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A THESIS SUBMITTED FOR THE DEGREE OF

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ASSOC. PROF. DR. FERRUH ÖZCAN

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

MONOSODYUM GLUTAMAT'IN 3T3-L1 PREADİPOSİT FARKLILAŞMASI ÜZERİNDEKİ ETKİLERİ

MEHMET SONER TÜRKÜNER

YÜKSEK LİSANS TEZİ

MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. FERRUH ÖZCAN

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2017

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GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 21/06/2017 tarih ve 2017/33 sayılı kararıyla oluşturulan jüri tarafından 30/06/2017 tarihinde tez savunma sınavı yapılan Mehmet Soner TÜRKÜNER 'in tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

JÜRİ

ÜYE
(TEZ DANIŞMANI) : Doç. Dr. Ferruh ÖZCAN

ÜYE : Yard. Doç. Can M. ERİŞTİ

ÜYE : Doç. Dr. İbrahim YAMAN

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

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İMZA/MÜHÜR

SUMMARY

Monosodium glutamate (MSG) is the sodium salt of the glutamic acid and generally used as a flavor enhancer in processed foods and the main ingredient of asian cusine. High none physiological concentrations of MSG when administered intraperitonally produces obese phenotype in rodents. The obesogenic effect of MSG in MSG obese animals is known to be mediated primarily by abrogated hyphothalamo pituitary axis (HPA) that control animal feding beheviour. On the other hand, recent epidemiologic studies correlated dietary MSG use with increasing body mass index (BMI) despite the absence of overt neuropaties in the hyphothalamic region. This would suggest that dietary MSG intake may contribute to etiopathogenesis of obesity without effecting appetite center in the brain. In these animal studies, MSG-fed mice and their offsprings demonstrated higher level of fat mass and increased expression of adipogenic genes compared to control mice. Furthermore, adipose precursor cells from MSG-obese mice displayed reduced proliferation capacity along with the hypertrophic adipocyte phenotype indicating MSG interfering with the adipogenic process.

In this study, we aim at investigating the effects of MSG on 3T3-L1 adipogenic differentiation. In order to do this proliferation, mitotic clonal expansion (MCE) and terminal lipogenic phases of adipogenesis were investigated in the presence of increasing concentrations of MSG by using proliferation assays, cell cycle analysis and conventional biochemical studies. Intriguingly, high dosages of MSG (≥ 2.5 mM) inhibited MCE and thus adipogenesis. This inhibitory effect is thought to be mediated by some key cell cycle regulatory proteins including CCAAT/enhancer-binding protein beta (CEBPβ), p27, and Cyclin B which appears to be independent of insulin receptor signaling. Based on the data presented we speculate that dietary use of MSG may potentially limit the MCE capacity of differentiating preadipocytes distrupting the delicate balance between adipocyte hyperplasia and hypertrophy contributing to obesity etiopathogenesis.

Key Words: Mono Sodium Glutamate, 3T3-L1, Mitotic Clonal Expansion, Cell Cycle, Adipogenesis.

ÖZET

Monosodyum glutamat (MSG) amino asit glutamat'a bağlı, tek sodyum atomlu serbest bir amino asit tuzu olup işlenmiş gıdalarda lezzet arttırıcı olarak sıkça kullanılan gıda takviyesidir. Bununla birlikte son dönemde yapılan epidemik ve hayvan çalışmaları sonucunda son yıllarda özellikle artış gösteren beden kitle endeksi, obezite ve insülin direnci gibi çeşitli metabolik hastalıklar ile ilişkili olduğu gösterilmiştir. Bu çalışmalarda, MSG ile beslenen farelerin, standart yüksek kalorili diyetle beslenenlere göre daha fazla yağ kitlesi biriktirdiği, çeşitli adipogenik genlerin artış gösterdiği ve iştah merkezi olan hipotalamik arkuat nükleus ile yağ depoları arasındaki leptin tabanlı bağlantının bozulduğu gözlemlenmiştir. Ancak son dönemde yapılan çalışmalarla MSG ile ortaya çıkan etkilerin birçoğunun ise nöronal patolojik durumlardan bağımsız bir şekilde gerçekleşmesi dikkat çekicidir (9,12). Bununla birlikte, MSG-obez farelerden elde edilen adipoz öncül hücrelerinin proliferasyon kapasitesini düşürdüğüne ve hipertrofik/hiperplazik dengeyi bozduğuna dair etkisinin gösterilmesi ise MSG ile öncül adipositler arasında olabilecek doğrudan etkileşimlerin araştırılabileceğini göstermiştir.

Bu doğrultuda yaptığımız çalışmada MSG'nin farklı konsantrasyonlarının 3T3-L1 adipogenezi üzerindeki olası etkilerinin araştırılması hedeflenmiştir. Bu amaçla, MSG' nin artan konsantrasyonlarında, 3T3-L1 öncül adipositlerinin farkılılaşma aşamaları olan proliferasyon, Mitotik Klonal Çoğalma (MKÇ) ve geç dönem lipojenik fazlarındaki olası etkileri, proliferasyon deneyleri, hücre döngüsü analizleri ve standart biyokimyasal metodları (western blotlama ve Oil Red O boyaması gibi) kullanılarak araştırılmıştır. Yapılan çalışmanın sonucu olarak, yüksek MSG konsantrasyonunun (≥2.5 mM) MKÇ ve adipogenezde potansiyel bir inhibitör olabileceği bulunmuştur. Bu engelleyici etkinin ise hücre döngüsünde anahtar role sahip CCAAT/enhancer protein beta (CEBPβ), p27 ve Siklin B gibi proteinler tarafından düzenlendiği düşünülmektedir. Dolayısıyla çalışmamız, MSG kullanımının farklılaşan öncül adipositlerin MKÇ'lerini kısatlayarak hipertrofi ve hiperplazi dengesini obezite patafizyolojisini geliştirecek şekilde bozduğunu ortaya koymuştur.

