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THE REGULATION OF BRN3B IN ADIPOCYTES BY GLUCOSE AND INSULIN

A Thesis in

Biomedical Sciences

By

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ABSTRACT

Obesity is the inappropriate accumulation of excess fat stored in the adipocytes of the adipose tissue. Understanding the molecular mechanisms that regulate the process of preadipocyte to adipocyte conversion, adipogenesis, is crucial when trying to design treatments for the disease. Known transcription factors, such as Peroxisome proliferator-activated receptor γ (PPARγ), play an essential role in preadipocytes differentiation into adipocytes. However, more recently novel nuclear receptors such as, Brn-3b (or POU4F2, second transcription factor in the fourth class of POU family), have been shown to be expressed in adipose tissue, but their link with adipogenesis is yet to be established. 3T3-L1s, a murine adipocyte cell-line, was used to investigate Brn3b gene expression during adipogenesis in high and low glucose conditions, in the presence and absence of dexamethasone, and compared to PPARγ gene expression. Two experiments with high glucose were performed one in the presence of dexamethasone and the other without dexamethasone. A third experiment was conducted using low glucose with dexamethsone. RNA extracted from cells at different time intervals was converted to cDNA before being analyzed by qPCR. Morphological examination of the cells confirmed the requirement of high glucose concentrations for cells to differentiate into adipocytes. There was significant increase in PPARγ gene expression during adipogenesis of cells grown in high glucose concentration in presence or absence of dexamethasone. However, no detectable expression of Brn-3b was apparent in either the preadipocytes or the adipocytes, under any of the experimental conditions studied. These results suggest that the expression of Brn-3b in adipose tissue derives from the non-adipocyte cells of the tissue, such as neuronal, endothelial, monocytic or epithelial.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
PPARγ	Peroxisome proliferator-activated receptor γ
Brn3b/ POU4F2	Second transcription factor in the fourth class of POU family
POU	(Pit-Oct-Unc) transcription factors family
WAT	White adipose tissue
BAT	Brown adipose tissue
RT/PCR	Reverse transcriptase polymerase chain reaction
DMSO	Dimethyl sulfoxide
PM-1-L-1	Preadipocytes media
TE	Trypsin EDTA
PBS	Phosphate buffer saline
AM-1-L-1	Adipocyte Maintenance media

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INTRODUCTION

As obesity is becoming more prevalent worldwide and associated with many metabolic disorders like type2 diabetes and cardiovascular diseases, many scientists turned their interest towards research into understanding the causes and consequences of obesity. Obesity has been studied not only clinically, but also at the molecular level. Many transcription factors have been investigated and some were linked to adipose tissue, like PPARγ. Brn3b is a transcription factor that has been studied extensively in nervous tissue and retinal ganglion cells. However, recent unpublished data, interestingly, revealed that Brn3b knockout mice had higher body weights and there was detectable expression of this nuclear receptor in murine adipose tissue. Furthermore, in a comparison between obese type 2 diabetic (T2DM) and healthy normal weight people, Brn3b mRNA was detected in peripheral blood cells and found to decrease more than one and half folds in diabetics. The findings of this unpublished study raised a question of the possibility of Brn3b involvement in obesity and diabetes.

Brn3b gene expression was investigated and compared to PPARγ gene, using 3T3-L1 cell line as a metabolically active adipocytes cellular model. 3T3-L1 cells were treated with high and low glucose concentrations in the presence of insulin. Data showed increased PPARγ gene expression during differentiation of preadipocytes into adipocytes in high glucose treatment, with or without dexamethasone, while no such changes were seen when using low glucose treatment. However, Brn-3b was not detected under any of these conditions in these cells.

LITERATURE REVIEW

Obesity

Obesity or overweight is defined according to Centers for Disease Control and Prevention (CDC) as: "Overweight and obesity are both labels for ranges of weight that are greater than what is generally considered healthy for a given height. The terms also identify ranges of weight that have been shown to increase the likelihood of certain diseases and other health problems." (CDC, 2012).

Obesity is also defined as the increase of body weight due to abnormal fat accumulation and is related to increased risk of many diseases such as diabetes mellitus (The Free Dictionary, 2014).

Obesity is becoming more prevalent all around the world, increasing the need for understanding the underlying etiologies. According to WHO, obesity has nearly doubled in the past thirty years (WHO, 2014).

High energy intake and with lower physical activity, and therefore energy expenditure, accounts for the overweight and obesity state spreading all over the world. Chronic obesity increases the risk for many diseases such as: cardiovascular hemodynamics (Lavie et al, 2014), kidney related diseases like obesity-related glomerulopathy (Ross & McGill, 2006), metabolic disorders such as type 2 diabetes mellitus and fatty liver disease, other morbidities like obstructive sleep apnea, dementia, and even several types of cancer (Blüher, 2013).

Fat cells are known as adipocytes, which contribute to around 50% of the cells of the adipose tissue. Adipose tissue is areolar connective tissue that maintains body temperature, attaches

skin to the beneath tissue, and shields body organs. Generally, adipose tissue includes two types, white adipose tissue (WAT) and brown adipose tissue (BAT). Each type has its specific characteristics; white adipose tissue serves as an energy reservoir, where triglycerides are stored in large lipid droplets (Lafontan, 2008). Also, recent studies have found that white adipose tissue can be considered as an endocrine and paracrine organ which produces chemicals similar to hormones called adipokines that can affect many body functions like thyroid, blood pressure, immune responses, and even reproductive systems (Trayhurn, 2005). On the other hand, brown adipose tissue is rich in mitochondria and mainly functions for heat generation by fatty acid oxidation. Unlike white adipose tissue, triglycerides in brown adipose tissue are distributed over multiple small lipid droplets (Cannon & Nedergaard, 2004). White and brown adipose tissues are also distributed differently. White adipose tissue (WAT) predominates in adult humans, but brown adipose tissue (BAT) mainly found in newborns and rodents. Studies have shown that white adipose tissue (WAT) is the one responsible for obesity status and its related complications like type2 diabetes mellitus. The reason for that is brown adipose tissue (BAT) produces less adipokines (Farmer, 2008). Furthermore, studies found that dysregulation of white adipose tissue (WAT) function leads to hyperglycemia and hyperlipidemia, which ultimately cause insulin resistance and chronic inflammation (Trayhurn, 2005).

Adipocytes

Adipocytes thought to be originating from mesenchymal stem cells, but still many steps among the commitment process are unclear (Guilak et al, 2006). However, other studies claim the potential of neuroepithelial cells to form mature adipocytes (Takashima et al, 2007). Even bone marrow progenitors may be converted into adipocytes that can be differently accumulated according to age and weight (Rodeheffer et al., 2008; Tang et al., 2009).

The process of fibroblast-like preadipocytes differentiation into mature lipid laden, insulin-responsive adipocytes, is known as "Adipogenesis" (Lefterova & Lazar, 2009). Preadipocytes have a similar morphology to fibroblasts; however, when they differentiate into mature adipocytes, they become round puffy cells with large fat droplet pushing nucleus to the border and making cytoplasm condensed in a thin layer (Ali et al, 2013).

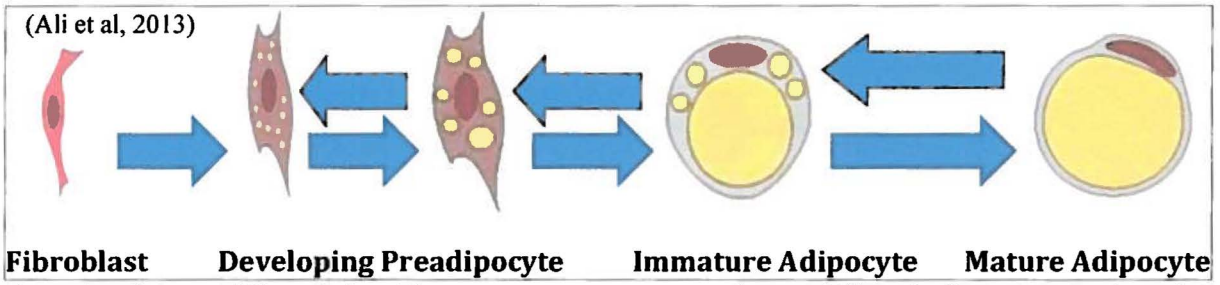


Figure 1 Adipocyte differentiation. The reverse arrows represent the possibility of adipocytes to reduce size when energy supply diminishes.

Adipogenesis process ends up with mature adipocytes that have gone through the main stages of differentiation. It starts with mesenchymal precursors becoming committed preadipocytes that undergo growth arrest until they start dividing again and clonally expand before they ultimately differentiate and become mature adipocytes (Lefterova and Lazar, 2009).

As adipogenesis is the main process in obesity state, scientists found adipogenesis an appealing subject for research and study. Adipogenesis has been studied extensively for the past 40 years (Ali et al, 2013); on different levels, in vivo and in vitro using preadipocytes cell line. The changes in preadipocytes, induced by their differentiation, on both their morphology and gene expression are studied. Several transcription factors involved in adipogenesis has been identified; however, many others are still unknown. Among the recognized transcription factors strongly associated with adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ), the transcriptional factor sterol-regulatory-element-binding-protein-1 (SREBP1), macrophage colony stimulating factor, insulin-like growth factor I (IGF-l), interferon regulatory factors IRFs (IRF3 and IRF4), fibroblast growth factors, pro-inflammatory cytokines, hypoxia and others. Moreover, in sake of preventing adipocytes accumulation,

adipogenesis inhibitors has been identified like glycoproteins, inflammatory cytokines, transforming growth factor-β (TGF-β), and growth hormone (Ali et al, 2013).

Peroxisome Proliferator-Activated Receptor γ (PPARγ)

Peroxisome proliferator-activated receptor γ (PPARγ) is considered one of the most famous transcription factors related to adipogenesis. PPARγ belongs to the nuclear receptor (NR) super-family of ligand-dependent transcription factors (TFs) (Poulsen et al, 2012). Early studies have shown that PPARγ is essential in adipogenesis (Tontonoz et al, 1994). A study has shown that PPARγ knockout mice cannot survive embryonic stage due to placental defects, while chimeric PPARγ mice showed inability of cells missing PPARγ for involvement in fat formation (Barak et al, 1999) (Rosen et al, 1999).

PPARγ is encoded by PPARg gene (PPARG/Pparg) that is translated into two proteins PPARγ1 and PPARγ2; and mainly expressed in adipocytes (Poulsen et al, 2012). PPARγ structure is divided into three domains: N-terminal domain, DNA binding domain, and C-terminal ligand-binding domain (Tontonoz & Spiegelman, 2008). PPARγ has the ability to bind to its binding site without a ligand; such binding leads to suppression of transcription. On the contrary, binding of PPARγ to its ligand leads to activation of gene transcription (Tontonoz & Spiegelman, 2008). Genes involved in fatty acid (FA) and glucose metabolism contain binding sites for PPARγ, which points to the involvement of PPARγ in metabolic pathways of adipogenesis (Mikkelsen et al, 2010).

In order for transcription factors, in general, to work; they need chromatin to be opened and nucleosome become free. Therefore, transcription factors accessibility to DNA is governed by chromatin remodeling complexes (Clapier & Cairns, 2009). In a study of adipocyte differentiation held on 3T3-L1 cells, a great change in chromatin structure has been observed during early adipogenesis (Siersbæk et al, 2011). Furthermore, big differences have been found, in another study, in comparison of chromatin structure of 3T3-L1 mature and preadipocytes (Waki et al, 2011). Transcription factors have an influence on the action of each other through chromatin remodeling. For example, C/EBPβ is an early acting transcription factor that can open sites for other transcription factors such as PPARγ. C/EBPβ can also bind to partially closed chromatin (Siersbæk et al, 2011).

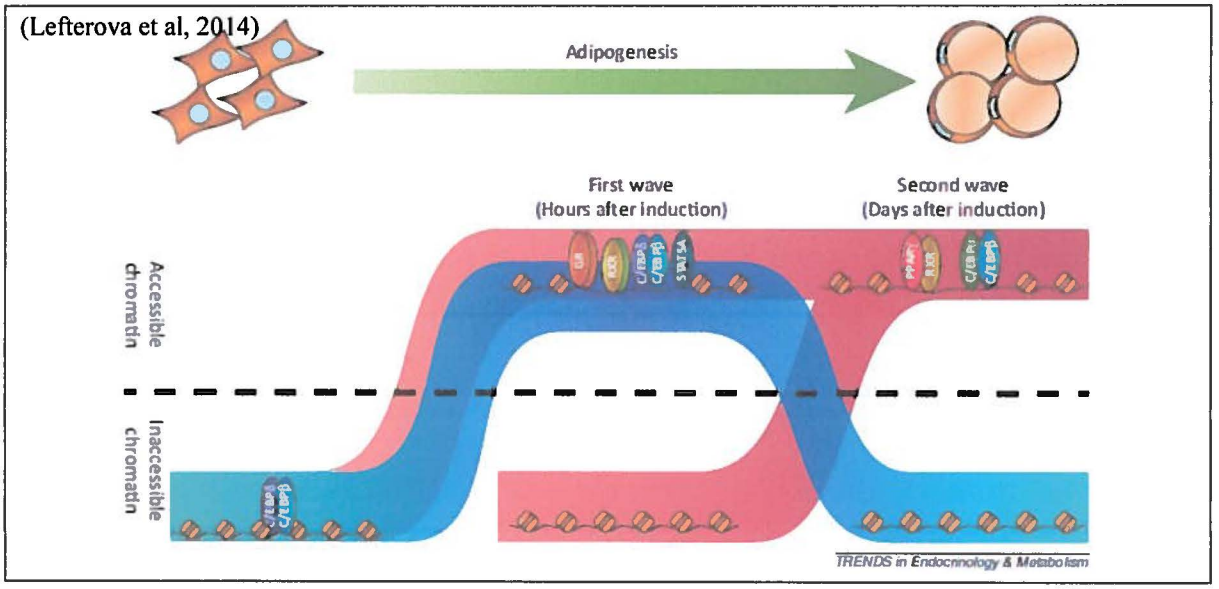


Figure 2 Transcriptional network during adipogenesis. Two waves of transcription factors (TFs) activation occur during adipocyte differentiation. Second wave TFs, including PPARγ are activated by first wave TFs that in their turn have been activated by adipogenic cocktail. Whole process is accompanied by chromatin remodeling to open sites for TFs.

