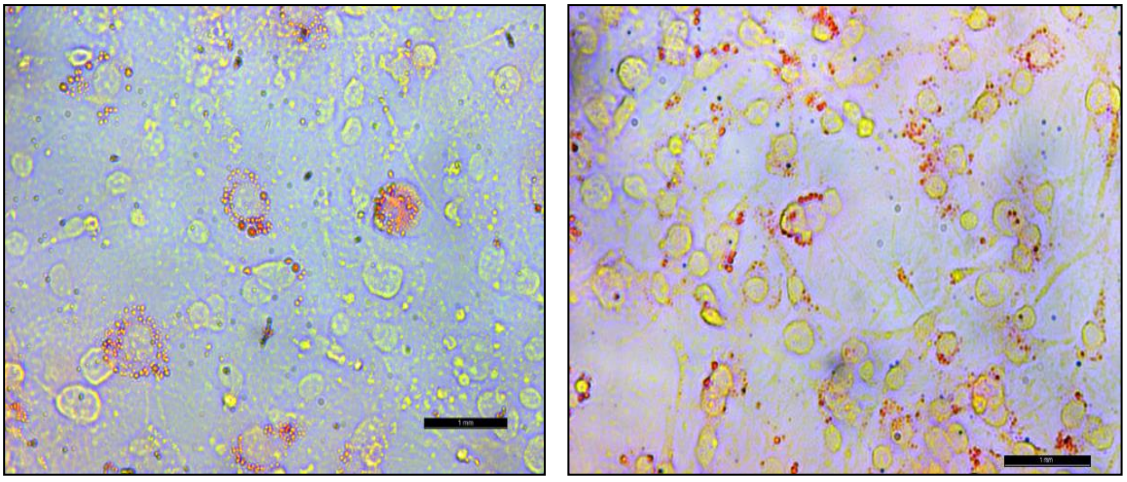
5.2 Assessment of Morphological Changes and Intracellular Lipid Accumulation by Oil Red O Staining

Oil Red O staining was performed to visualize intracellular lipid droplets. The differentiated cells exhibited intense red staining due to lipid accumulation as indicated in Fig. 10. The staining intensity increased with differentiation time, indicating successful adipogenesis (Fig. 11a - Fig. 11c). The presence of abundant red lipid droplets thus confirmed the transformation of preadipocytes into mature adipocytes.

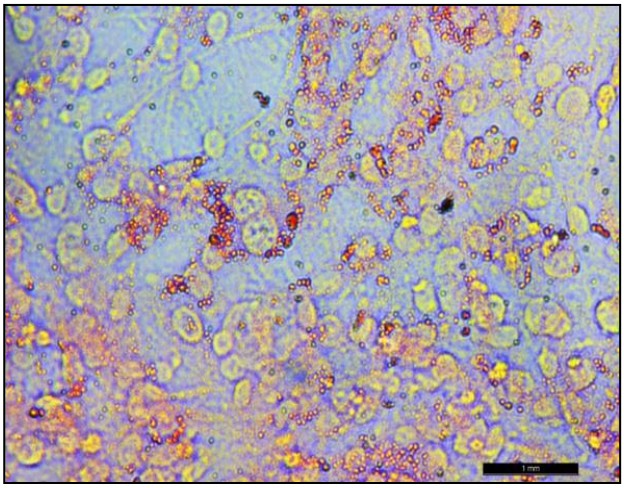


(a) **(b)**

Figure 10: Representative images of Oil Red O staining of differentiated 3T3-L1 cells demonstrating intracellular lipid accumulation, A) 10x magnification; B) 40x magnification.



(a) **(b)**



(c)

Figure 11: Microscopic Images of Oil Red O staining of differentiated 3T3-L1 cells demonstrating intracellular lipid accumulation, a) Day 4 (20x magnification); b) Day 8 (20x magnification); c) Day 12 (20x magnification).

5.3 Assessment of Concentration and Purity of RNA by Bio-photometric Analysis

The quantification and purity check of the isolated RNA was done using a biophotometer by measuring the absorbance. The A260/A280 and A260/A230 absorbance ratio of preadipocytes and mature adipocytes was found to be ~ 2 (Table 7), indicating high-quality RNA suitable for downstream applications and isolated RNA was subsequently used for cDNA synthesis.