Anahtar Kelimeler: Mono Sodyum Glutamat, 3T3-L1, Mitotic Klonal Çoğalma, Hücre Döngüsü, Adipogenez.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		Explanations
µl	:	Mikrolitre
µM	:	Mikromolar
kg	:	Kilogram
mg	:	Miligram
ml	:	Mililitre
mM	:	Milimolar
ADSCs	:	Adipose Drive Stem Cells
ANOVA	:	Analysis of Variance
APCs	:	Adipose Precursor Cells
APS	:	Amonium Persulfate
ARC	:	Arcuate Nucleus
BAT	:	Brown Adipose Tissue
BCA	:	Bicinchoninic Acid
BMI	:	Body Mass Index
BSA	:	Bovine Seum Albumine
CDK	:	Cyclin Dependent Kinase
CEBP-α	:	CCAAT-Enhancer-Binding Proteins Alpha
CEBP-β	:	CCAAT-Enhancer-Binding Proteins Beta
CEBP-γ	:	CCAAT-Enhancer-Binding Proteins Gamma
DEX	:	Dexamethasone
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl Sulfoxide
ECL	:	Enhanced Chemiluminescence
EU	:	European Union
FABP4	:	Fatty Acid Binding Protein 4
FACS	:	Fluorescence-Activated Cell Sorting
FBS	:	Fetal Bovine Serum
GAB-1	:	GRP 2 Associated Binding Protein 1

GMP:	:	Guanosine- 5- Monophosphate
HPA	:	Hypothalamic Pituitary Adrenal
HRP	:	Horseradish Peroxidase
IBMX	:	Isobutylmethylxanthine
IL-6	:	Interleukin-6
IRS-1	:	Insulin Receptor Substrate 1
MCE	:	Mitotic Clonal Expansion
MDI	:	Methylisobutylxanthine, Dexamethasone, Insulin
MEFs	:	Mouse Embriyonic Fibroblasts
MKÇ	:	Mitotik Klonal Çoğalma
MSG	:	Monosodium Glutamate
PBS	:	Polybutylene Succinate
Pen/Strep	:	Penicillin/Streptomycin
PI	:	Propidium Iodide
PPAR-γ	:	Peroxisome Proliferator Activated Receptor Gamma
PVDF	:	Polyvinylidene Fluoride
RPM	:	Rotation Per Minute
SDS	:	Sodium Dodeocyl Sulfate
siRNA	:	Small Interference RNA
SVF	:	Stromal Vascular Fraction
TBS-T	:	Tris Buffered Saline with Tween
TEMED	:	Tetramethylethylenediamine
TNF- α	:	Tumor Necrosis Factor Alpha
WAT	:	White Adipose Tissue
WHO	:	World Health Organisation
WST-1	:	Water Soluble Tetrazolium Salts

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1.INTRODUCTION

1.1 Adipose Tissue

Adipose tissue is a type of connective tissue composed of adipocytes and stromal vascular fraction (SVF) including adipose derived stem cells (ADSCs), preadipocytes, fibroblasts, vascular endothelial cells and immune cells. It is one of the most sensitive and significant physiological regulators. In respose to physiological changes, it regulates homeostatic energy balance via storing energy in the form of lipids or burning of them. It also protects body against external damages and cold via insulation. In recent studies, adipose tissue is defined as a major endocrine organ. It produces different hormones (e.g., leptin, estrogen etc.) and cytokines/adipokines (e.g, Tumor Necrosis Factor Alpha (TNF-α), Interleukin-6 (IL-6) etc.). Thus it plays role in different processes, like inflammation and related metabolic pathways [1], [2].

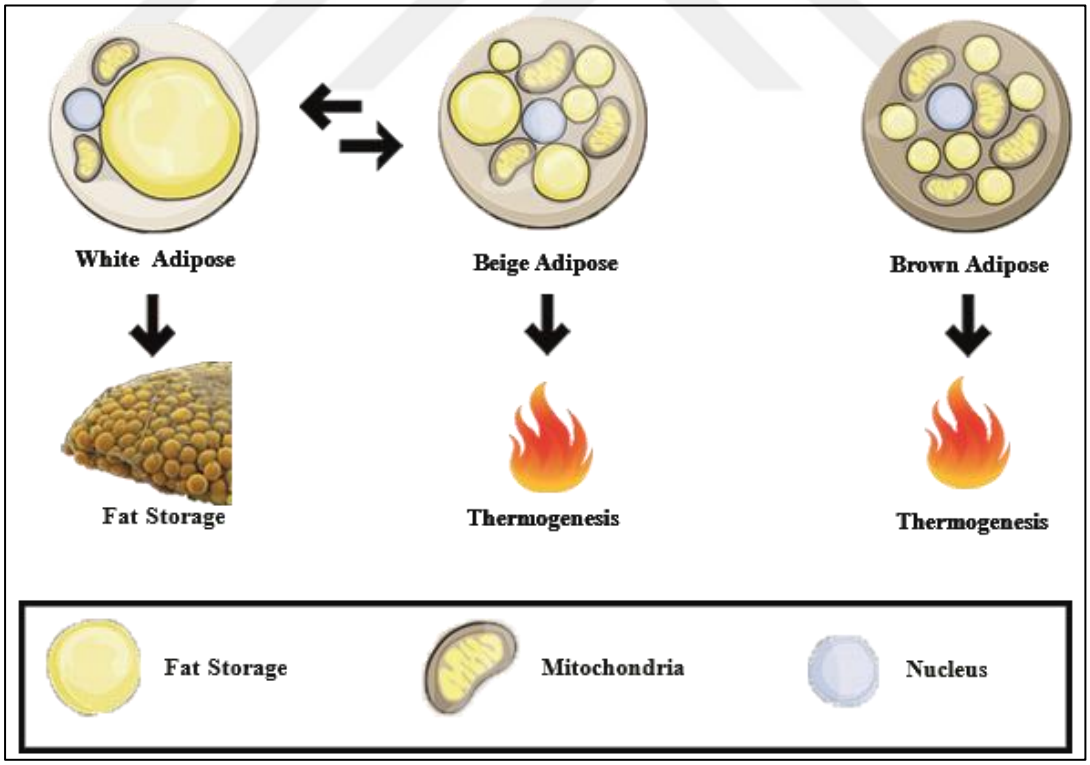

Figure 1.1: Types and functions of adipocytes.