It has been noticed that, in a number of cell types, PPARγ binding sites exhibit open chromatin, on the contrary, sites specific for PPARγ role in adipocytes are made available subsequently throughout adipogenesis (Oger et al, 2014). Whole PPARγ locus and other

adipocyte gene loci in 3T3-L1 preadipocytes are repressed by H3K9me2, a histone mark; such repression is lifted by the adipogenic cocktail (Wang et al, 2013).

In a comparison between mouse and human PPARγ binding sites in adipocytes, studies showed that only few sites are shared, estimated to be about 9%-30% (Soccio et al, 2011) (Mikkelsen et al, 2010).

In an experiment for diabetes management, synthetic PPARγ agonists have been used; nevertheless, that experiment was not quit successful owing to dangerous consequences leading to activation of PPARγ in non adipose tissues (Cariou et al, 2012).

POU (Pit-Oct-Unc) Transcription Factors

There is multiple transcription factors involved in many vital processes in body organs; Brn3b is one of those transcription factors. Brn3b belongs to POU (Pit-Oct-Unc) transcription factors family, which are expressed in a range of tissues especially nervous tissue and embryonic cells. POU name comes from the first transcription factors discovered in the family; Pit1, Oct1, and Oct2. Oct2 is found in mammalians, which is the same as Unc86 found in *Caenorhabditis elegans* (Herret et al., 1988). Pit1 stands for pituitary and is involved in pituitary growth; whereas Oct1 is known as that because it binds to octamer motif of DNA and promotes gene transcription of histones and immunoglobulins. Brn3b also known as POU4F2 since it is the second transcription factor in the fourth class of POU family. Brn3b, known also as Brn-3.2 : 6, originally was isolated, using reverse transcriptase polymerase chain reaction (RT/PCR), from ND7 neuronal cell line (Lillycrop et al, 1992). The significant function of Brn3b in visual system development is shown when a Brn3b knockout in mice resulted in great retinal abnormalities (Erkman et al, 1996). In another study on retina, 70 % of retinal ganglion cells were missing in Brn3b knockout mice due to abnormal axon growth and ultimate cell death (Wang et al., 2000). Although Brn3b initially discovered and

predominantly present in neuronal cells, Brn3b is also found in many other cell types such as: breast cells, testis, retina, and peripheral blood cells (Ounzain et al, 2011) (Budhram-Mahadeo et al, 2001) (Xiang et al, 1993) (Niculescu et al, 2007).

Brn3b, as a transcription factor, can control the expression of many genes responsible for variable cellular processes. Brn3b found to promote cell cycle progression genes such as cyclin-dependent kinase 4 (CDK4) (Samady et al, 2004), and a regulator cyclin D1 (Budhram-Mahadeo et al, 2008). In general, Brn3b found to promote cell division and even increase tumor growth (Irshad et al, 2004).

3T3-L1 Cell Line

Cell lines have been used by scientists for decades in research and scientific experiments. Multiple cell lines are available with a variety of characteristics that can be specified by scientists according to the scientific experiment to be held.

3T3-L1 cell line has been used for over three decades now in research. 3T3-L1 cells are derived from Swiss mouse fibroblasts 3T3 cells, and have the potential, in appropriate conditions, to differentiate into mature adipocytes. 3T3-L1 cells were originally discovered by Green and Kehinde in 1974, from 3T3-M cells that have been isolated and frozen from Swiss embryo cells. Green and Kehinde noticed that 3T3-L1 cells, when in resting state, can accumulate triglycerides. In addition, lipid distribution in 3T3-L1 cells was similar to that of brown adipose tissue; where small distributed fat droplets were seen in a plentiful cytoplasm with central nucleus (Green & Meuth, 1974).

Through their experiments, Green and Meuth, noticed that in order for the cells to go into resting state that allow them to start differentiation, cells need to be in a confluent monolayer first. However, trypsin could be used to arrest cells at any time along with using methyl cellulose in medium and calf serum at concentration of 30% (Stoker, 1968).

An advantage of 3T3-L1 cell line is that they are immortalized, and can be passaged and frozen to keep a constant source of cells through experiments. Nevertheless, the negative side of 3T3-L1 cells is that after many passages their potential to differentiate drops. Another drawback not only of 3T3-L1 alone, but generally when applying experimental changes on a cell line, cells are in a biological and molecular condition differs from that of living tissues (Ntambi & Young-Cheul, 2000).

Since that time until now 3T3-L1 cells have been used extensively in research as preadipocytes that can differentiate into adipocytes. More specifically, 3T3-L1 cells have been used in experiments related to obesity and diabetes.

Hypothesis

Building upon the unpublished interesting findings by Dr. Vidya Mohamed-Ali and colleagues, which found that Brn3b knockout mice had a higher body weights; and, diabetic obese patients had reduced Brn3b mRNA in peripheral blood (Hindle *et al.*, 2010) (see appendix IV). We hypothesize that Brn3b transcription factor may contribute for the regulation of metabolic processes linked to obesity and its consequences. Brn3b gene expression change might be associated with the differentiation of preadipocytes into adipocytes; hence Brn3b might be involved in obesity.

Aim

The main aim of this study is to investigate if the adipocytes-*in vitro*- has the expression of Brn3b in a model of metabolically active cell line, murine 3T3-L1 adipocytes, and to compare it with the expression of PPARγ; in sake of elucidating mechanisms by which Brn3b may influence adipogenesis and obesity.

Specific Aims:

1- To study and compare mRNA gene expression of both Brn3b and PPARγ during 3T3-L1 cell differentiation from preadipocytes into adipocytes at different time intervals (at days 0, 3, 5, and 7).

2- To examine the effect of low glucose concentration on gene expression of Brn3b and PPARγ during adipogenesis at the same time intervals (days 0, 3, 5, and 7).

3- To explore whether the presence or absence of dexamethasone may affect cell differentiation, adipogenesis, and gene expression of Brn3b and PPARγ.

	Seven	85.88 (±25.55)	1.70 (±0.111716)

As shown in the table, the concentration and purity indicated high yield with 260/280 ratios from 1.66 up to 2.04.

NanoDrop graphs:

An example of a NanoDrop graph from the third experiment tube no.1 of day zero representing a good quality and high concentration represented by the peak of RNA absorbing at 260nm wavelength see Figure 14.

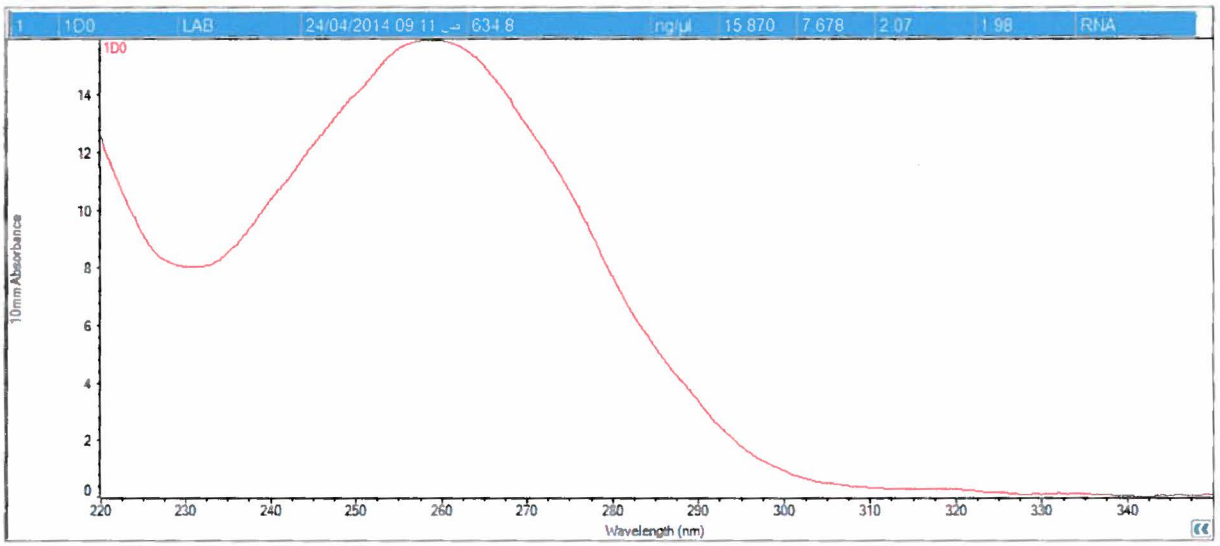


Figure 14 a good NanoDrop graph

An example of relatively low standard quality NanoDrop graph (Figure 15) from the third experiment tube no.5 of day three, showing an Ethanol contamination peak at about 230nm wavelength.

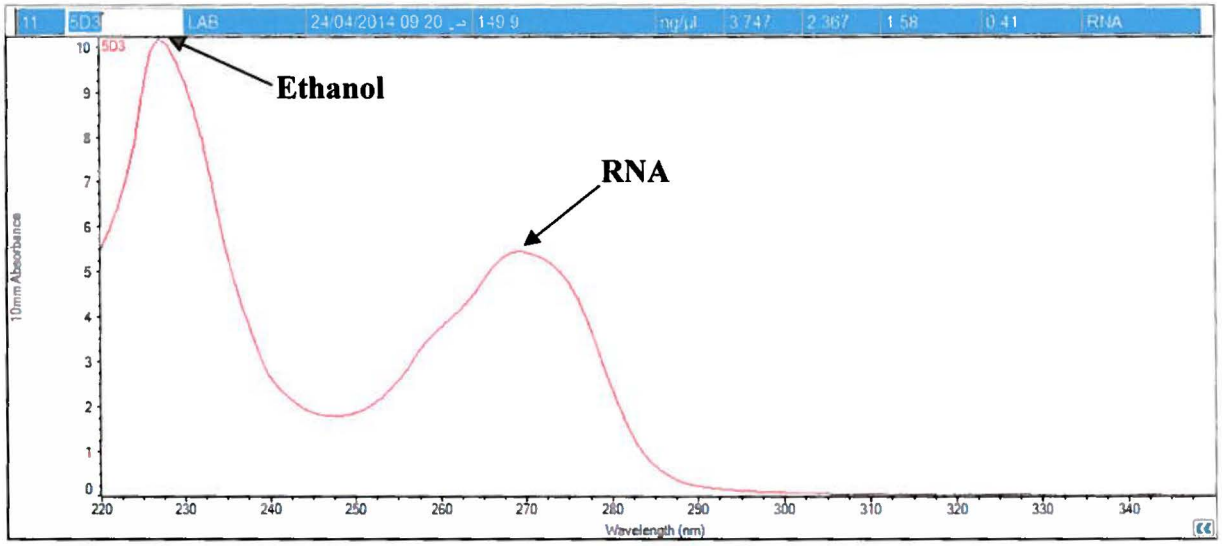


Figure 15 a low quality RNA NanoDrop graph

RESULTS

Morphological Changes of 3T3L-1 Cells

The following images demonstrate morphological changes of 3T3L-1 Cells in different groups in response to different media:

Confluency of preadipocytes

Primarily, cells were resurrected and proliferated; the following images (Figures 4A, 4B) show preadipocytes growing in a culture flask:

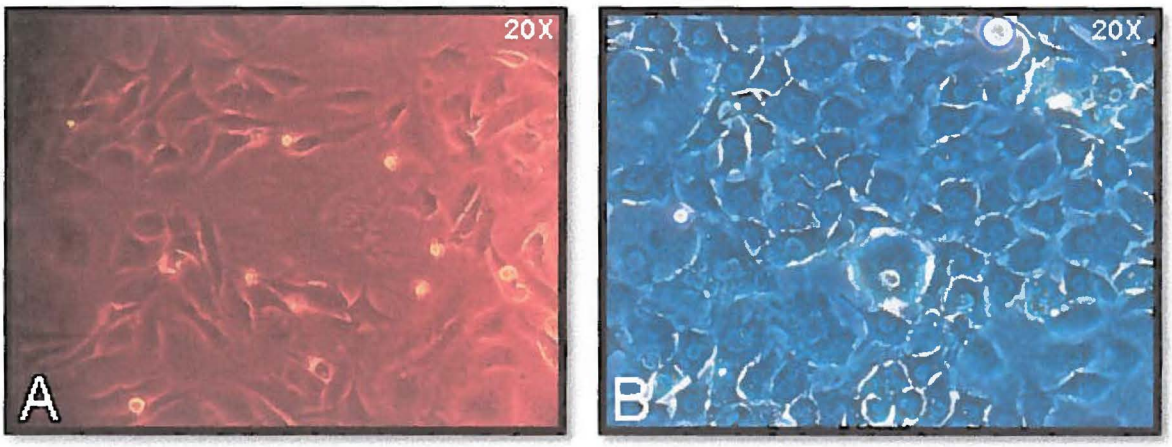


Figure 4 preadipocytes nearly confluent (A), preadipocytes 100% confluent (B), high glucose preadipocyte media used. magnification 20X .

Images have been taken for cells at all different days except day zero, since cells at day zero look the same as ordinary preadipocytes.

A- Effect of high glucose medium without dexamethasone on differentiation of 3T3L-1 cells

3T3-L1 cells at different time points of differentiation from preadipocytes to adipocytes using different magnification (5, 10 and 20X) were illustrated in figures 5-7. Figures clearly indicate well differentiation over time points studied and changes in cell morphology from fibroblast like spindle shape to fat laden round cells.

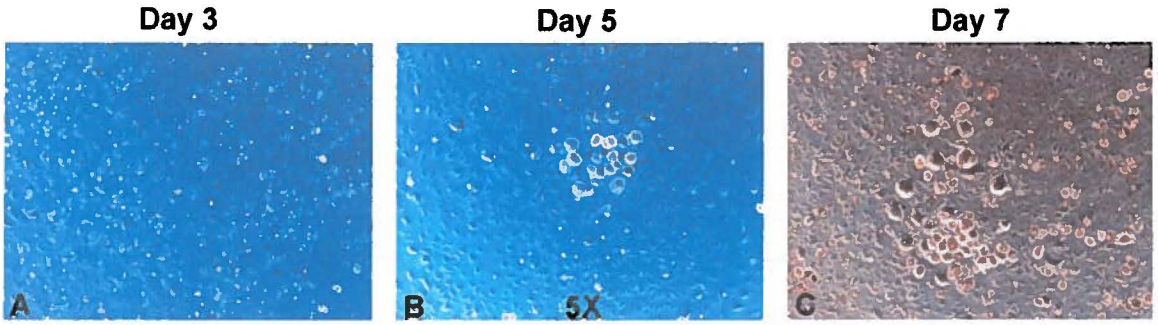


Figure 5 cells of the first experiment. At day 3 after adding the high glucose differentiation media (A), at day 5 (B), and at day7 (C), Maintenance media used without Dexamethasone, magnification 5X.