Table 7: Bio photometric Estimation of Concentration and Purity of Isolated RNA

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

5.4 Assessment of RNA Integrity by Agarose gel electrophoresis

RNA integrity was assessed by 1.2 % agarose gel electrophoresis and intact RNA was obtained with distinct 28S and 18S ribosomal RNA bands (Fig. 12).

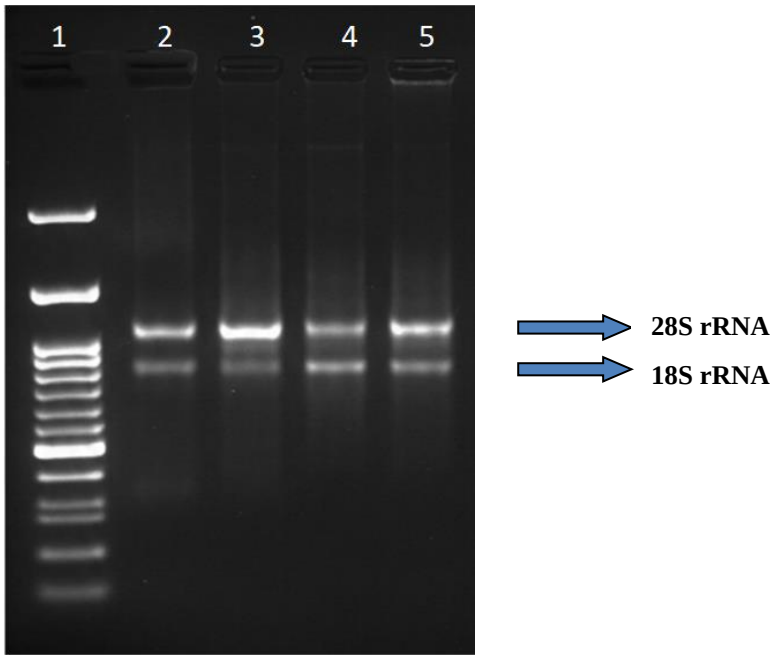


Figure 12: Total RNA isolated from cells at various stages of differentiation
Lane 1: 100bp DNA ladder; Lane 2: RNA from preadipocytes (3T3L-1); Lane 3: RNA from the cells at 2nd day of differentiation; Lane 4: RNA from the cells at 6th day of differentiation;Lane 5: RNA from the cells at 12th day of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

5.5 cDNA Quality Analysis

To evaluate the quality and synthesis efficiency of the cDNA, qPCR was performed using primers specific to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene and the same was used as the endogenous control in all the experiments (Fig. 13). Amplification of *GAPDH* yielded highly reproducible results with low quantification cycle values (Fig. 14), indicating substantial and consistent template abundance across samples, confirming its suitability for downstream gene expression analysis.