There are two major types of adipose tissue; White adipose tissue (WAT) and Brown adipose tissue (BAT). Under the particular conditions, WAT become BAT and form beige adipose performing similar function with BAT (Figure 1.1). White adipocytes store energy in a large lipid droplet. Brown and beige adipocytes are composed of tiny droplets and many mitochondria for burning energy and providing heat (thermogenesis) [3], [4].

Expansion of adipose tissue is performed by either increasing adipocyte number (hyperplasia) or increasing adipocyte size (hypertrophy) [5]- [7]. Adipogenesis provide formation of large number of hyperplastic adipocytes that produce more adiponectin and less inflammatory cytokines. On the other hand, existed hypertrophic adipocytes produce less adiponectin and more inflammatory cytokines. In adipose tissue, high number of hypertrophied adipocytes cause hypoxia and macrophage infiltration. Cytokines secreted from these macrophages inhibit hyperplastic process. So there is a physiological balance between hypertrophy and hyperplasia within healthy individuals (Figure 1.2).

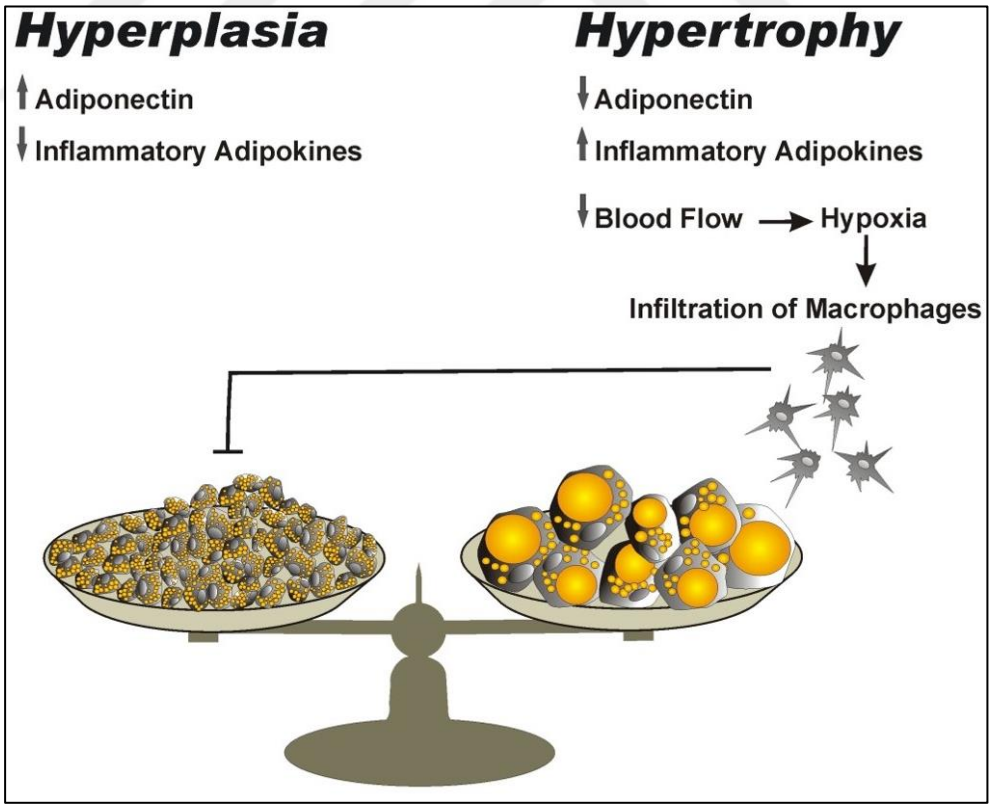

Figure 1.2: Balance between hyperplasia and hypertrophy of adipose tissue.

Adipogenesis is the differentiation process from preadipocytes to mature adipocytes. Preadipocytes are highly proliferative progenitor cells. They can be isolated from the SVF of adipose tissue and then differentiated into adipocytes by a combination of adipogenic medium [8]. In this study, 3T3-L1 mouse preadipocyte cells were used for this. In vitro murine adipogenesis model (Figure 1.3); subconfluent preadipocytes proliferate quickly until reaching confluence (100% cell density). They continue proliferation for approximately 48 hours and become postconfluent. At this state, they signal each other to stop cell cycle at G1 phase, which is called contact inhibition. Upon adipogenic induction with methylisobutylxanthine (IBMX), dexamethasone (DEX) and insulin (MDI) medium triggers cells to re-enter cell cycle by repressing cell cycle inhibitors (such as p27 and p21). This two to three rounds of additional cycle is referred as mitotic clonal expansion and required for the chromosomal reorganisation and the expression of adipogenic genes [9], [10].

During this period, inducers of MDI medium promote different transcription factors; DEX triggers CCAAT-Enhancer-Binding Proteins Beta (CEBP-β) and IBMX triggers to CCAAT-Enhancer-Binding Proteins Gamma (CEBP-γ). CEBP-β / γ are early transcription factors of adipogenic differentiation. They promote master regulators (Peroxisome Proliferator Activated Receptor Gamma (PPAR-γ) and CCAAT-Enhancer-Binding Proteins Alpha (CEBP-α)) of adipogenesis. Especially CEBP-β is required for both MCE and terminal differentiation. [11], [12]. For instance, mouse embryonic fibroblasts (MEFs) from CEBP-β (-/-) mice, neither undergo MCE nor differentiate into adipocytes [9]. Furthemore, knockdown of CEBP-β by siRNA in 3T3-L1 prevents MCE and differentiation [13]. Unlike these inducers, insulin directly promotes master regulators. Thus it can be used alone for induction. But this causes weak differentiation potential. All of these early and late activated transcription factors have anti-mitotic properties. Thus they stop MCE after two to three round and cause cells to enter G0 phase. After that, master regulators (PPAR-γ and CEBP-α) trigger adipogenic genes (such as Fatty Acid Binding Protein 4 (FABP4), adiponectin etc.) and hence terminal differentiation containing formation of lipid droplets [14], [15].