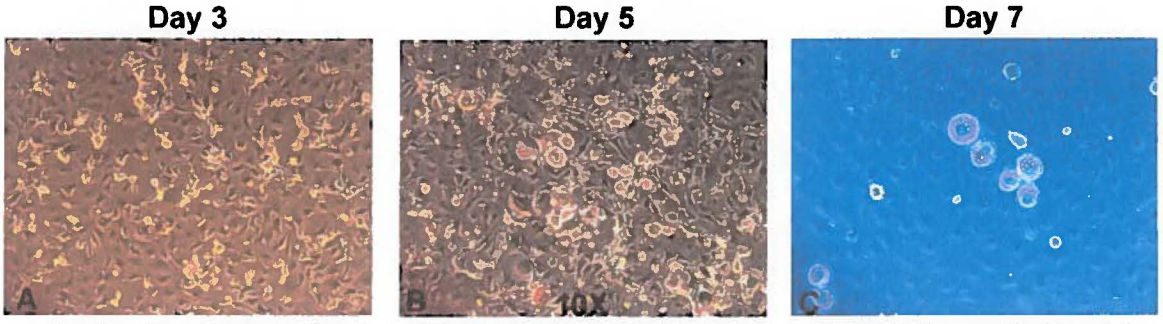


Figure 6 cells of the first experiment. At day 3 after adding the high glucose differentiation media (A), at day 5 (B), and at day7 (C), Maintenance media used without Dexamethasone, magnification 10X.

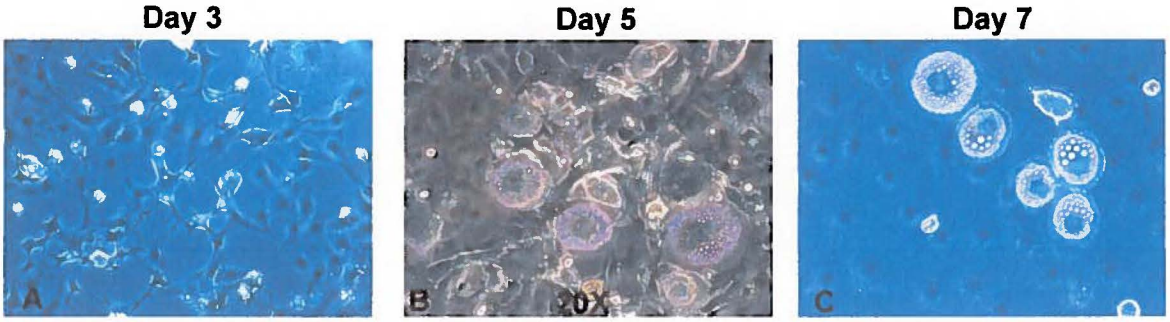


Figure 7 cells of the first experiment. At day 3 after adding the high glucose differentiation media (A), at day 5 (B), and at day7 (C), Maintenance media used without Dexamethasone, magnification 20X.

B- Effect of high glucose medium with dexamethasone on differentiation of 3T3L-1 cells

Cells shown in figures 8-10 represent 3T3-L1 cells at different time points of differentiation from preadipocytes to adipocytes. Figures clearly indicate well differentiation over days of the experiment.

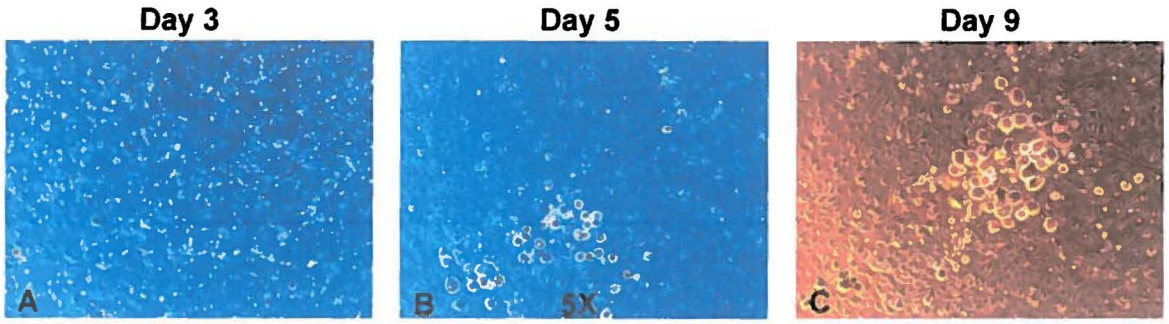


Figure 8 cells of the third experiment. At day 3 after adding the high glucose differentiation media (A), at day5 (B), and at day9 (C), Maintenance media used with Dexamethasone, magnification 5X.

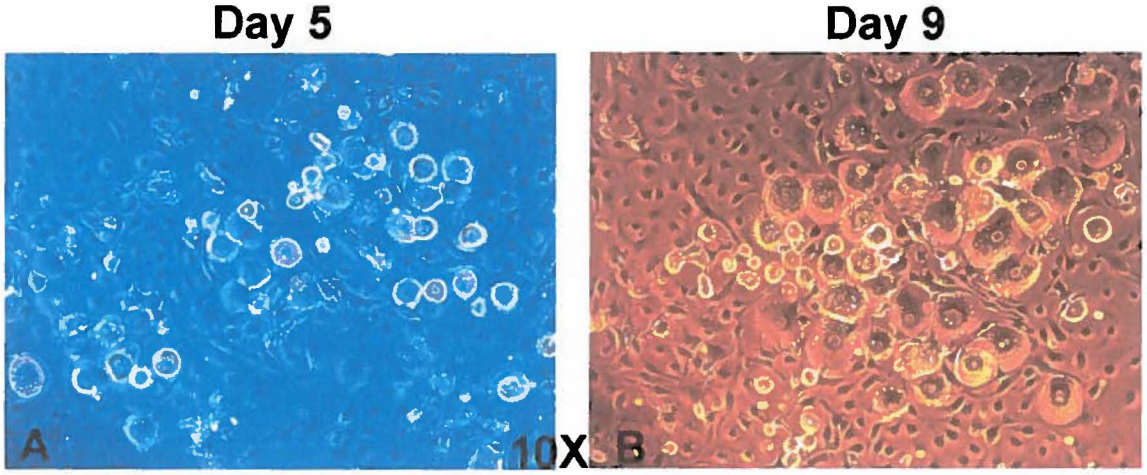


Figure 9 cells of the third experiment. At day5 (A), and at day9 (B), Maintenance media used with Dexamethasone, magnification 10X.

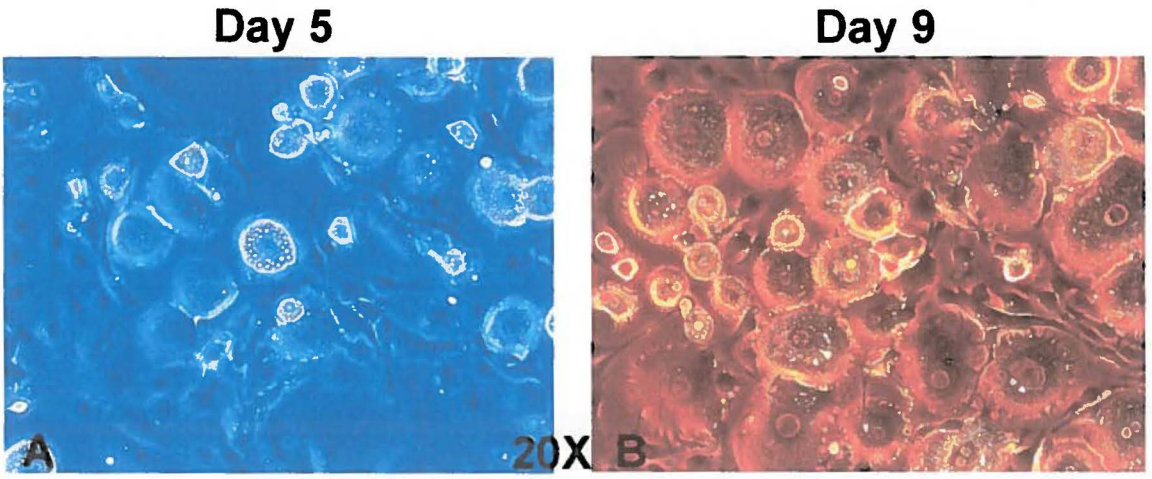


Figure 10 cells of the third experiment. At day5 (A), and at day9 (B), Maintenance media used with Dexamethasone, magnification 20X.

C- Effect of low glucose medium with dexamethasone on Differentiation of 3T3L-1 cells

Cells in figures 11-13 illustrate 3T3-L1 cells at different time points of the low glucose experiment. The figures clearly indicate poor and lack of differentiation over time points studied.

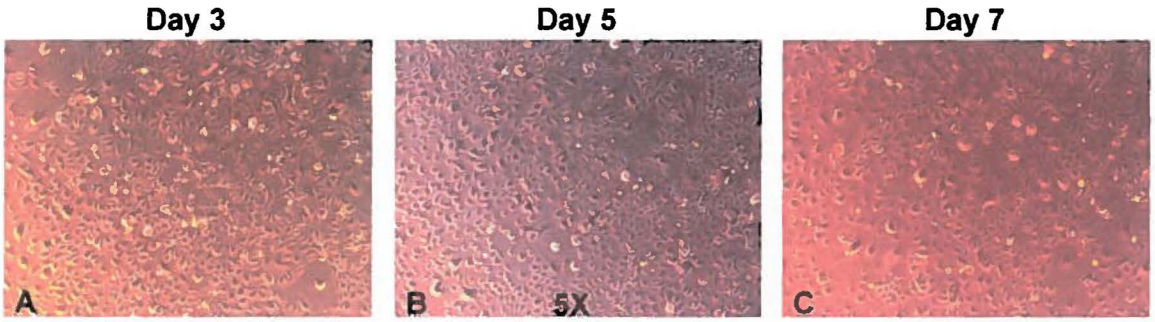


Figure 11 cells of the fourth experiment. At day3 after adding low glucose differentiation media (A), at day5 (B), and at Day7 (C), Maintenance media used with Dexamethasone and low glucose, magnification 5X.

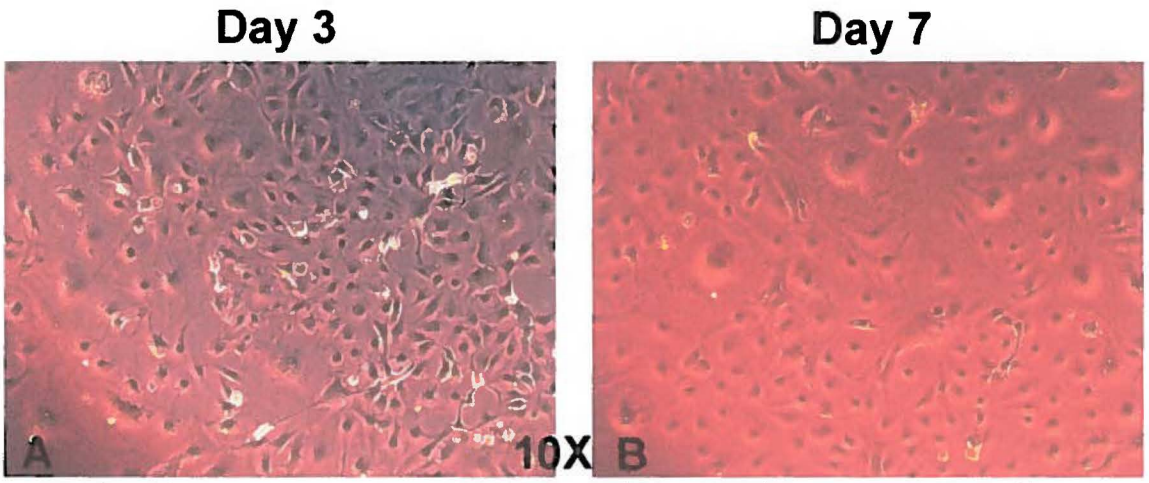


Figure 12 cells of the fourth experiment. At day3 (A), and at day7 (B), Maintenance media used with Dexamethasone and low glucose, magnification 10X.

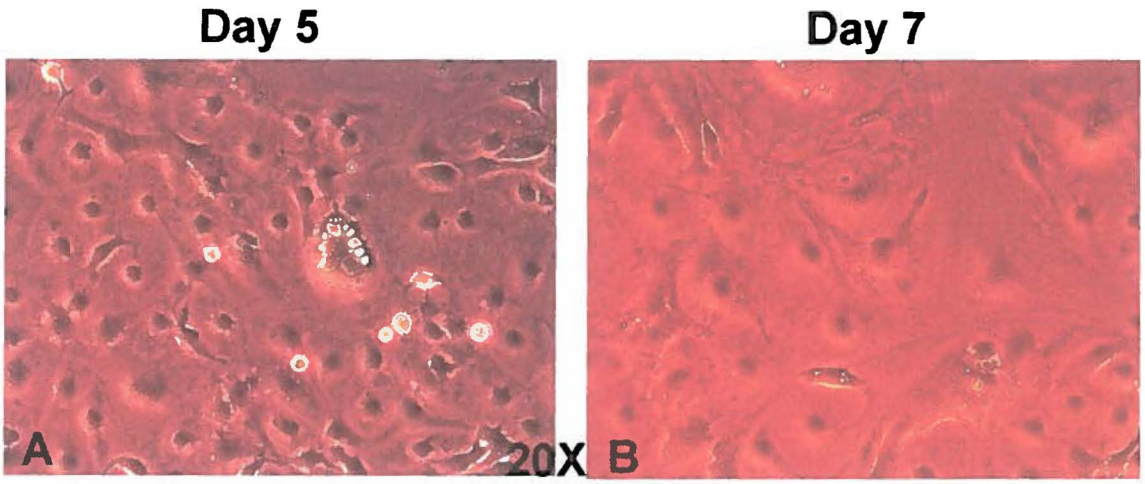


Figure 13 undifferentiated preadipocytes of the fourth experiment at day 5 (A), with only one possibly differentiating cell in the middle of the image. At day7 (B), Maintenance media used with Dexamethasone and low glucose. 20X magnification.