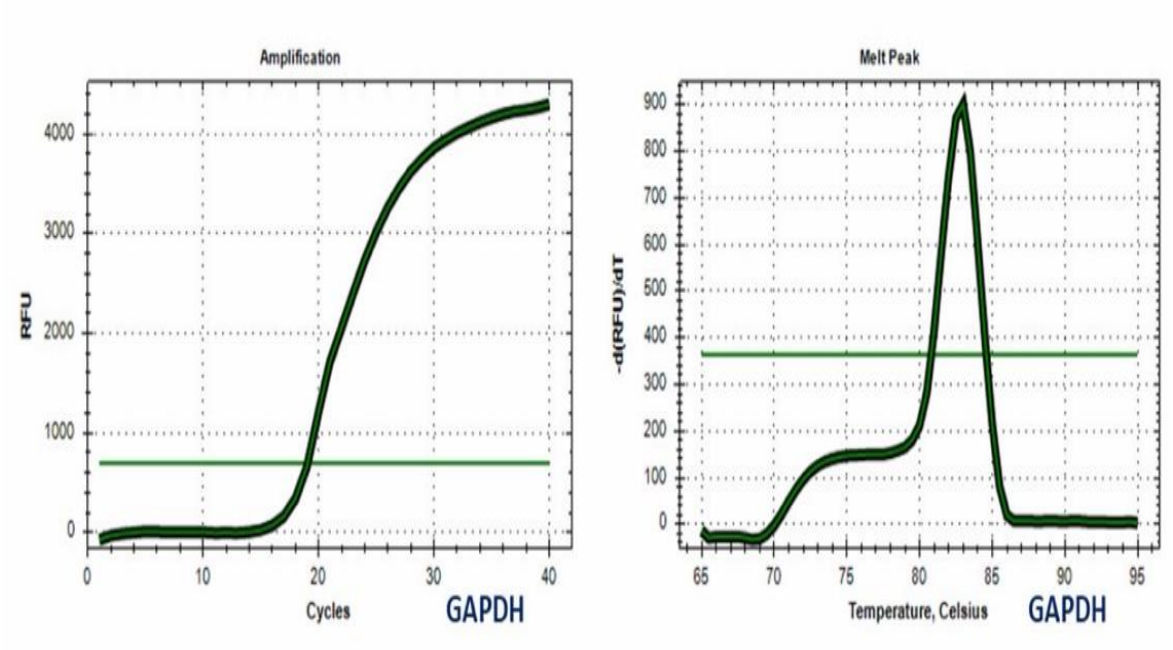


Figure 13: Representative qRT-PCR amplification and melt curve analysis of the *GAPDH* reference gene used for cDNA quality assessment.

5.6 Amplification Curve Analysis

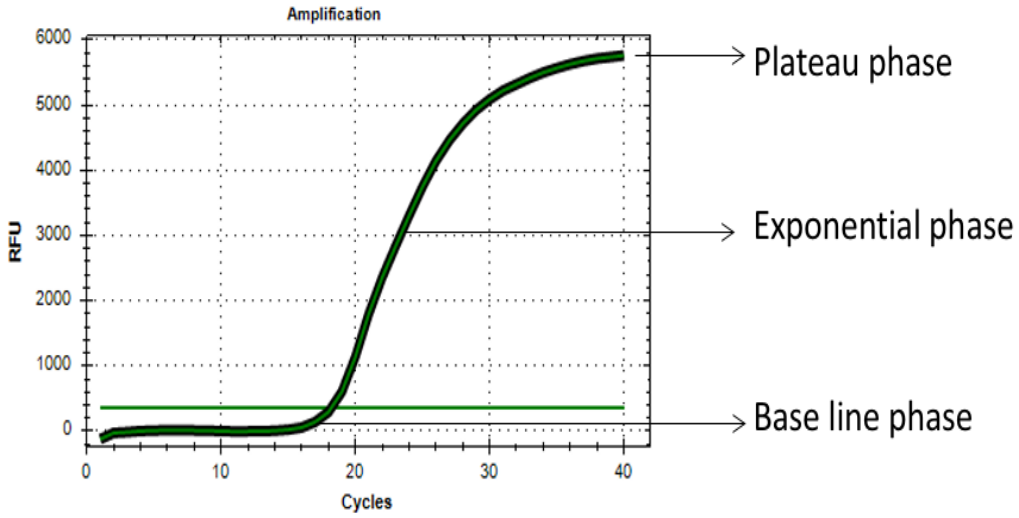
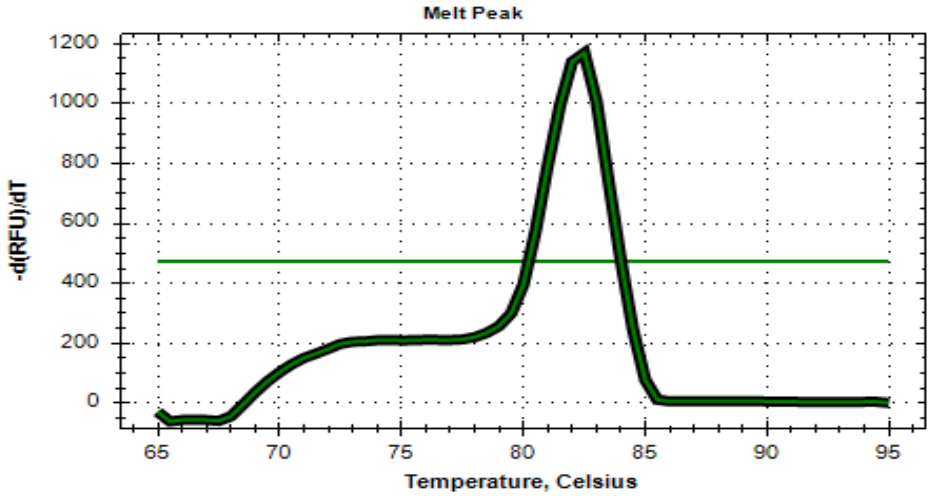