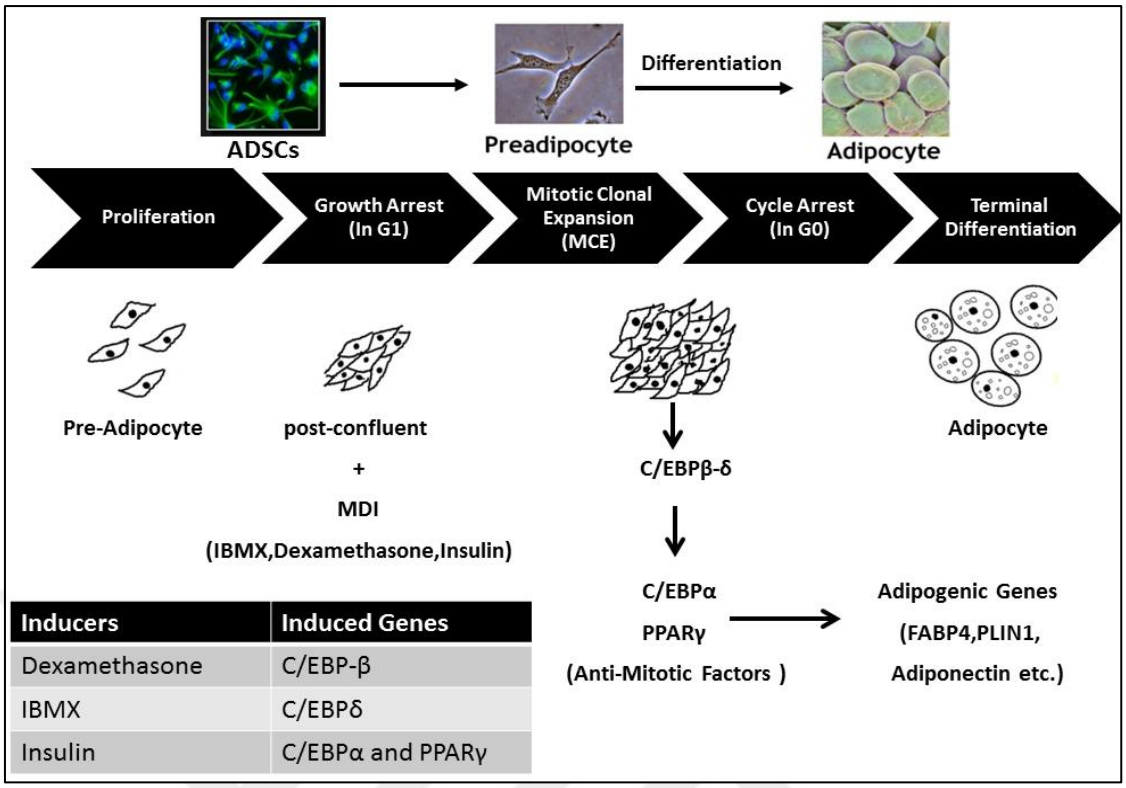

Figure 1.3: In vitro murine adipogenesis model.

1.2. Obesity

Obesity is a global epidemic disease based on accumulation of excess body fat (adipose tissue), which may have effect on health. It is determined by body mass index that is a measure of body fat obtained by dividing weight by the square of height. It is defined as BMI greater than or equal to 30 kg/m2 and its classification is performed according to BMI ranges (Table 1.1) [16], [17].

Table 1.1: The International Classification of adult weight according to BMI.

BMI(kg/m2) thresholds	Classifications
<18.5	Underweight
18.5-25	Normal Weight
25-30	Overweight
30-35	Class I obesity
3-40	Class II Obesity (Severe)
>40	Class III Obesity (Morbid)