RNA Extraction Results

Table (3) shows the mean RNA concentration and purity for each day from each experiment obtained by NanoDrop, data are shown as mean (± Standard Deviation):

Table 3. Mean RNA concentration and purity

Group	Day	Concentration ng/µl	260/280
First group (High glucose, without Dex.)	Zero	1567.5 (±658.76)	2.00 (±0.015986)
	Three	383.01 (±71.69)	1.92 (±0.008165)
	Five	377.03 (±68.21)	1.92 (±0.05647)
	Seven	299.1 (±36.98)	1.96 (±0.046188)
Second group (High glucose, with Dex.)	Zero	264.6 (±294.69)	1.72 (±0.24683)
	Three	94.03 (±44.54)	1.55 (±0.10976)
	Five	128.26 (±50.19)	1.86 (±0.152394)
	Nine	126.36 (±62.60)	1.79 (±0.109202)
Third group (Low glucose, with Dex.)	Zero	272.06 (±130.78)	1.75 (±0.181177)
	Three	130.78 (±37.95)	1.66 (±0.1027)
	Five	139.31 (±40.95)	1.74 (±0.114078)

METHODOLOGY

Materials

Cells were purchased from Zen Bio Company, (Chapel Hill-Nelson Blvd., Suite 104, PO Box 13888/ Research Triangle Park, NC 27709, USA). Cryopreserved 3T3-L1 preadipocytes were received in 2ml vial containing 0.5 million cells of passage 8, Cat# SP-L1-F, and media were purchased alongside the cells from the same company (Zen Bio). The media were: 3T3-L1 Preadipocyte Media, Cat# PM-1-L-1 (contains 4.5g/L D-glucose, equals ~25 mM); 3T3-L1 Adipocyte Maintenance Media, Cat# AM-1-L-1; 3T3-L1 Adipocyte Maintenance Media without dexamethasone, Cat# AM-1-L-1-DF; 3T3-L1 Adipocyte Differentiation Medium, Cat# DM-2-L-1; and Cryopreservation Medium for 3T3-L1 cells, Cat# FM-1-L-1-100. All media except Preadipocyte Media, Cat# PM-1-L-1, contained 3.15g/L D-glucose, (equals ~ 17.5 mM).

3T3-L1 Adipocyte Differentiation Medium *containing low glucose (1g/L= ~5.5 mM)*, Cat# CO-5; and 3T3-L1 Adipocyte Medium *containing low glucose (1g/L= ~5.5 mM) DMEM*, Cat# CO-5 were also obtained from Zen Bio Company.

Other materials and reagents used in this study were: Trypsin/ EDTA (TE) solution (1X), REF# R-001-100, was from Gibco (Life Technologies, UK). Phosphate Buffered Saline (PBS) pH 7.4 (1X), was from Gibco (Life Technologies, UK). TRIzol Reagent Ambion RNA [REF #15596026] was purchased from Life Technologies USA. Chloroform HPLC grade, Code: C/4966/15, was purchased from Fisher Scientific. RNase free water was from PreAnalytiX/ Qiagen, # 1057099 Hilden, Germany. Agilent RNA 6000 Pico Kit was from Agilent Technologies, Waldbronn- Germany. High Capacity RNA-to-cDNA kit was purchased from Applied Biosystems P/N 4387406. TaqMan Gene Expression Master Mix (P/N 4369016); Pre-Developed TaqMan Assay Reagents, Mouse ACTB (20X) (Ref#

4352933E, UK); TaqMan® Gene Expression Assays, Ref# 00440945 (Mouse PPARγ primer); and TaqMan® Gene Expression Assays, Ref# Hs00960964_g1, PN4351372 (Mouse POU4F2/Brn3b primer), were all purchased from Applied Biosystems Foster, USA.

All other materials used in this study were of high quality and purchased from Sigma (St. Louis, MO, USA)

Ethical and Biosafety Approvals

Qatar University institutional review board has exempted this study, exemption number: QU-IRB 269-E/13; see Appendix II. Qatar university institutional Bio-safety committee has approved this study, approval number: QU-IBC 3/13-14, see Appendix III.

Cell Culture

Cryopreserved 3T3L-1 preadipocytes Passage 8 were received and kept in -80°C. They were transported in liquid Nitrogen from Qatar University campus, biomedical research labs to Anti Doping Laboratory- Qatar (ADL-Q), where all work had been conducted. 3T3L-1 cells were resurrected before work by thawing them in a water bath at 37°C. Cells were washed in pre-warmed 5ml preadipocytes media (PM-1-L-1) in a 15ml tube, then centrifuged at 1000 rpm for 2 minutes. Washing purpose was performed to get rid of all Dimethyl sulfoxide (DMSO) that was used in the cryopreserving media. After centrifugation, the supernatant was discarded and the cells were resuspended in 2.5 ml pre-warmed PM-1-L-1 media and were seeded in a small (25 CM^3) culture flask containing 2.5ml warm PM-1-L-1 media, and labeled as Passage Number 9 (P9). The cells were checked under the microscope (Leice Microscope DMI 3000 B, 5X magnification) to make sure that sufficient amount of cells had been transferred to the flask before placing it in the humidified incubator (37°C, 5% CO_2).

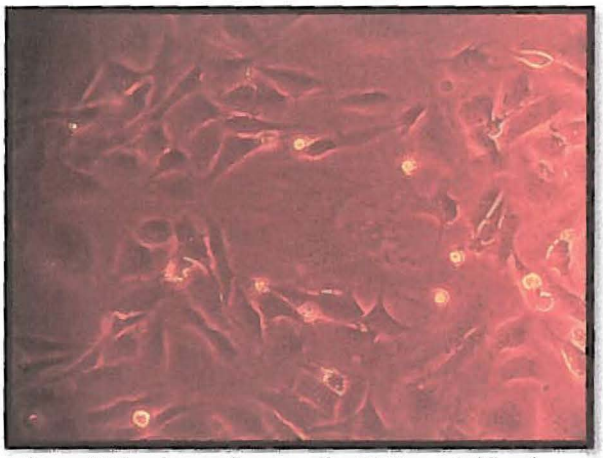


Figure 3 Nearly confluent cells. 20X maginification

After 18 hours incubation, the 3T3-L1 preadipocytes have adhered to the flask and started its division. Figure 3 to the left is showing cells nearly confluent. Cells were re-fed by removing old media (without touching the surface containing the cells) and replacing it with fresh 7.0 ml pre-warmed PM-1-L-1 media. The media had been changed always in the next day of seeding to remove debris and any dead unstuck cells.

Cell Passage

Each time cells had to be used in an experiment, cells had to be taken off the plate in order to grow and multiply. Pre warmed Trypsin EDTA (TE) was used to peal cells off the plate, after removing the media and washing the cells with phosphate buffer saline (PBS) 5ml in case of using $75CM^3$ flask and 2.5 ml in case of using $25CM^3$ flask. Cells were incubated with TE for 2 minutes at 37°C; after visualizing the cells under microscope and making sure they were dislodged, cells were mixed and placed in 15 ml tube. The flask was washed again with 5ml warm PM-1-L-1 to take any remaining cells and added them on top of the TE washed ones. The tube containing TE treated cells was then centrifuged at 1000 rpm for 5 minutes. The resulting supernatant was discarded, only small amount (~ 0.5- 1ml) was left to resuspend the cells. The cells pellet became ready to add PM-1-L-1 warm media to reseed or count them.

In case of freezing cells away, after treating cells with TE (the procedure described above), the cell pellet was resuspended in 0.5-1 ml warm cryopreserving media (amount depends on the number of cells); then 500µl of the cell suspension was placed in different 2ml cryopreserving tubes, which were stored straight in -80°C freezer.

Cell Count

Cell count was performed routinely and before the differentiation procedure. Cell to cell contact may enhance differentiation, and by counting cells we ascertain cells in all wells start differentiation at the same time. When counting cells was required; cells were treated with TE (the way described before). Upon re-suspending cells with PM-1-L-1 media, a small amount was taken (~ 20 µl) and placed it on hemacytometer (Bright Line) and was covered with cover slip. The number of cells counted in the middle 25 squares was multiplied by 10^4 per ml:

For example: in one experiment 56 cells in the 25 middle squares was gotten, then:

$56 * 10^4 = 5.6 * 10^5$ /ml

Cell Differentiation

Four 6-well plates for each differentiation experiment were used. All four plates were labeled and 1.5 warm PM-1-L-1 media was placed in each of their 6 wells. The same quantity of cells was seeded in each well. Then, the plates were kept in 37°C, 5% CO_2 incubator, and in the next day PM-1-L-1 media was changed. When the cells became ~ 100 % confluent, the PM-1-L-1 media was replaced with warm differentiation media DM-2-L1 (3.15 g/L glucose), 1.5 ml / well; and the cells in these plates were labeled day zero (D0). Thereafter, one of these plates was taken at day zero and its media was removed and cells were washed with 0.5 ml TRIzol reagent and placed in a microcentrifuge tube labeled with number, date, and day number (a tube for each well). Another 0.5 ml TRIzol was added in each well to wash any cell remnants and again transferred to the corresponding tube. Tubes were then kept in -80°C until initiation of RNA extraction.

For differentiation of the cells, they were exposed to the differentiation media for certain number of days followed by the maintenance medium, as shown in details in the next paragraph.

For the other plates differentiation media was left on the cells for three days; after that, it was replaced with Adipocyte Maintenance media AM-1-L-1; and these plates were labeled day three (D3). One of the plates labeled day three (D3) was imaged under the microscope (Leice Microscope DMI 3000 B, 5X, 10X, and 20X magnification). Following that, its media was removed and the cells were harvested in TRIzol reagent the way described before. Two days later, Adipocyte Maintenance media AM-1-L-1 was again refreshed in the remaining plate, which was labeled day 5 (D5) and processed exactly as described for day 0 and day3. The other plate was processed exactly as described for day 0 and day3. Two more days, on day seven (D7), the remaining plate was taken out, and processed exactly as described for day 0, day3 and day 5 and the cells treated with TRIzol reagent and kept in -80 °C waiting for RNA extraction.

The Study Groups

There were three groups in this study based upon the contents of the Differentiation and Adipocyte Maintenance media. Each group was repeated two times.

A- 3T3L-1 in high glucose Adipocyte Maintenance media without dexamethasone

It was performed exactly as described above in the previous paragraph, but the only difference that on day three, Adipocyte Maintenance media was used without dexamethasone (AM-1-L-1-DF) and continued thereafter for day 5 and day 7.

B- 3T3L-1 in high glucose Adipocyte Maintenance media with dexamethasone

In the third differentiation experiment, on day three, the Adipocyte Maintenance media AM-1-L-1 was used (already containing dexamethasone) and our experiment was performed

exactly as described in the previous paragraph. In addition, the cells of the last plate were kept for additional time until day nine (D9).

C- 3T3L-1 in low glucose (1g/L) Adipocyte Maintenance media with dexamethasone

In this experiment, differentiation media with low glucose was used (1g/L) CO-5 on day zero (D0), and Adipocyte Maintenance media with low glucose DMEM CO-5 (already contained dexamethasone) on day three (D3). the cells were kept until day seven (D7) only; and TRIzol reagent was added to the cells by pipetting up and down, and then the cells were kept at -80° C for further RNA extraction.

Total RNA Extraction

Total RNA was extracted from the cells that were preserved in TRIzol reagent at -80° C, according to the protocol provided by the manufacturer (REF #15596026, Ambion RNA by Life Technologies, USA). The tubes containing cell extract in 1ml TRIzol each were taken out of freezer and left to be thawed. Then, 200µl chloroform was added to each tube; then the tubes were shaken and incubated in room temperature for 2-3 minutes. After that, the tubes were centrifuged for 15 minutes at 13000 rpm and 4°C, whereas the mixture separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The colorless upper phase contained RNA, which was harvested and placed in a clean labeled microcentrifuge tubes. 500µl of 100% isopropanol was added on each tube and incubated in -20°C for 1 hour or overnight. After that, the tubes were centrifuged for 10 minutes at 13000 rpm and 4°C. RNA formed a pellet on the bottom and side of the tubes. The supernatant was discarded without disturbing the pellet. 1ml 70% Ethanol was then added on each tube for washing and mixed slightly. Again, the tubes were centrifuged for 5 minutes at 13000 rpm at 4°C. The supernatant was removed completely without disturbing the pellet, and then left to air dry. RNA pellet was then resuspended in 30µl RNase- free water and mixed well then

placed in heat block at 55°C for 10 minutes. After that, the tubes were placed on ice and RNA concentration was measured using NanoDrop.

RNA Quality Estimation

Apart from measuring RNA samples by NanoDrop, some randomly chosen samples were analyzed using Agilent RNA Bioanalyzer to check its quality and purity. The Procedures were followed according to the Agilent RNA 6000 Pico Kit Quick Start Guide provided by the company. RNA gel matrix was prepared first by pipetting 550 µl into a spin filter (provided with the kit); and centrifuged at 1500 g for 10 minutes at room temperature. Out of the filtered 550 µl, only 65 µl was needed in an RNase-free microcentrifuge tube. 1µl of RNA dye concentrate was vortexed for 10 seconds, after equilibrated to room temperature for 30 minutes, then spun down and added to the filtered 65 µl of RNA gel matrix. The tube then was vortexed and centrifuged at 13000g for 10 minutes at room temperature. 9 µl of the gel-dye mix was added in the third "G" marked well of the chip. The plunger was set at 1ml position and chip priming station was closed. The plunger was pressed until held by the clip, and then released after waiting for exactly 30 seconds. After waiting another 5 seconds, the plunger was repositioned to 1ml and the chip priming station was opened for adding another 9 µl of the gel-dye mix on each of the two "G" labeled wells. 9 µl of the RNA conditioning solution was pipetted into the well marked CS. Then, 5µl of RNA marker was added on all 11 remaining wells plus the ladder well. following that, 1µl of the pre-heated (70°C for 2 min) RNA samples was pipetted each into its corresponding well, along with 1 µl of the heat denatured ladder in the well marked ladder. The chip then was vortexed in the special chip vortexer for 1min at 2400 rpm; then chip was placed in Agilent 2100 Bioanalyzer instrument. Run took around 20-30 minutes to finish.

cDNA Synthesis

cDNA was prepared according to the manufacturer of High Capacity RNA-to-cDNA kit (P/N 4387406, Applied Biosystems, USA). 2 tubes from each day of the four experiments (of the three groups) were selected according to RNA quality and concentration. The reaction mix was prepared then distributed on all labeled PCR tubes. After that, RNA and water were added with different quantities each according to RNA concentration. Generally, the cDNA synthesis mixture had the following components, as shown in the following table:

Table 1. cDNA synthesis mixture

Component	**Volume/Reaction (µl)**
2X RT Buffer	10.0
20X Enzyme Mix	1.0
RNA Sample	1-9 (according to concentration)
Nuclease-free H_2O	1-8 (according to RNA sample volume)
Total per Reaction	20

The tubes were caped and mixed by vortex then spun down before placing them in thermo-cycler. Reverse transcription reaction was performed according to manufacturer's protocol: 37°C for 60 minutes followed by 95°C for 5 minutes then hold at 4°C. cDNA tubes were kept in the freezer at -20°C.