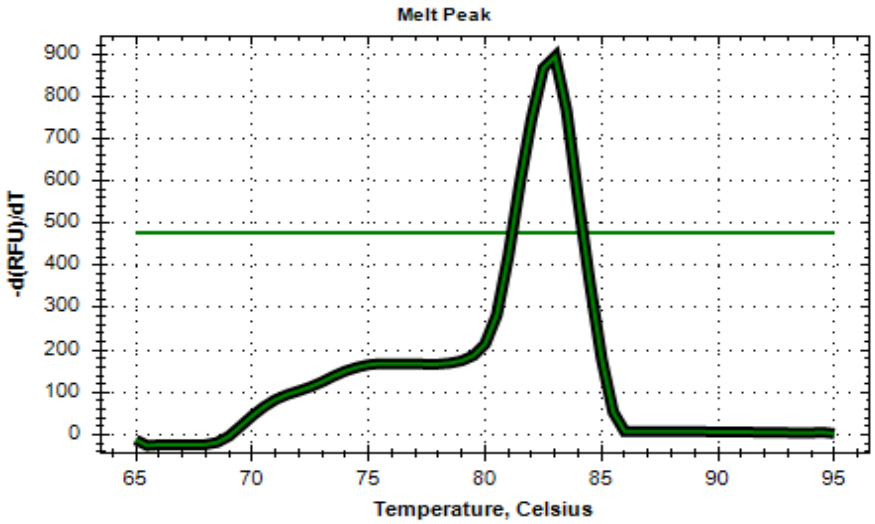
Figure 14: Representative amplification curve demonstrating efficient qPCR amplification, characterized by an early quantification followed by a strong exponential phase.

5.7 Melt Curve Analysis

Melt curve analysis further confirmed the high specificity of the reaction, characterized by a single sharp melt peak indicating specific amplification of the target gene with no significant primer-dimer formation or non-specific PCR (Fig. 15).



(A)



(B)

Figure 15: Melt curve analysis: Single, sharp melt peak showing accurate amplification (A) *Adipoq;* (B) *KLF4.*

5.8 Relative Gene Expression Analysis of *KLF4*

Quantitative real-time PCR analysis revealed that *KLF4* expression increased significantly during the early stages of adipocyte differentiation. The expression of *KLF4* was up regulated during the early stages of adipocyte differentiation, with the highest expression observed on Day 6 with a fold change of 4.37, followed by a marked decrease on Day 12 with a fold change of 0.82 (Table 8), confirming its role as an early adipogenic transcription factor (Fig. 16). The fold change was calculated relative to the undifferentiated control cells, which were normalised to 1 using the $2^{(-\Delta\Delta Ct)}$ method.

Table 8: Relative Fold change of *KLF4* during adipocyte differentiation in 3T3-L1 cells determined by qRT-PCR using the $2^{(-\Delta\Delta Ct)}$ method.