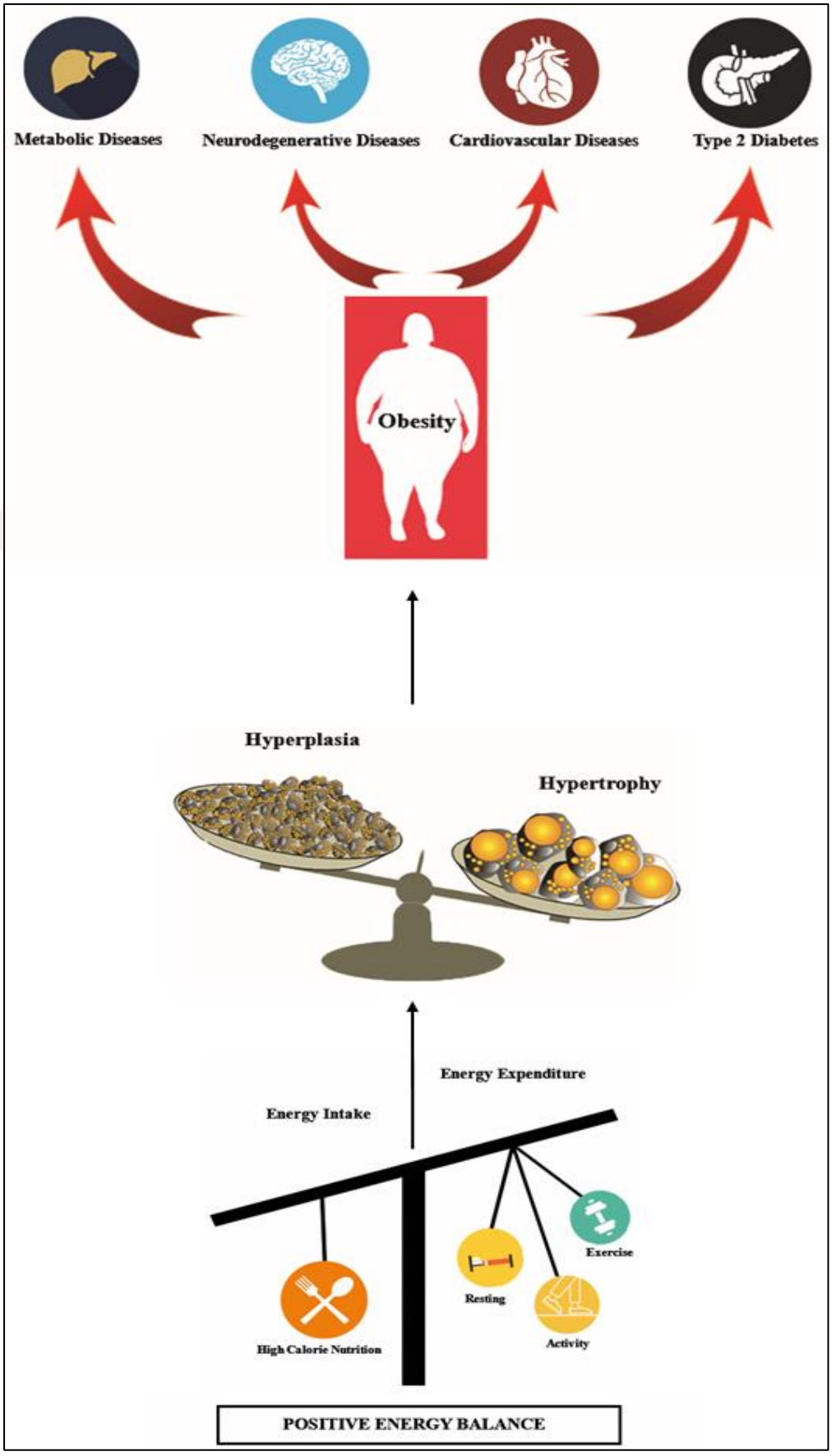

Figure 1.4: Development of obesity and related diseases.

Obesity can be expressed as positive energy balance resulting from the combinations of high calorie intake, lack of physical activity (sedentary lifestyle) and genetic susceptibility [18], [19]. This excess energy is stored in different fat depots by adapting storage capacity of pre or existing adipocytes via increasing hyperplasia or hypertrophy [5]. Upon disrupting balance between these two processes, excess hypertrophy trigger local inflammatory response leading to development of insulin resistance and related type II diabetes [20]. Because of this expanded adipose tissue (especially in visceral), obesity become a risk factor for development of various metabolic, cardiovascular, neurodegenerative and cancer diseases [21], [22] (Figure 1.4). Furthermore, this is confirmed by latest statistics of World Health Organization (WHO). All of these show the importance of obesity epidemiology and related studies for treatment of it.

1.3. Monosodium Glutamate

Monosodium glutamate is the sodium salt of the glutamic acid and generally used as flavor enhancer especially for processed foods (Figure 1.5a). It is also naturally found in various nutrients (such as fish, tomato and cheese etc.). It triggers umami receptors of tongue and is perceived independently without combination of classical taste receptors by working together with Guanosine-5′-monophosphate (GMP). Umami means savory taste and one of the five basic tastes (together with sweet, sour, salty and bitter) (Figure 1.5b). Because its receptors overlap other taste centers, it has combinatory effect of all tastes and provide satisfactory and addictive effect on individuals [23], [24]. Therefore MSG is preferred by many food manufacturers as aromatic palatability enhancer. However, because of its controversial effects, European Union (EU) classifies MSG as a food additive permitted in certain foods with quantitative limits.

MSG has been causally related to obesity, insulin resistance, hepatic steatosis and dyslipidemia in neonatal rodent models [25], [26]. In previous studies, the relationship between high doses of neonatal MSG administration and obesity is thought to be mediated by disrupting the hypothalamic signaling cascade of leptin action within brain [27], [28]. Furthermore, in MSG-obese rats, communication between hypothalamic-pituitary-adrenal (HPA) axis and visceral fat depots has been

disconnected by neuropathies in hypothalamic arcuate nucleus [29]- [31]. However, recent epidemiological studies indicated a strong correlation between MSG consumption and obesity even in the absence of neuronal injury of hypothalamic region [32]- [35].

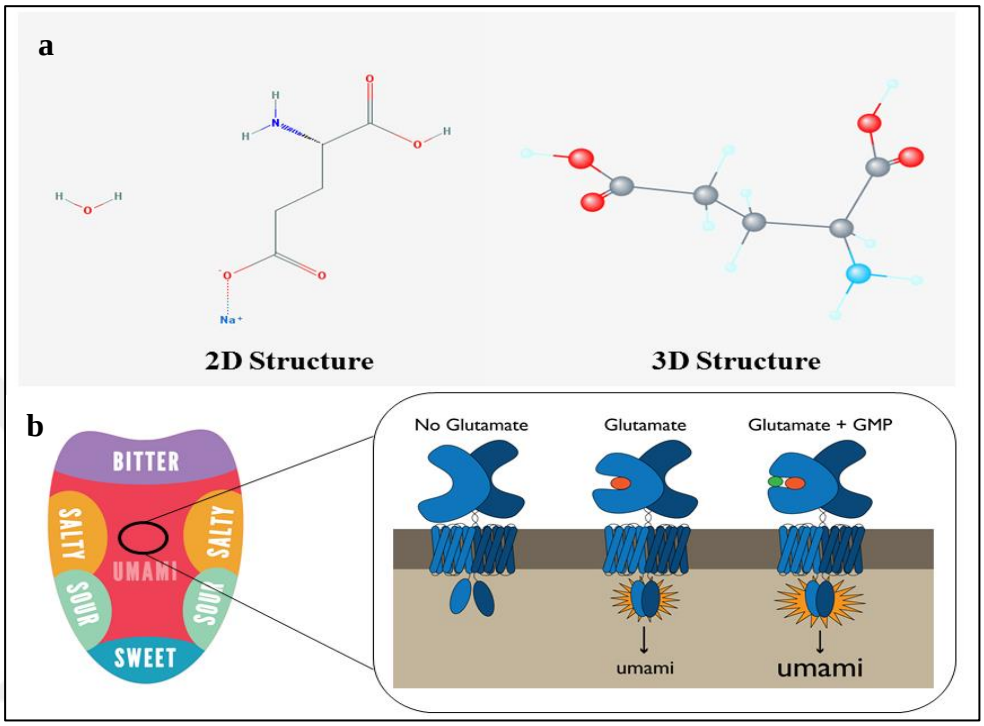

Figure 1.5: Structures of MSG and related UMAMI receptors. a) Two and three dimension of MSG. b) UMAMI receptors of MSG on tongue.