For details about calculations done for the chosen 32 samples see Appendix I

Real Time PCR

For gene expression experiments, Applied Biosystems real-time quantitative PCR ViiA 7 instrument was used. TaqMan Gene Expression Master Mix (P/N 4369016) from Applied Biosystems was used as well.

After preparing all cDNA samples and unifying their concentrations to the recommended 200 ng/µl (as illustrated in Appendix I), the PCR reaction mix was prepared. A tube of PCR reaction mixture enough for 32 samples was prepared each time for beta-actin and PPARγ genes. One negative control well containing water instead of cDNA was prepared as well. The reaction mixture contained the following components as shown in the following table:

Table 2. qPCR mixture for β-actin and PPARγ or Brn3b

Content	Amount per sample	β-actin for 34 samples	PPARγ/Brn3b for 34 samples
Master Mix	10 µl	340 µl	340 µl
Primer	1 µl	34 µl (β-actin primer)	34 µl (PPARγ primer)
RNA free H_2O	8 µl	272 µl	272 µl
cDNA (200ng)	1 µl	-	-
Total	**20 µl**	**646 µl**	**646 µl**

19 µl from each mixture were distributed in the corresponding wells in the optical 96-well plate, and then 1 µl from each cDNA sample was added. The plate was then sealed with adhesive cover and centrifuged, and then was placed in the real-time PCR instrument. Another mixture was performed exactly the same for Brn3b and β-actin as endogenous control.

Cycle threshold (C_T) defined as the cycle number at which a significant increase in the fluorescence signal compared to background emission is first detected. Calculating C_T values for each sample was performed to quantify unknown samples. ΔC_T defined for each sample by subtracting the β-actin gene C_T from each selected gene C_T. Relative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ methods, i.e. as the negative exponential power of 2 of each sample's ΔC_T minus the ΔC_T of a reference sample, which kept constant throughout experiments.

Statistical Data Analysis

Data were explored for outliers, skewness, and normality using Shapiro-Wilk test and log transformed when necessary for the statistical analysis, if normality assumption was violated, and thereafter back transformed for data presentation.

Continuous data are expressed as median and inter-quartile range [25% - 75%] with standard deviation, as shown in table 9.

Each experiment was independently repeated at least two times. All values in the figures are expressed as median and inter-quartile range and/or mean with standard deviation. To determine statistical significance within each group exposed for the same medium, the values were compared versus day 0 (as a control or reference day) with paired Student's t -tests. To determine the statistical significance between the different groups exposed for different media at days 3, 5 and 7, unpaired Student's t -tests were used for this comparison. All statistical analyses were performed using the SPSS program for Windows (version 21 statistical software; Texas instruments, IL, USA), and Microsoft excel (version 2010 stat software) were used for graphs. Two-tailed P value < 0.05 is statistically significant.

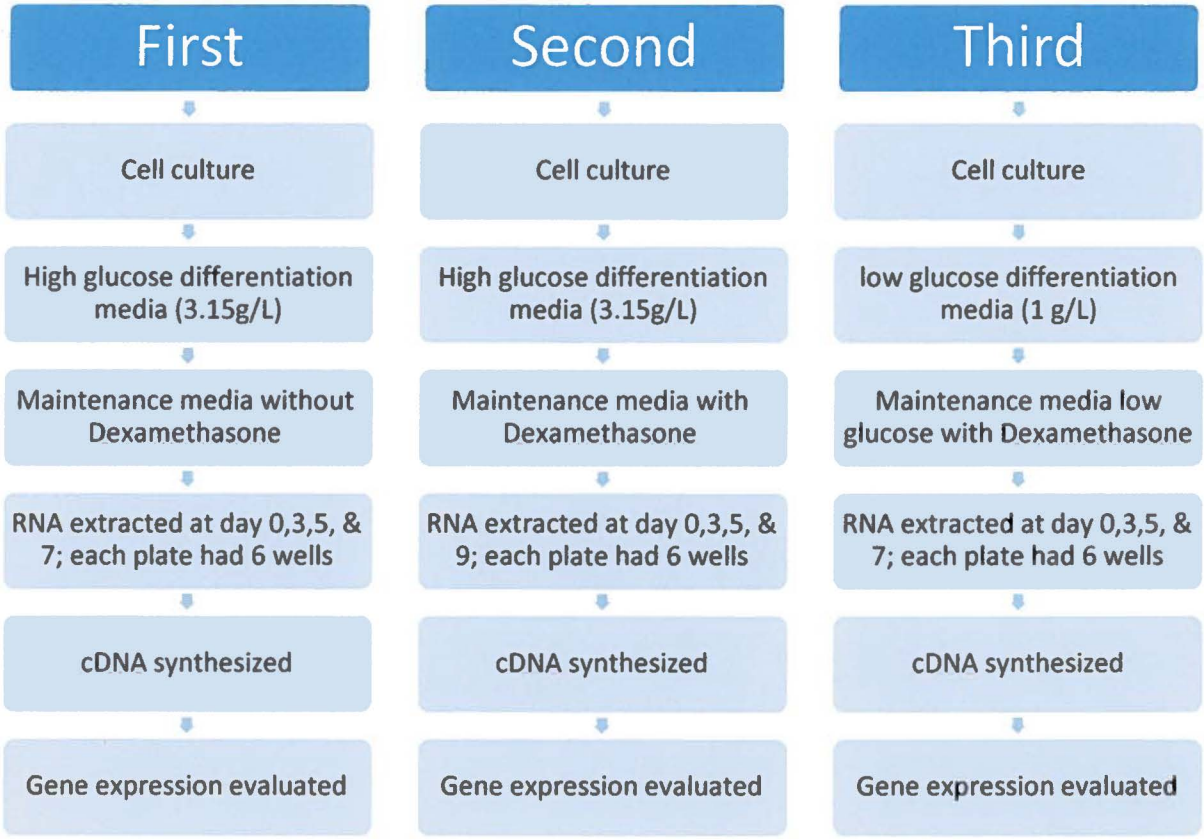
Work Scheme

First, second and third groups are shown in the following scheme. All were repeated twice.

First experiment (group): High glucose without dexamethasone (HG-DEX).

Second (group): High glucose with dexamethasone (HG+DEX).

Third (group): Low glucose with dexamethasone (LG + DEX).


First
Cell culture
High glucose differentiation media (3.15g/L)
Maintenance media without Dexamethasone
RNA extracted at day 0,3,5, & 7; each plate had 6 wells
cDNA synthesized
Gene expression evaluated
Second
Cell culture
High glucose differentiation media (3.15g/L)
Maintenance media with Dexamethasone
RNA extracted at day 0,3,5, & 9; each plate had 6 wells
cDNA synthesized
Gene expression evaluated
Third
Cell culture
low glucose differentiation media (1 g/L)
Maintenance media low glucose with Dexamethasone
RNA extracted at day 0,3,5, & 7; each plate had 6 wells
cDNA synthesized
Gene expression evaluated

Agilent RNA Bioanalyzer Results

RNA quality and concentration estimation by NanoDrop is not sufficient; therefore, RNA samples were run on Agilent 2100 Bioanalyzer. Some samples gave a good yield and pure RNA, while others were of poor quality. Figure 16 shows an example of the good (16.A) and a poor (16.B) quality RNA.

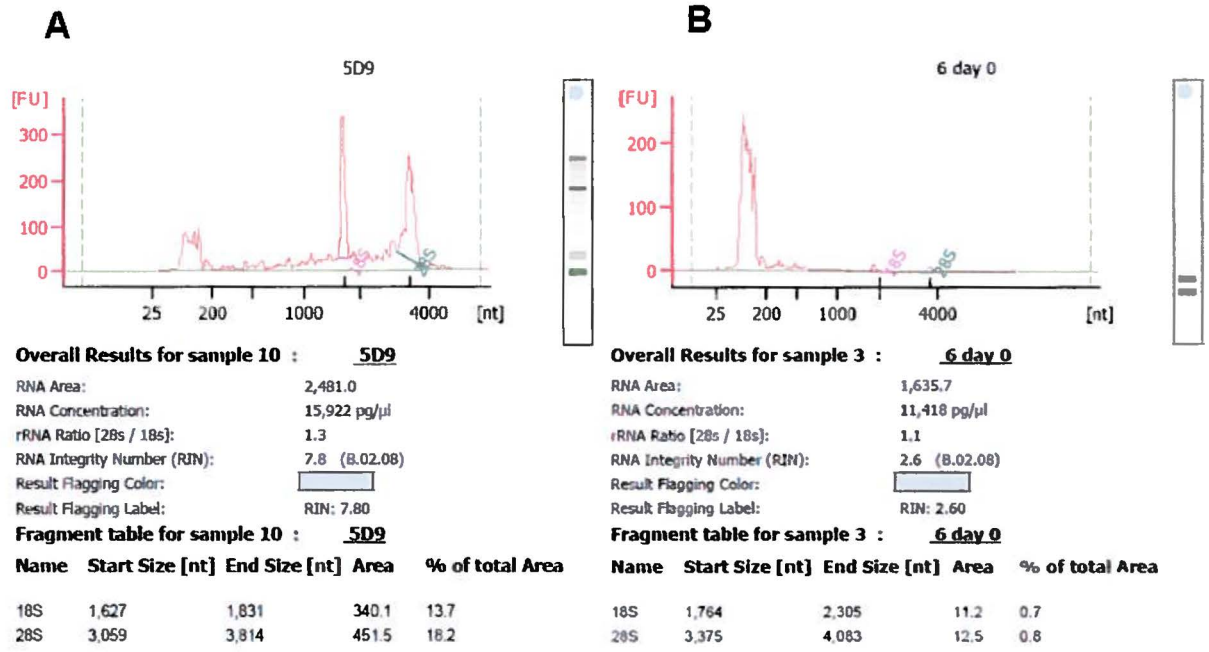


Figure 16. Agilent Bioanalyzer RNA quality estimation results; A good quality RNA (A), and a poor quality RNA (B). RNA area represents the total RNA area. RNA concentration is calculated from the area. rRNA ratio is the ribosomal RNA ratio of 28s/ 18s. RIN number better be above 6.

Gene Expression Results

A- PPARγ gene expression in different groups (Figures 17-21)

PCR was performed on 2 tubes of each time point for the 3 studied groups and repeated twice. Cycle Threshold (C_T) values for PPARγ gene were obtained and mean C_T values were calculated; and then referring first to the C_T values for endogenous control β-actin then referring to day zero tube of each experiment $\Delta\Delta C_T$ values were calculated. Fold differences were then obtained by getting ($2^{-\Delta\Delta C_T}$); Delta C_T = $C_{T\ PPAR\gamma} - C_{T\ \beta\text{-actin}}$ / Delta Delta C_T = ΔC_T

sample# – $\Delta C_{T\ Day0}$. Fold change range was established by subtracting or adding standard deviation to ($2^{-\Delta\Delta C_T}$) .In the following figures, bars are showing fold difference ± standard deviation of PPARγ expression in different days in the three groups.

PPARγ mRNA expression in response to HG-DX media:

3T3-L1 cells exposed to high glucose without dexamethasone showed progressive and significant increase in mRNA expression of PPARγ as shown in figure 17 and table 4. PPARγ expression increased about 6.7 folds from day 0 to day 3; and from day 0 to day 5 by 18.1 folds; and increased from day 0 to day 7 by 19.8 folds.

Data were explored for normality distribution by histogram and Sharipo-Wilk test for normal distribution and accept normality; hence, paired sample t-test was used to compare the gene expression in different days of adipogenesis within each treatment group versus day 0, results obtained for high glucose without dexamethasone were illustrated in table 4.

Table (4) is displaying the statistical details of fold increase for the high glucose without dexamethasone group with P- values calculated using Paired T-test compared to day0 of the same group.

Table 4. Mean C_T values for PPARγ and β-actin in 3T3-L1 cells with calculations of ΔC_T, $\Delta\Delta C_T$, fold change ± standard deviation, and P values compared to day0 (as a control) for high glucose without dexamethasone group

Day	PPARγ mean C_T	β-actin mean C_T	ΔC_T (PPARγ-β-actin)	$\Delta\Delta C_T$ (ΔC_T day# - ΔC_T day0)	Fold difference in PPARγ relative to β-actin	P-value compared to day0
Day0	28.14±0.56	19.60±0.53	8.54±0.78	0.00±0.78	1.0 (0.5-1.7)	-
Day3	27.34±0.57	21.56±0.25	5.78±0.62	-2.75±0.62	6.7 (4.3-10.4)	0.0103

Day5	25.59±0.45	21.23±0.19	4.36±0.49	-4.17±0.49	18.1 (12.8-25.5)	0.0007
Day7	24.82±0.31	20.60±0.36	4.22±0.47	-4.31±0.47	19.8 (14.2-27.7)	0.0014

Data illustrated in figure 17 is showing column graph of the values displayed in table 4; fold change of PPARγ mRNA expression relative to β-actin endogenous gene are shown with error bars representing standard deviation. The figure shows the progressive increase in PPARγ expression during adipogenesis.