Day	Fold change
Day 2	4.37
Day 6	5.69
Day 12	0.82

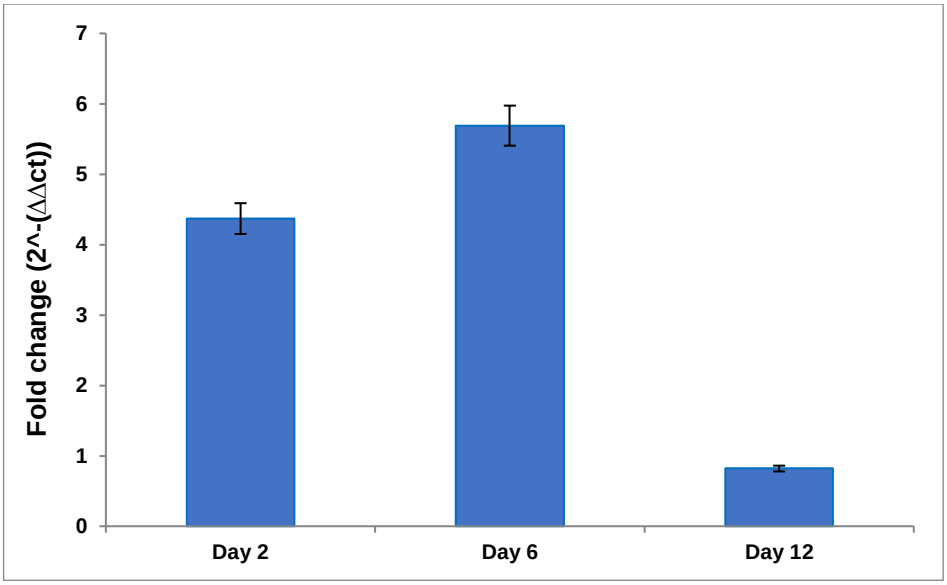


Figure 16: Relative expression of *KLF4* during adipocyte differentiation in 3T3-L1 cells determined by qRT-PCR using the $2^{(-\Delta\Delta Ct)}$ method.

5.9 Relative Gene Expression Analysis of *Adipoq*

The results indicated that the expression of *Adipoq* was found to be minimal on initial days (Fig. 17), and on day 12 a marked increase was observed with a relative fold change of 2625.42 (Table 9). This confirms *Adipoq* as a marker of terminal adipocyte differentiation and maturation, reflecting the development of functionally mature adipocytes. Gene expression levels were quantified relative to the undifferentiated control cells, which were normalized to a value of 1, and fold changes were calculated using the $2^{(-\Delta\Delta Ct)}$ method.

Table 9: Relative Fold change of *Adipoq* during adipocyte differentiation in 3T3-L1 cells determined by qRT-PCR using the $2^{(-\Delta\Delta Ct)}$ method.

Day	Fold change
Day 2	0
Day 6	0.529
Day 12	2625.42

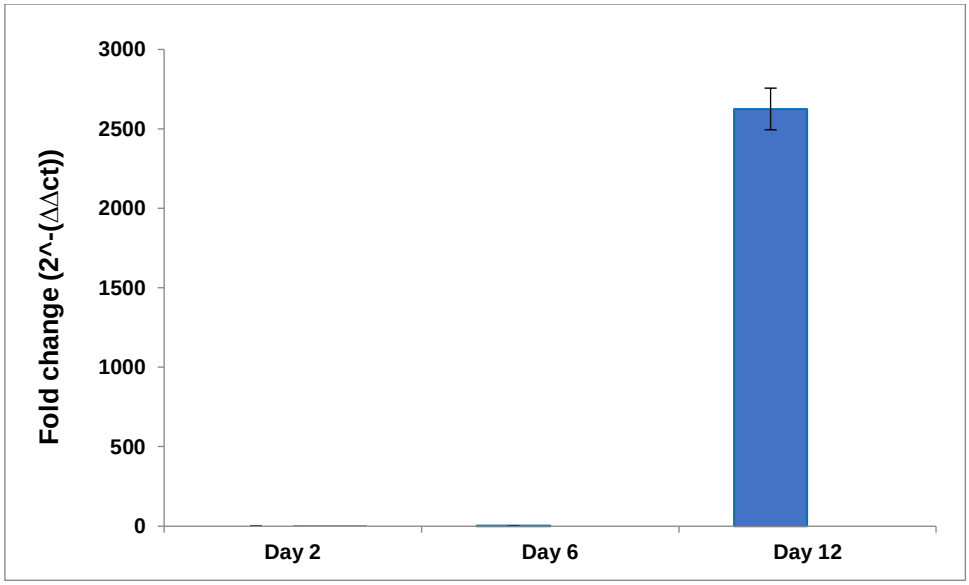


Figure 17: Relative expression of *Adipoq* during adipocyte differentiation in 3T3-L1 cells determined by qRT-PCR using the $2^{(-\Delta\Delta Ct)}$ method.