In the literature, there are different controversial data for obesogenic effect of MSG both in human and animal sudies. Dietary MSG consumption increases the expression of adipogenic genes, triglycerides, serum free fatty acids, insulin and bile synthesis in rodent models [30], [36]. Furthermore, the offspring of MSG fed rats shows higher level of abdominal fat and body weight when compared to offspring of high caloric chow fed rats [37]. Interestingly, MSG-obese rats also demonstrate reduced *de novo* adipogenesis accompanying with adipocyte hypertrophy [38], [39]. Because of limited entry of ingested MSG into circulation, these animal models do not exactly proper for results from epidemiological studies [40]- [43]. From all of these, we hypothesized that dietary MSG consumption may interact with adipogenesis. To test this hypothesis we investigated the possible effects of MSG on different phases of 3T3-L1 adipogenesis in vitro.

2. MATERIALS AND METHODS

2.1. Experimental Outline

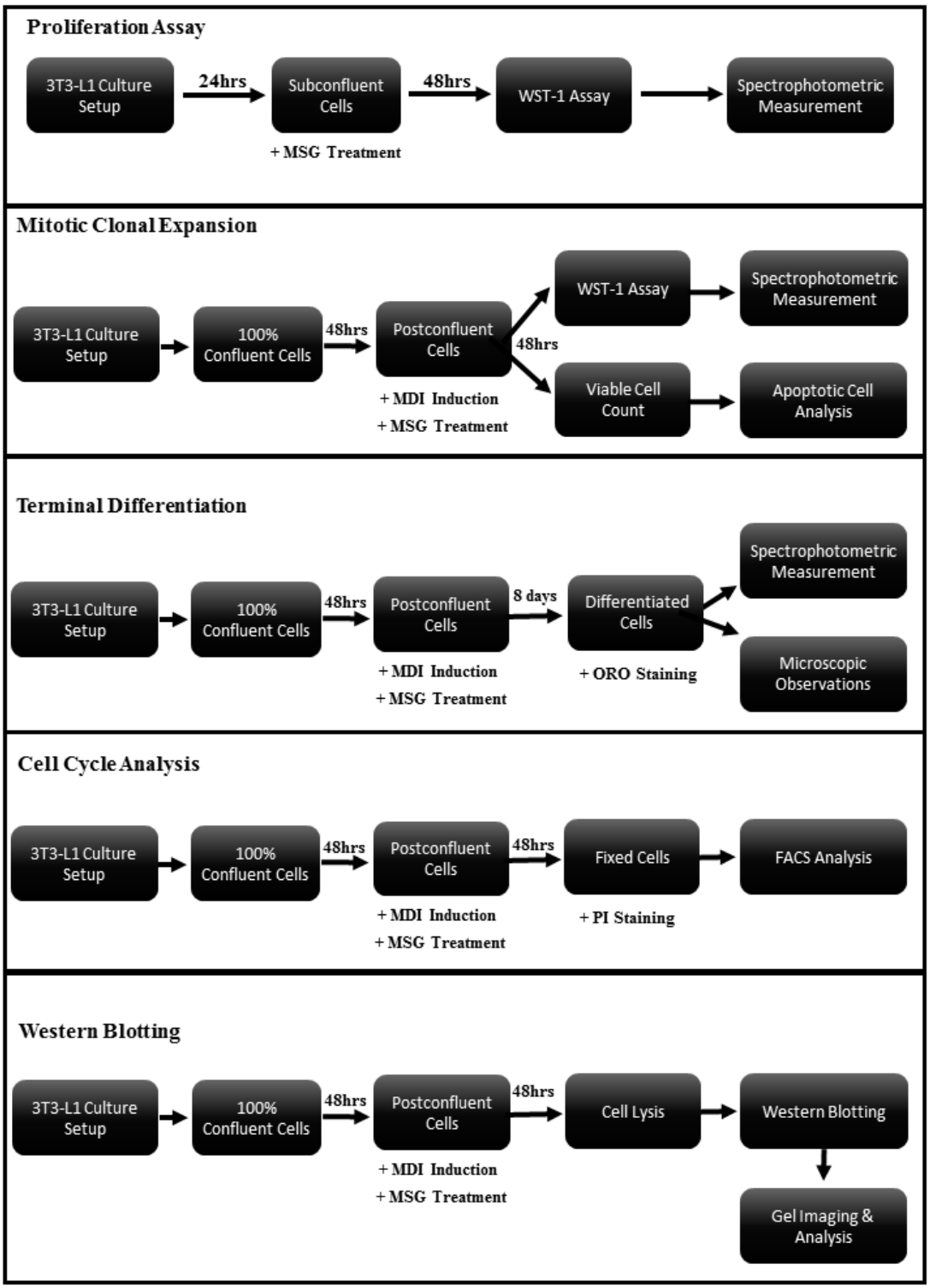

Figure 2.1: General experimental setup of study.

In this study, possible effects of MSG on different adipogenic phases (proliferation, mitotic clonal expansion and terminal lipogenesis) of 3T3-L1 preadipocytes were investigated by using proliferation assays, cell cycle analysis and standart biochemical methods such as; western blotting and Oil Red O staining etc (Figure 2.1).