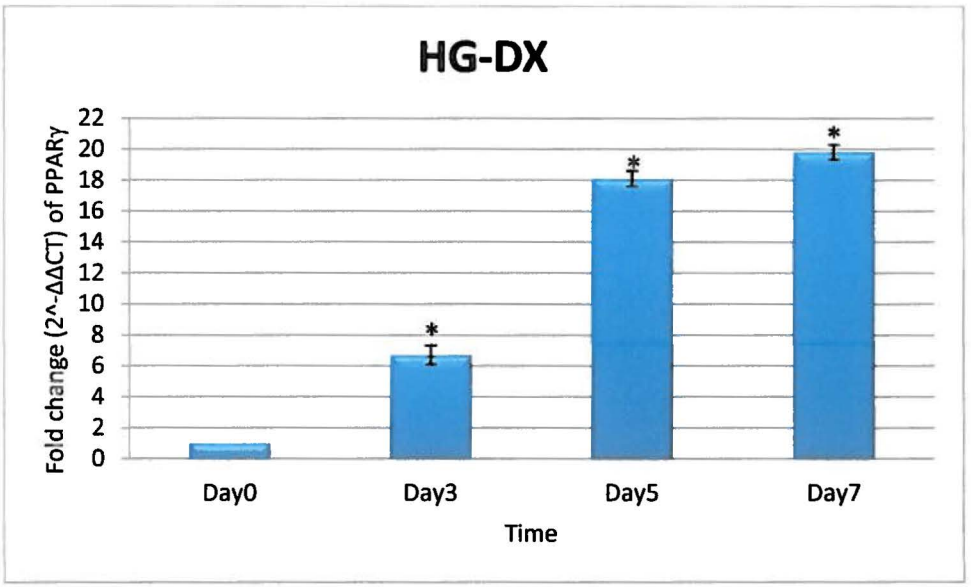


Figure 17 . Column graph of PPARγ mRNA expression among the days of differentiation for high glucose without dexamethasone group, represented by the fold change ($2^{-\Delta\Delta C_T}$) ± SD. (n=4 for each day). Two tailed p value is significant at * P<0.05 vs. adipocytes at day 0 as a control.

PPARγ mRNA expression in response to HG+DX media:

Similar observations were observed for 3T3-L1 cells that were exposed to high glucose media with dexamethasone, whereas progressive increase in mRNA expression of PPARγ was noticed as shown in figure 18 and table 5. The increase in the PPARγ expression from day 0 to day 3 was 10.7 folds; and from day 0 to day 5 was 17.2 folds; and from day 0 to day 9 by 17.4 folds. Table 5 illustrates the statistical details of fold increase for the high glucose with

dexamethasone group and P-values obtained by Paired T-test compared to day0 of the same group.

Table 5. Mean C_T values for PPARγ and β-actin in 3T3-L1 cells with calculations of ΔC_T, $\Delta\Delta C_T$, fold change ± standard deviation, and P values compared to day0 for high glucose with dexamethasone group

Day	PPARγ mean C_T	β-actin mean C_T	ΔC_T (PPARγ-β-actin)	$\Delta\Delta C_T$ (ΔC_T day# - ΔC_T day0)	Fold difference in PPARγ relative to β-actin	P-value compared to day0
Day0	27.66±0.24	18.81±0.36	8.85±0.43	0.00±0.43	1.0 (0.7-1.3)	-
Day3	28.71±1.25	23.29±1.33	5.41±1.83	-3.43±1.83	10.7(3.0-38.5)	0.0006
Day5	25.65±0.40	20.90±0.40	4.74±0.56	-4.10±0.56	17.2(11.6-25.5)	0.033
Day9	25.77±0.24	21.05±0.32	4.72±0.40	-4.12±0.40	17.4 (13.1-22.9)	0.0002

Data shown in figure 18 represent column graph of the values displayed in table 5; fold change of PPARγ mRNA expression relative to β-actin endogenous gene are shown with error bars representing standard deviation. The figure shows the progressive increase in PPARγ expression during adipogenesis with a noticeable high increase in day3.

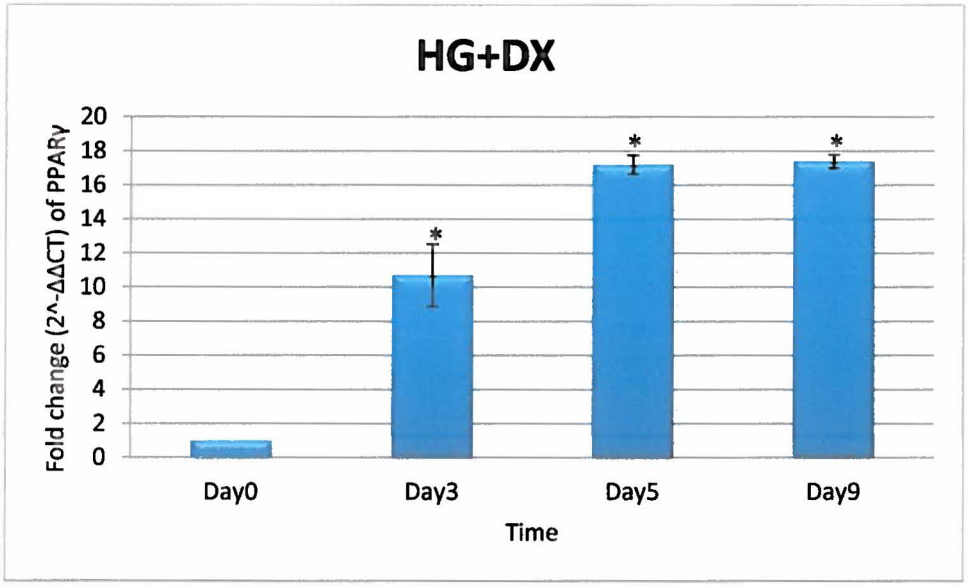


Figure 18. Column graph of PPARγ mRNA expression among the days of differentiation for high glucose with dexamethasone group, represented by the fold change ($2^{-\Delta\Delta C_T}$) ± SD. (n=4 for each day). * P<0.05 vs. adipocytes at day 0 as a control.

PPARγ mRNA expression in response to LG+DX media:

3T3-L1 cells grown in low glucose media with dexamethasone exhibited limited mRNA gene expression of PPARγ as shown in figure 19 and table 6. There were slight increase in PPARγ expression; from day 0 to day 3 increased by 2.3 folds; and increased from day 0 to day 5 by 3.7 folds; and from day 0 to day 7 increased only by 3.3 folds. Table (6) reveals the slight statistical change of PPARγ expression for the low glucose with dexamethasone group along with P-values calculated using Paired T-test compared to day 0 of the same group.

Table 6. Mean C_T values for PPARγ and β-actin in 3T3-L1 cells with calculations of ΔC_T, $\Delta\Delta C_T$, fold change ± standard deviation, and P values compared to day0 for low glucose with dexamethasone group

Day	PPARγ mean C_T	β-actin mean C_T	ΔC_T (PPARγ-β-actin)	$\Delta\Delta C_T$ (ΔC_T day# - ΔC_T day0)	Fold difference in PPARγ relative to β-actin	P-value compared to day0
Day0	28.19±0.74	20.26±0.85	8.64±1.13	0.00±1.13	1.0 (0.4-2.1)	-
Day3	29.55±0.40	22.10±0.32	7.44±0.51	-1.20±0.51	2.3 (1.6-3.2)	0.0798
Day5	28.04±0.49	21.30±0.42	6.73±0.65	-1.91±0.65	3.7 (2.3-5.9)	0.0053
Day7	28.46±0.27	21.56±0.22	6.89±0.35	-1.74±0.35	3.3 (2.6-4.2)	0.0126

The graph shown in figure 19 presents the values displayed in table 8; fold change of PPARγ mRNA expression relative to β-actin endogenous gene are shown with error bars representing standard deviation. The figure shows the slight increase in PPARγ expression during adipogenesis with low glucose medium (1g/L).

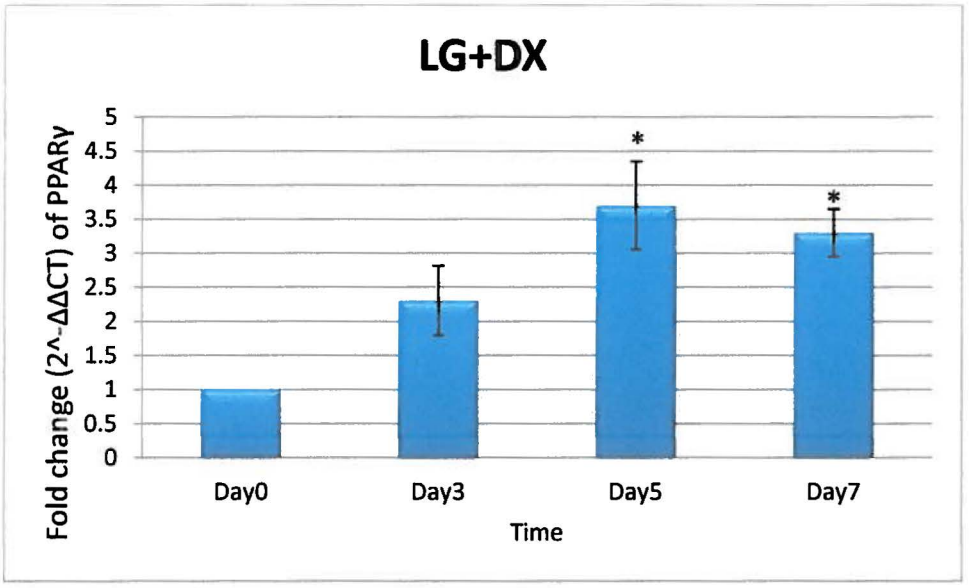


Figure 19. Histogram of PPARγ mRNA expression in low glucose with dexamethasone experiment, represented by the fold change ($2^{-\Delta\Delta C_T}$) ± SD. (n=4 for each day). * P<0.05 vs. adipocytes at day 0 as a control. 3T3L1 cells were treated with low glucose with dexamethasone as indicated in methods section. Total RNA extracted as indicated at the time points and reverse transcribed using qPCR .

PPARγ mRNA expression in response to HG+DX versus LG+DX media:

A comparison of PPARγ mRNA expression between high glucose (3.15g/L) and low glucose (1 g/L) groups both with dexamethasone is shown in figure 20. Statistical analysis demonstrated significant difference in the PPARγ gene expression between high glucose compared to low glucose at different time points. Table (7) demonstrates P values for days 3, 5, and 7/9 between high glucose and low glucose groups.

Table 7. P-values for the comparison between high glucose and low glucose both with dexamethasone groups at different days obtained by Paired T-test

Paired T- test		
HG+DEX vs. LG+DEX		
Day	**P value**	**Significance**
day 3	0.0001	Significant
day 5	0.0209	Significant
day 7	0.0001	Significant

The higher PPARγ gene expression in high glucose group compared to low glucose group is represented in figure 20. Data are represented by fold change ($2^{-\Delta\Delta C_T}$)

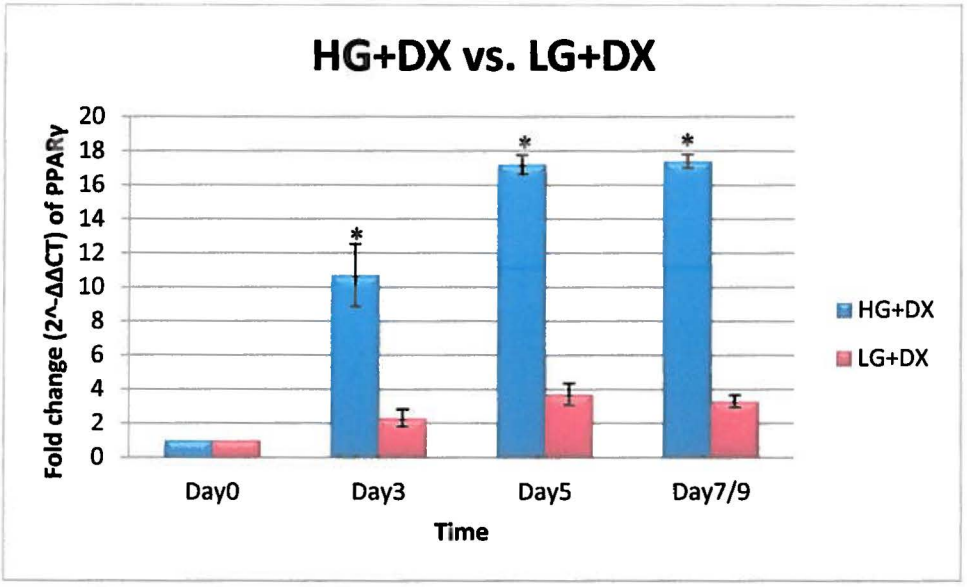


Figure 20 a comparison of relative PPARγ mRNA expression (fold change $2^{-\Delta\Delta C_T}$) in response to high (blue) versus low glucose (red) media with dexamethasone. Data are presented as mean with SD of PPARγ in different days (n=4 for each day in each group). * P<0.05 is significantly different between exact days (3, 5, and 7/9) in two groups, while day 0 is used as control for both groups after normalization and calibration as a reference.

PPARγ mRNA expression in response to high glucose media in the presence and absence of dexamethasone

Statistical analysis demonstrated significant difference in the PPARγ gene expression between high glucose groups using dexamethasone or without using dexamethasone only at day3 of the experiment as shown in figure 21. Relative PPARγ gene expression at day3 of the high glucose with dexamethasone was significantly higher than PPARγ gene expression in the high glucose without dexamethasone group at the same day. No significant difference has been detected in days 5 and 7/9 between groups with dexamethasone and without dexamethasone. Table (8) demonstrates P values for days 3, 5, and 7/9 between high glucose groups using or not using dexamethasone.

Table 8. P-values for the comparison between high glucose with and without dexamethasone groups at different days obtained by Unpaired T-test

Unpaired T- test		
HG with DEX vs. HG without DEX		
Day	**P value**	**Significance**
day 3	0.0024	significant
day 5	0.0918	Not significant
day 7/9	0.8589	Not significant

The difference in PPARγ gene expression between high glucose group with dexamethasone (red) and without dexamethasone (blue) at different time points is illustrated in figure 21.