Chapter 6

Discussion

Adipogenesis is a highly coordinated process involving sequential activation of several transcription factors and adipocyte-specific genes that collectively transform fibroblast-like preadipocytes into mature lipid-laden adipocytes. Understanding the molecular mechanisms involved in adipogenesis is essential for elucidating the pathogenesis of obesity, insulin resistance, type 2 diabetes mellitus, and other metabolic disorders. The present study investigated adipocyte differentiation by evaluating morphological changes, lipid accumulation and the expression patterns of *KLF4* and *Adipoq* genes. The findings obtained in this study indicate successful adipocyte differentiation and demonstrate the differential expression patterns of *KLF4* and *Adipoq* during distinct stages of adipogenesis.

Microscopic examination revealed characteristic morphological changes associated with adipocyte differentiation. Undifferentiated 3T3-L1 preadipocytes exhibited fibroblast-like morphology and formed a confluent monolayer before induction. Following treatment with the adipogenic induction medium containing Insulin, Dexamethasone, and IBMX, the cells gradually lost their spindle-shaped appearance and acquired a rounded morphology accompanied by intracellular lipid accumulation.

By the terminal stage of differentiation, abundant lipid droplets were observed throughout the cytoplasm, indicating successful conversion into mature adipocytes. These observations are in agreement with the findings of Gregoire *et al*., (1998), who demonstrated that adipogenesis involves growth arrest, clonal expansion, and terminal differentiation leading to lipid accumulation. The morphological changes observed in the present study thus confirmed the effectiveness of differentiation protocol and indicate successful adipocyte maturation.

Oil Red O staining further validated adipocyte differentiation by visualizing intracellular lipid droplets. The differentiated cells exhibited intense red staining due to triglyceride accumulation, whereas undifferentiated control cells showed little or no staining. The increased intensity of Oil Red O staining observed during the later stages of differentiation indicates enhanced lipid storage capacity and maturation of adipocytes.

Similar observations were reported by Green and Kehinde (1975), who established the 3T3-L1 cell line as a model system for studying adipogenesis and demonstrated that mature

adipocytes are characterized by significant intracellular lipid accumulation. Therefore, the positive Oil Red O staining observed in this study confirms successful adipocyte differentiation and terminal maturation.

Quality RNA is essential for reliable gene expression analysis. In the present study, RNA isolated from differentiated adipocytes exhibited satisfactory purity with A260/A280 ratios ranging from 1.8 to 2.0. Agarose gel electrophoresis revealed distinct 28S and 18S ribosomal RNA bands without significant degradation, indicating good RNA integrity. The absence of smearing further confirmed the quality of isolated RNA. Successful cDNA synthesis and efficient amplification of target genes during quantitative real-time PCR demonstrated that the extracted RNA was suitable for downstream molecular analyses. High-quality RNA is critical for obtaining accurate and reproducible qRT-PCR results and ensuring reliable quantification of gene expression.

Gene expression analysis revealed distinct temporal patterns of *KLF4* and *Adipoq* during adipocyte differentiation. *KLF4* expression increased significantly during the early stages of adipogenesis and subsequently declined during terminal differentiation. The transient increase in *KLF4* expression suggests that it functions as an early adipogenic transcription factor responsible for initiating the differentiation process. Birsoy *et al*., (2008) reported that *KLF4* directly activates the expression of *C/EBPβ*, thereby initiating the adipogenic transcriptional cascade. *C/EBPβ* subsequently induces *PPARγ* and *C/EBPα*, which serve as master regulators of adipogenesis. Therefore, elevated *KLF4* expression observed during the early phase of differentiation in the present study confirms its role in promoting adipogenic commitment and activating downstream transcription factors required for adipocyte maturation.