2.2. Cells and Culture Conditions

3T3-L1 mouse preadipocyte cell line was supplied as commercial from ATCC (CL-173, LGC, Germany). These cells were cultured and stored according to suggestions from the supplier. *Dulbecco's Modified Eagle Medium* (DMEM) (41966-Gibco) supported with 10% Fetal Bovine Serum(FBS) (10270-Gibco) and 1% penicillin/streptomycin(Pen/Strep) (17- 602E- Lonza) was used for culture maintenance under 5% CO_2 condition. Subculturing of cells was performed under 60% cell density (subconfluent) and did not allow to reach 100% confluency for protecting their differentiation capacity under normal conditions. Storage of cells was performed by using growth medium described above supplemented with 5% (v/v) Dimethyl Sulfoxide (DMSO) (D2650-Sigma) at nitrogen vapor phase.

2.3. MSG Concentrations and Preparations

In this study, the range of using low (10-100 μM) and high (0.25-25 mM) MSG concentrations were determined based on the scientific reports containing the intracellular glutamate concentration (2.5mM), average dietary use especially in Asian countries (5-7 g/day) and the extreme intraperitoneal injection (10 mg/kg) into newborn rodents [27], [44], [45]. L-Glutamic acid monosodium salt hydrate was obtained as commercially (G5889-Sigma Aldrich). MSG was dissolved in dH2O to 1M and 1mM stock solutions and then stored at 20 ^{o}C until use. Because glutamine is different from MSG (glutamic acid) [46] and required for the adipogenic differentiation, we used DMEM containing 4 mM glutamine for all experimental procedures.

2.4. Proliferation Assay

3T3-L1 cells were seeded into 96-well tissue culture plate (TPP) at densities of ~6,000 cells per well. 24 hours after seeding, various concentrations of low and high dosages of MSG were added to wells except DMEM controls. Cells were allowed to double for 48 hours incubation and then the proliferation rates were quantified by Water Soluble Tetrazolium Salts (WST-1) proliferation assay as suggested in the manual (05 015 944 001- Roche). For this assay, the spectrophotometric measurements were performed at 450 and 690 nm after optimized incubation periods (30 min-1 hrs) by using Varioskan Flash Reader (Thermo Scientific, USA).

2.5. Mitotic Clonal Expansion Assay

The cells were seeded into 96-well plate as described in the proliferation assay. After two days of confluency, postconfluent preadipocytes (day 0) were induced to differentiate by using adipogenic medium (MDI) [47] supplemented with methylisobuthylxantine (SC-201188A, Santa Cruz), dexamethasone (A2153, AppliChem) and insulin (I6634, Sigma Aldrich) either in the presence or absence of low and high dosages of MSG. Additionally, MAPK kinase inhibitor U0126 was added to another group as positive control. Viable cell count with Trypan Blue/Hemocytometer and WST-1 assay were performed to quantify cell proliferation of MCE as described in previous section.

2.6. Terminal Differentiation and Oil Red O Staining

Differentiation experiment was performed by seeding cells into 24-well tissue culture plate (TPP) at densities of ~40,000 cells per well. Postconfluent preadipocytes were induced to differentiate as described before with or without addition of various MSG concentrations. Two days after MDI induction, cells were cultured in adipocyte maintenance medium for remaining days. This medium was changed every other day (Table 2.1). Morphologically differentiated adipocytes were obtained after 8 days of culture. At the end of day 8, cells were stained with Oil Red O (O0625-Sigma Aldrich)

as described in standart protocol [48]. Total lipid content of adipocytes was quantified by spectrophotometric measurements of Oil Red O at 500 nm with Varioskan Flash Reader (Thermo Scientific, USA). Adipocyte imaging was performed following Oil Red O staining under the bright field microscopy with NIS-element software attached to Nikon Ti fluorescent microscope.

Table 2.1: Adipogenic differentiation procedure of 3T3-L1 preadipocytes.

DAYS	PROCESSES
0	**Postconfluent cells undergo MDI induction.**
0-2	**Mitotic Clonal Expansion within MDI medium (Adipogenic Medium).**
2-4	**Adipoycte maintenance medium (Groth medium +insulin (1:1000)).**
4-8	**Normal Growth Medium (DMEM+FBS+P\S).**
8-10	**Oil Red O Staining and Analysis.**

2.7. Cell Cycle Analysis

3T3-L1 cells were seeded into 6mm tissue culture plate (TPP) at densities of ~300,000 cells per plate. Postconfluent cells were induced to differentiate with MDI in the presence or absence of various MSG concentrations. Two days after MDI induction, cells were washed with 1 ml 1xPBS and then were trypsinized for 2-3 minutes. After trypsinization, cells were collected with centrifuge 1500 rpm for 5 minutes. Supernatants were discarded and pellets were solved completely with 200 µl PBS. Cold 70% Ethanol was added cells drop by drop on the vortex and they were incubated for 30 minutes on the ice. After incubation, 3 ml PBS were added and centrifuged at 3000 rpm for 5 minutes at +4 °C. During centrifuge, Propidium Iodide (PI) solution was prepared; (0,001 g PI (P4170-Sigma Aldrich) is solved in 500 µl 10% Triton X-100 and total volume was completed 50 ml with PBS). After centrifuge supernatants were discarded, pellets were solved with 3 ml PBS and then again centrifuged at 3000 rpm for 5 minutes at +4 °C. Pellets were solved with 700 µl RNase added PI Staining Solution and incubated at 37 °C for 30 minutes. After staining, cells were centrifuged at 3000 rpm for 5 minutes at +4 °C and pellets were again solved with PBS (500 µl). Samples were transfered to Fluoresence Activating Cell Sorting (FACS) tubes. Following, the cell cycle analysis was performed by using BD-Accuri

C6 flow cytometer (BD Biosciences). Obtained results were analyzed with FlowJo v10 software (LLC, USA).

2.8. Western Blotting

For the immunological detection of cell cycle and adipogenic signaling pathway components, preadipocytes were seeded into 10mm tissue culture plate (TPP) at densities of ~600,000 cells per plate. Cell lysates were collected after 24 and 48 hours of adipogenic induction with or without different MSG concentrations by scraping within NP-40 lysis buffer. Concentrations of obtained total protein samples were measured by Bicinchoninic Acid (BCA) assay based on Bovine Serum Albumine standarts (BSA) as suggested in manual (23225-Thermo Scientific) at 562 nm with Varioskan Flash Reader (Thermo Scientific, USA).