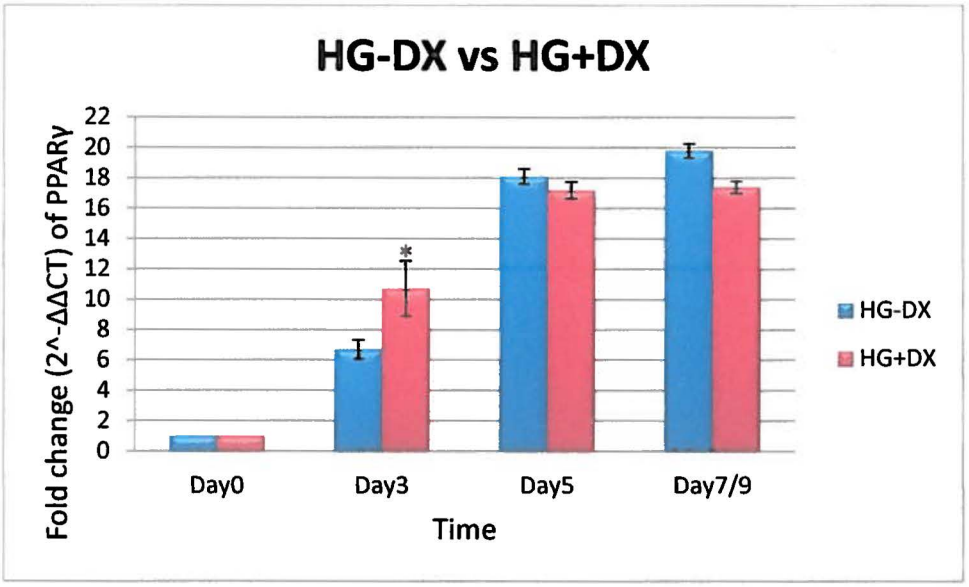


Figure 21 a comparison of PPARγ mRNA expression fold change ($2^{-\Delta\Delta C_T}$) in response to high glucose media with (red) and without dexamethasone (blue). Data are presented as mean of PPARγ in different days (n=4 for each day in each group). * P<0.05 is significantly different between the exact day of differentiation (3) in the two groups, while day 0 is used as control for both groups after normalization and calibration to day 0 for each group as a reference.

PPARγ gene expression in all studied groups

In another way of representing the results in one block, figure 22 show the three groups high glucose without dexamethasone, high glucose with dexamethasone, and low glucose with

dexamethasone in single view. Table 9 illustrates the numerical details of the median and inter-quartiles (25%-75%) with standard deviation for the three studied groups. Figure 22 represents fold change of all groups, where rectangles represent inter-quartiles (25%-75%) with median at 50% and error bars represent SD of the median.

Table 9. A comparison between the three groups in median and inter-quartiles (25%-75%) with SD

Day & group	Median	25 - 75 P	Standard Deviation
Day0-HG-Dex	0.978	0.74-1.00	0.16
Day3-HG-Dex	5.820	4.80-7.96	1.69
Day5-HG-Dex	16.809	9.92-26.85	8.85
Day7-HG-Dex	16.898	11.47-30.58	10.52
Day0-HG+Dex	1.074	0.96-1.16	0.10
Day3-HG+Dex	11.119	10.65-12.90	1.31
Day5-HG+Dex	18.397	17.76-18.91	0.61
Day9-HG+Dex	18.648	17.07-20.14	1.66
Day0-LG+Dex	1.288	0.69-2.07	0.71
Day3-LG+Dex	2.770	2.36-3.25	0.46
Day5-LG+Dex	4.538	4.21-4.83	0.33
Day7-LG+Dex	4.030	3.47-4.73	0.66

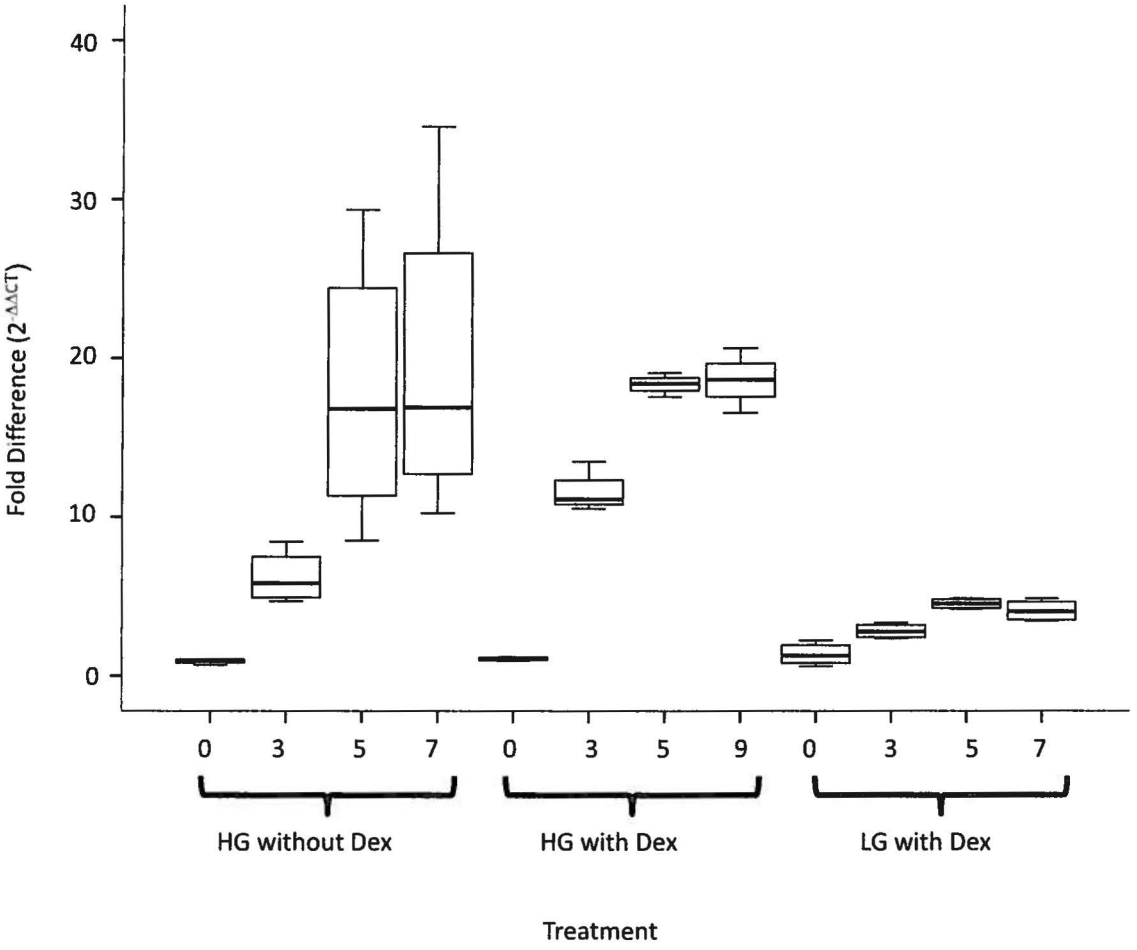


Figure 22 Comparison of the three treatment groups showing PPARγ gene expression expressed by fold change ($2^{-\Delta\Delta C_T}$). Data presented as median (interquartile range), with error bars representing SD.

Figure 23 shows amplification plot of PPARγ versus β-actin.

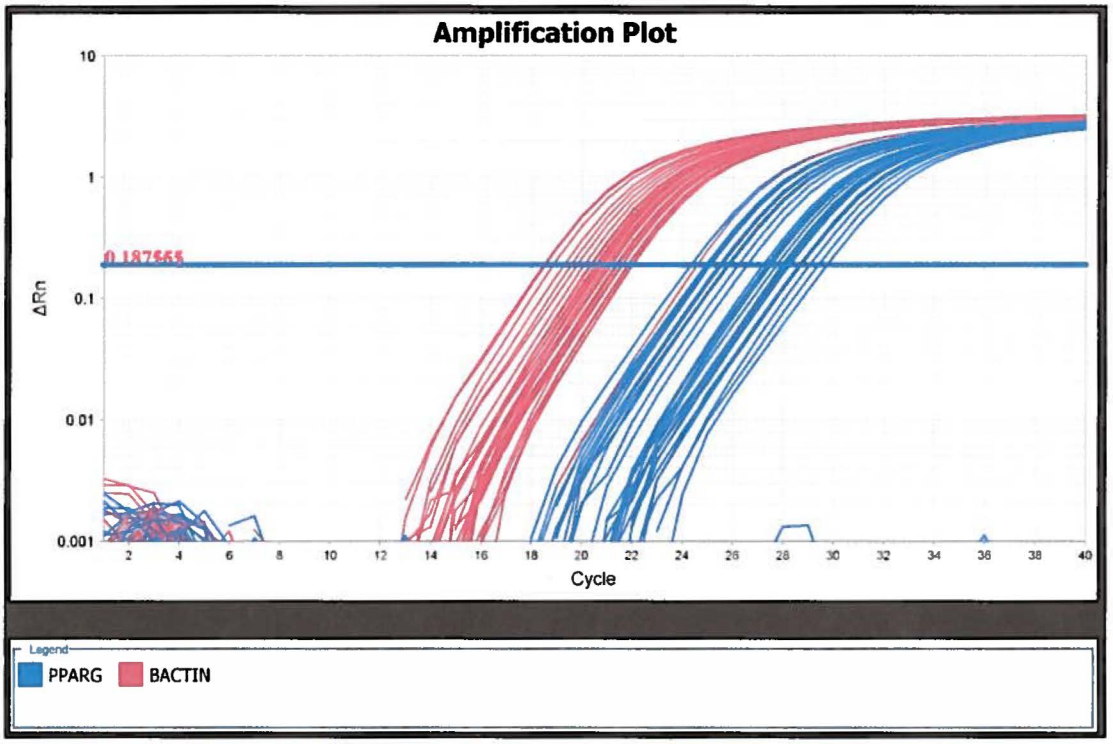


Figure 23 amplification plot of PPARγ gene (blue) versus β-actin (red).

Of note, the housekeeping gene β-actin is used as control gene in our study showed consistent results in response to different media at different time points of adipogenesis without any significant or marked changes in expression.

B- Brn3b gene expression

PCR was also performed for the same samples used in our study, for PPRA gamma gene expression from different groups, for Brn3b along with β-actin gene as endogenous control. No amplification of Brn3b mRNA gene expression was detected in all experiments (for the three different groups) and in all time points (days 0,3,5,7 or 9) as shown in figure 24. No amplification of Brn3b gene was observed between preadipocytes or adipocytes.

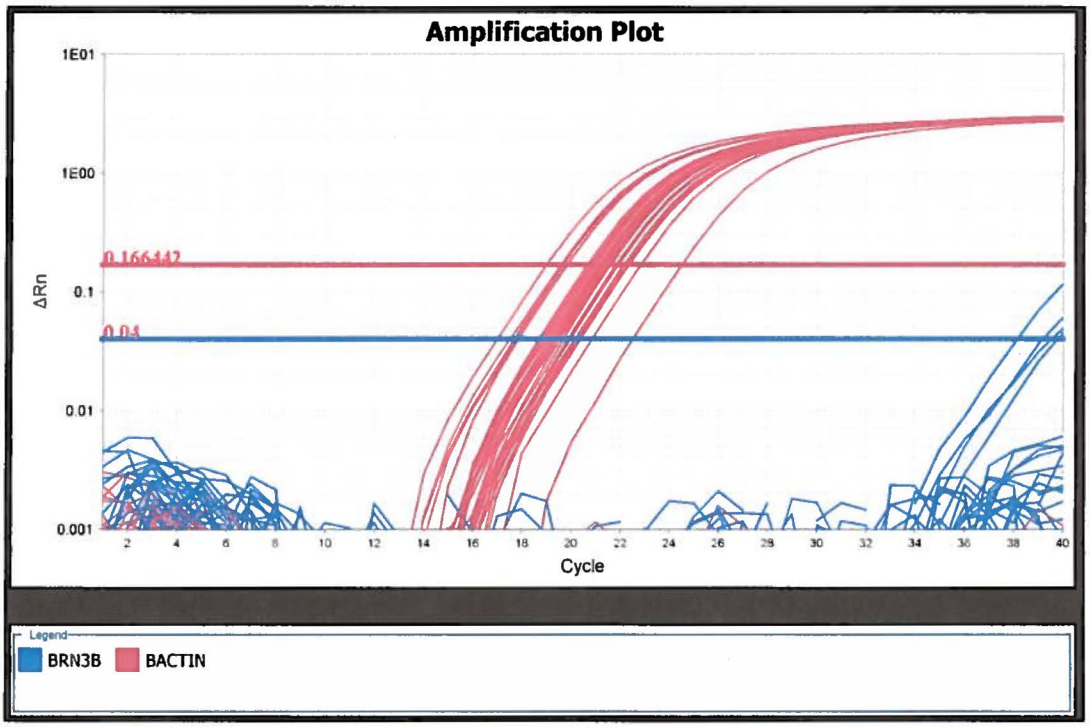


Figure 24 amplification plot of Brn3b gene (blue) versus β-actin (red). No amplification was seen in Brn3b.

DISCUSSION

As obesity is spreading and gaining more attention, studies focusing on understanding mechanisms leading to obesity are being conducted. In this study, 3T3-L1 preadipocytes were successfully resurrected and grown in different glucose concentrations in the presence of insulin, as well with and without dexamethasone; intended for studying the expression of Brn3b transcription factor and its impact during adipogenesis compared with the well-known transcription factor PPARγ. Three groups have been studied; in the first group cells were treated with high glucose media without dexamethasone, in the second group cells were treated with high glucose media with dexamethasone, and in the third group cells were treated with low glucose media with dexamethasone. The cell extract were obtained at days 0, 3, 5, 7 and/or 9 and the gene expression at these days were evaluated for the expression of Brn3b and PPAR gamma by qPCR. The major finding of this study is that neither preadipocytes nor adipocytes at different points of differentiation with perturbations of glucose and dexamethasone show or demonstrate expression of Brn3b transcription factors at the mRNA level, while PPARγ expression was expressed in preadipocytes as well as adipocytes under these conditions. The implications of this finding are discussed in the following paragraphs.