In contrast to *KLF4*, adiponectin expression was relatively low during the initial stages of differentiation and increased progressively during the later stages. Maximum expression was observed in mature adipocytes, indicating successful terminal differentiation. Adiponectin is an adipocyte-specific hormone predominantly secreted by mature adipocytes and is known to enhance insulin sensitivity, regulate glucose metabolism, and promote fatty acid oxidation. Scherer *et al*., (1995) first identified adiponectin as a novel adipocyte-derived protein and demonstrated its role in maintaining metabolic homeostasis. The elevated adiponectin expression observed in the present study indicates the acquisition of metabolic functionality

and confirms successful adipocyte maturation. Increased adiponectin expression is generally associated with improved insulin sensitivity and protection against metabolic disorders.

The sequential expression pattern observed in the present study highlights the coordinated regulation of adipogenesis. *KLF4* expression predominated during the early stages, whereas *Adipoq* expression increased during terminal differentiation. These findings are consistent with the transcriptional hierarchy proposed by Rosen and Spiegelman (2000), who described adipogenesis as a cascade involving early transcription factors followed by activation of adipocyte-specific genes. The temporal regulation of *KLF4* and *Adipoq* observed in this study demonstrates that these genes function as markers of early and late stages of adipocyte differentiation, respectively. Their coordinated expression reflects the successful progression from preadipocytes to fully differentiated adipocytes.

The findings of the present study are also supported by Cristancho and Lazar (2011), who emphasized that adipogenesis is controlled by complex transcriptional and epigenetic mechanisms. They proposed that early transcription factors activate master regulators, which in turn induce adipocyte-specific genes responsible for metabolic functions. The increased adiponectin expression observed in mature adipocytes further confirms activation of adipocyte-specific pathways involved in lipid metabolism and insulin sensitivity.

Overall, the results obtained in the present study demonstrate successful adipocyte differentiation as evidenced by morphological changes, positive Oil Red O staining and differential expression of *KLF4* and *Adipoq* genes. *KLF4* acts as an early transcription factor involved in initiating adipogenesis, whereas *Adipoq* serves as a marker of terminal differentiation and mature adipocyte function. These findings provide valuable insights into the molecular mechanisms regulating adipogenesis and suggest that *KLF4* and adiponectin may serve as potential biomarkers and therapeutic targets for obesity, insulin resistance, diabetes mellitus, and other metabolic disorders. Further investigations involving additional adipogenic genes and signaling pathways may provide a more comprehensive understanding of adipocyte biology and contribute to the development of novel strategies for the prevention and treatment of metabolic diseases.

Summary and Conclusion

The present study aimed to evaluate the expression of *KLF4*, an early adipogenic transcription factor, and *Adipoq*, a marker of mature adipocytes, during adipocyte differentiation in 3T3-L1 cells. Adipocyte differentiation was successfully induced using IBMX, dexamethasone, and insulin. Morphological analysis using an inverted microscope revealed a gradual transition of fibroblast-like preadipocytes into rounded adipocytes with progressive lipid droplet accumulation. Oil Red O staining further confirmed successful adipocyte differentiation by demonstrating increased intracellular lipid accumulation, with maximum staining observed on Day 12. The quality of RNA and cDNA was validated using spectrophotometry, agarose gel electrophoresis, amplification curves, and melt curve analysis, confirming the suitability of the samples for qRT-PCR analysis.

Relative gene expression analysis using the $2^{(-\Delta\Delta Ct)}$ method showed that *KLF4* expression was significantly up-regulated during the early stages of adipocyte differentiation, reaching its highest level on Day 6 before declining on Day 12, indicating its role in the initiation of adipogenesis. In contrast, *Adipoq* expression remained low during the early stages but increased markedly on Day 12, confirming its role as a marker of terminal adipocyte differentiation and maturation. Overall, the combined morphological, histological, and molecular findings confirmed the successful differentiation of 3T3-L1 preadipocytes into mature adipocytes. These results enhance our understanding of the molecular events regulating adipogenesis and suggest that *KLF4* and *Adipoq* may serve as potential biomarkers and therapeutic targets for obesity and related metabolic disorders. Further studies involving additional adipogenic markers, protein-level validation, and functional analyses are required to strengthen these findings and explore their clinical relevance.

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