Brn3b is known to be expressed abundantly in nervous tissue (Lillycrop et al, 1992), and other tissues such as retina (Wang et al., 2000), breast cells (Ounzain et al, 2011), testis (Budhram-Mahadeo et al, 2001), and peripheral blood cells (Niculescu et al, 2007), but there is no currently available published data regarding Brn3b expression in preadipocytes and adipocytes of the adipose tissue. The current data of this study revealed that Brn3b transcription factor is not expressed in the preadipocytes or into the mature adipocytes after differentiation whether in normal media or even with perturbations of glucose and dexamethasone in the growth differentiation and maintenance media used. To the best of our

knowledge, this is first study to look for Brn3b expression in preadipocytes and adipocytes in 3T3-L1 murine cell model. These results correlate well with a parallel-unpublished study (Ms. Moza Alkwari, Dr. Vidya and Dr. Nasser) that was conducted on human adipose tissue of obese subjects. The unpublished study revealed that Brn3b was not expressed in isolated human adipocytes, but only expressed at the mRNA level in the stromal vascular cells obtained from the same subject particularly in subcutaneous and omental adipose depots. Therefore, the Brn3b expression that has been shown in stromal vascular fraction of the human adipose tissue probably originates from non-adipocytic lineage cells such as hematopoietic, endothelial, and stromal cells. These data are in agreement with our findings that neither preadipocytes nor mature adipocytes express Brn3b. Such findings need to be further investigated and confirmed by cellular and molecular experiments.

Our results demonstrate that in glucose rich media (high glucose, 3.15 g/L), 3T3-L1 preadipocytes started to differentiate, compared with cells exposed to low glucose media (1g/L) that has been shown by morphological change at different days. When cells are left to grow in glucose rich media (high glucose, 3.15 g/L), they started to differentiate. Cells differentiation started to be noticed at day three, which also can be confirmed by the gradual increment of the adipogenic gene expression PPARγ. Later, at day five and day seven or nine, differentiated cells can be distinguished easily in the images by their intra-cellular fat droplets. The morphological results were further correlated with the significant increase of PPARγ gene expression by many folds. Noticeably, cells grown in low glucose media (1g/L) were arrested and failed to differentiate, as confirmed by the lack of lipid accumulation in the cells and the limited changes in the adipogenic PPARγ gene expression. Obviously, results obtained point to the necessity of high glucose concentration to facilitate cells to differentiate. Glucose is an important nutrient and is important factor for differentiation of preadipocytes and adipogenesis (Aguiari *et al.*, 2008) (Yue *et al.*, 2010). Strong association of PPARγ with

adipogenesis and differentiation of preadipocytes into adipocytes was observed in this study as well as previous reports in literature (Schoonjans *et al.*, 1996) (Spiegelman *et al.*, 1997) (Spiegelman, 1998). However, dexamethasone seemed to exert its effect on cells differentiation and PPARγ expression at only the early stage of differentiation. As shown in the current results, PPARγ expression at day3 in the group of high glucose with dexamethasone had higher fold change than that of the high glucose without dexamethasone group. Such findings are in parallel with the previous published data that point to the role of dexamethasone or glucocorticoids in adipogenesis. Several published reports indicated that dexamethasone stimulate C/EBP transcription factors family early during differentiation that in turn stimulates PPARγ expression. At later stage, C/EBPβ and C/EBPδ expression decreases while C/EBPα is induced by PPARγ (Ali *et al.*, 2013) (MacDougald & Mandrup, 2002) (Wu, Puigserver & Spiegelman, 1999). In addition, the morphology of 3T3-L1 cells that have been grown with or without dexamethasone differentiated well and looked similar under microscope. Although graphs for PPARγ mRNA expression in cells grown with or without dexamethasone looked different, statistically there was no significant difference at the later stages, only at day 3 (early stage) dexamethasone induced PPARγ mRNA expression and accelerate the adipogenesis, consistent with some previous studies (Hamm *et al.*, 1999) (Lai *et al.*, 1982).

This study has several limitations; first only one cell type (3T3-L1) was used in this study. Second, Since Brn3b was found not to be expressed in preadipocytes, other known Brn3b expressing cells needed to be included as positive control. However, all other quality control measures have been taken, such as negative blank control and RNA quality check.

In summary, Brn3b transcription factor was not expressed in adipocytes or their precursors preadipocytes. Glucose concentration is a significant factor in adipogenesis. PPARγ is indeed an adipogenesis associated transcription factor. The presence of dexamethasone stimulated

adipocytes differentiation and the expression of PPARγ at early stage, probably accompanied by the actions of other PPARγ activating transcription factors (Wu *et al.*, 1996).

The findings of this study open up for many other studies and much further work to be done. Brn3b needs to be studied in different cell types and compared to a known Brn3b expressing type. Moreover, Brn3b at the level of protein and post transcriptional and translational change require examination.

CONCLUSION

Adipogenesis was examined successfully in this study using the 3T3-L1 cell line. Media containing high glucose concentration was confirmed to be a crucial factor in adipogenesis, along with other factors such as dexamethasone, which were found to stimulate adipogenesis especially at early stage. As expected, PPARγ gene expression was elevated during preadipocytes differentiation into adipocytes. On the contrary, mRNA of a novel transcription factor Brn3b was not detected in 3T3-L1 preadipocytes or in their differentiated adipocytes. Further studies are needed with other adipose cell lines and in vivo studies to determine the presence /absence of Brn3b expression. The effect of Brn3b, originating from other cell types within the adipose tissue need to be investigated in the future.

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APPENDICES

Appendix I

The following table demonstrates the chosen 32 samples and the calculations of cDNA synthesis and dilution to 200ng/µl in preparation for PCR, 1 µl from each synthesized cDNA sample was mixed with the amount of water shown in the table:

Sample ID	Concentration	Quantity of RNA	Final concentration	Water for dilution to 200 ng/µl
1D0-1	~1000 ng/µl	1.5 µl	1500 ng/µl	6.5 µl
3D0-1	~1000 ng/µl	1.5 µl	1500 ng/µl	6.5 µl
4D3-1	538.6 ng/µl	4 µl	2154.4 ng/µl	9.7 µl
6D3-1	353.5 ng/µl	6 µl	2121 ng/µl	9.6 µl
2D5-1	499.5 ng/µl	4 µl	1998 ng/µl	8.99 µl
5D5-1	433.3 ng/µl	4 µl	1733.2 ng/µl	8.66 µl
4D7-1	305.3 ng/µl	6 µl	1831.8 ng/µl	8 µl
5D7-1	265.7 ng/µl	7 µl	1859.9 ng/µl	8.3 µl
3D0-2	505 ng/µl	4 µl	2020 ng/µl	9 µl
5D0-2	490 ng/µl	4 µl	1960 ng/µl	8.8 µl
3D3-2	209.8 ng/µl	9 µl	1888.2 ng/µl	8.4 µl
6D3-2	96.2 ng/µl	9 µl	865.8 ng/µl	3.3 µl
1D5-2	256.4 ng/µl	8 µl	2051.2 ng/µl	9 µl
3D5-2	272.6 ng/µl	7 µl	1908.2 ng/µl	8.5 µl
2D7-2	274.9 ng/µl	7 µl	1924.3 ng/µl	8.5 µl
4D7-2	262.4 ng/µl	7 µl	1836.8 ng/µl	8 µl

1D0-3	634.8 ng/µl	3 µl	1904.4 ng/µl	8.5 µl
6D0-3	723.7 ng/µl	2.5 µl	1809 ng/µl	8 µl
5D3-3	149.9 ng/µl	9 µl	1349.1 ng/µl	5.7 µl
6D3-3	142.8 ng/µl	9 µl	1285.2 ng/µl	5.4 µl
2D5-3	147.7 ng/µl	9 µl	1329.3 ng/µl	5.6 µl
5D5-3	208 ng/µl	9 µl	1872 ng/µl	8.3 µl
4D9-3	152.9 ng/µl	9 µl	1376 ng/µl	5.8 µl
5D9-3	223.2 ng/µl	9 µl	2008.8 ng/µl	9 µl
4D0-4	348.6 ng/µl	6 µl	2091.6 ng/µl	9.4 µl
6D0-4	357.4 ng/µl	6 µl	2144.4 ng/µl	9.7 µl
1D3-4	172 ng/µl	9 µl	1548 ng/µl	6.7 µl
4D3-4	179 ng/µl	9 µl	1611 ng/µl	7 µl
5D5-4	175 ng/µl	9 µl	1575 ng/µl	6.8 µl
6D5-4	198 ng/µl	9 µl	1782 ng/µl	7.9 µl
2D7-4	114 ng/µl	9 µl	1026 ng/µl	4.1 µl
6D7-4	123 ng/µl	9 µl	1107 ng/µl	4.5 µl

Appendix II

جامعة قطر
QATAR UNIVERSITY

Qatar University Institutional Review Board
QU-IRB

December 16, 2013

Nasser Rizk
Department of Health Sciences
College of Arts and Sciences
Qatar University
Tel.: 4403 4786
Email: nassrizk@qu.edu.qa

Dear Dr. Nasser Rizk,

Sub.: Research Ethics Review Exemption
Ref.: Project titled, "The regulation of Brn-3b in adipocytes by glucose and insulin"

We would like to inform you that your application along with the supporting documents provided for the above proposal, is reviewed and having met all the requirements, has been exempted from the full ethics review.

Please note that any changes/modification or additions to the original submitted protocol should be reported to the committee to seek approval prior to continuation.

Your Research Ethics Approval No. is: **QU-IRB 269-E/13**
Kindly refer to this number in all your future correspondence pertaining to this project.

Best wishes,

K. Hlali

Dr. Khalid Al-Ali
Chairperson, QU-IRB

جامعة قطر
Institutional Review Board
(IRB)
Office Of Academic Research

Appendix III

جامعة قطر
QATAR UNIVERSITY

Qatar University
Institutional Bio-safety Committee

To: Dr. Nasser Rizk
Department of Health Sciences
College of Arts and Science

28th Dec, 2013

Dear Dr. Rizk,

Subject: Research grant # QUST-CAS-FALL-13/14-5
Ref: Project titled "The regulation of Brn-3b in adipocytes by glucose and insulin"

We would like to inform you that your application along with supporting documents provided for the above proposal have been reviewed by QU-IBC, and having met all the requirements, has been granted approval for a period of one year and renewable for each year thereafter, should be sought and approved by QU-IBC period to continue.

Please note that QU-IBC approval is contingent upon your adherence to the following QU-IBC Guidelines:

- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of QU Safety Plans and applicable regulations/guidelines, or any significant research-related accidents and illnesses to the QU-IBC. Also, ensuring personnel receive appropriate orientation and specific training for the safe performance of the work.

Your research approval No. is: **QU-IBC 3/13-14**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes.

Dr. Marawan Abu-Madi
Chairperson, QU-IBC
Department of Health Sciences
College of Arts & Sciences
Qatar University
Tel: +974 4403 4791
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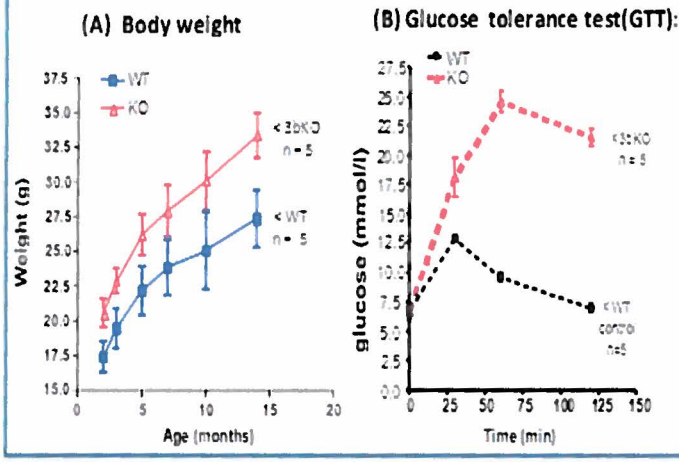
Institutional Bio-safety Committee
(IBC)
Office Of Academic Research

Appendix IV

Results of preliminary studies found that Brn3b knockout mice had a higher body weights compared to normal mice.

Figure 1: Loss of Brn-3b cause increased body mass and high blood glucose in KO mutant mice

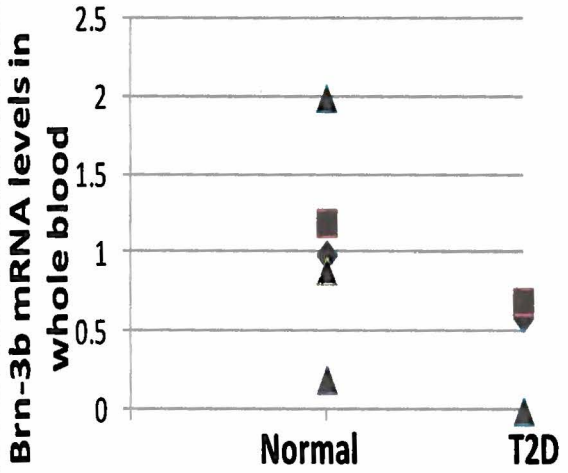


A. Brn-3b KO mutants have increased body weight *(red line; n=5) that is more pronounced in older animals compared with age-matched wild-type (WT) control littermates (blue; n=5) measured at intervals up to 14 mths.*

B. Brn-3b KO mutant mice are profoundly glucose intolorant as shown by the elevated blood glucose *after glucose tolerance testing (GTT) (red line) when compared with WT controls (black line). Baseline blood glucose was measured after overnight fasting and a glucose bolus was administrated. Half-hourly blood glucose levels were measured. Baseline blood glucose (BG) was similar in both groups. 30 min after glucose bolus, WT mice showed the expected increase in BG, which was reduced to baseline by 2 hours. However, Brn-3b KO mutants had significantly higher blood glucose levels after 30 min which peaked at 1 hr remained elevated at 2 hours. In contrast, control groups demonstrated increased blood glucose at 30 min following bolus dose followed by a reduction to baseline by 2 hrs.*

Results of preliminary studies also have shown that Brn-3b mRNA expression extracted from peripheral blood is significantly reduced in patients with T2D compared with normal non-diabetic controls.

Fig 6: Quantification of Brn-3b from whole human blood



Blood was collected from normal subjects or T2D patients using PAX gene Blood RNA system (Qiagen). Following RNA extraction and cDNA synthesis, quantitative real-time PCR was undertaken using Taq man probes for POU4F2/Brn-3b. Differences in RNA were adjusted using house-keeping gene, PPIA.