According to concentrations, protein samples were prepared with 4X SDS Loading Dye and denaturated at 95°C or 5 minutes. After preparation, protein samples were loaded into the jel which had prepared according to values on the below table (Table 2.2). Proteins were run at 80V and then were transferred to PVDF membrane (IPVH00010-Merck) for 2 hours at 100V in the cold room to avoid overheating. After transfer step, blocking of membrane was occured with 5% nonfat dried milk within the shaker for 1 hour at room temperature. Then blocked membranes were washed with TBS-T and they were incubated with primer antibody on shaker for overnigt at +4 °C for specifying interested proteins. After overnight incubation, secondary HRP conjugated antibodies were incubated with membranes on shaker for 2 hours at room temperature and they were again washed with TBS-T. Immunoblotting images were captured with ChemiDoc XRS+ (Biorad, USA) by using ECL reagents (7003-CST). Quantification of images was performed by Image J software (NIH, USA). All of using antibodies were obtained as commercially (CST and Santa Cruz).

Table 2.2: SDS-PAGE Gel Components

SDS Stacking Gel	
dH2O	4,55 ml
Tris-HCL (1,5M Ph:6,8)	750 µl
10% SDS	60 µl
40% Acrylamide	601 µl
10% APS	30 µl
TEMED	6 µl
	6 ml
SDS Seperating Gel	
dH2O	4,7 ml
Tris-HCL (1,5M Ph:8,8)	2,6 ml
10% SDS	100 µl
40% Acrylamide	2,5 ml
10% APS	100 µl
TEMED	10 µl
	10 ml

2.9. Statistical Analysis

The data for each experiment were obtained from three independent experiment performed in triplicates. Statistical significance of the differences was calculated by one or two-way ANOVA using GraphPad Prism 5 Software (USA). These significancies were represented as p-values and asteriks within the figures.

3.RESULTS

3.1. The Effects of MSG on Proliferation of 3T3-L1 Preadipocytes

To investigate the effects of MSG on proliferation of subconfluent 3T3-L1, cells were cultured in the presence of either low or high dosages of MSG for 48 hours and then proliferation rates were quantified by WST-1 assay. When compared to control cells treated with only DMEM, low dosages of MSG had no effect on cell proliferation (Figure 3.1a). However, at the high concentration treatment, a significant decrease in proliferation rate was observed above 10mM MSG (Figure 3.1b). Furthermore, at these high dosages of MSG, abnormal cell morphology and apparent cell death was observed during the microscopic examinations (Figure 3.1c).

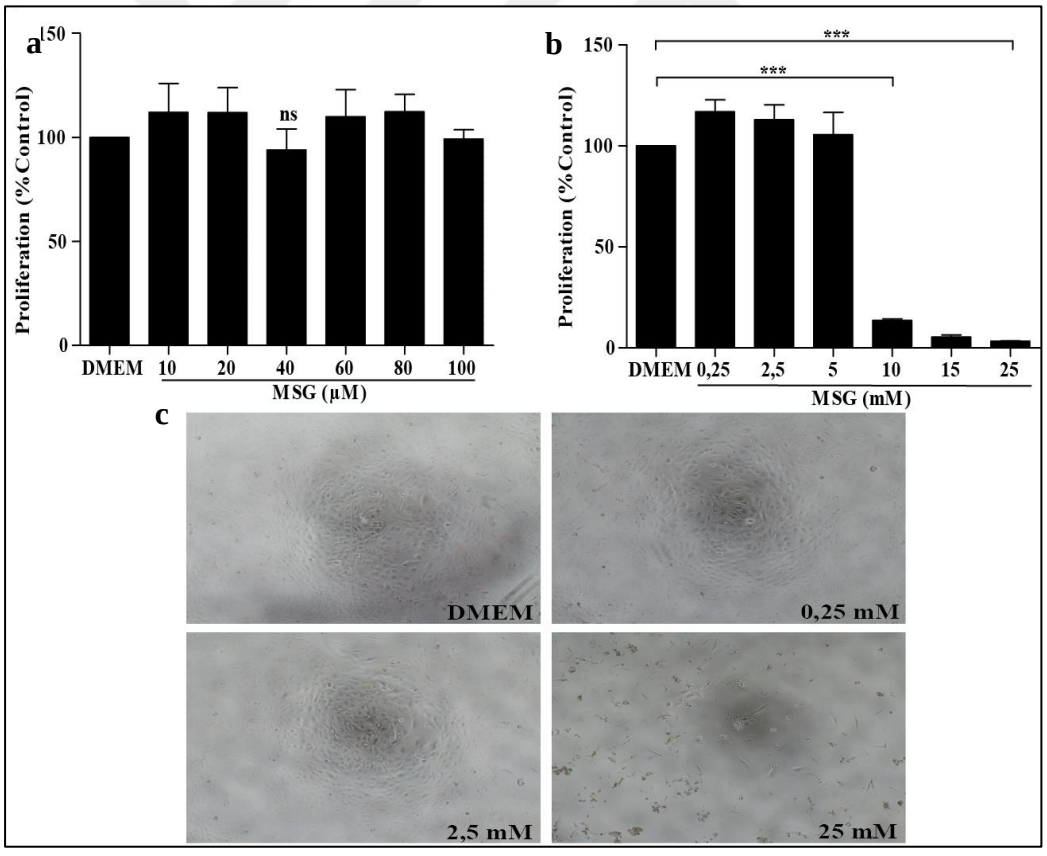

Figure 3.1: Changes in proliferation rate and cell morphology upon low and high dosages of MSG treatment. a) Low dose MSG. b) High dose MSG. c) Morphologic changes of 3T3-L1 cells under the high dosages MSG.

3.2. The Effects of MSG on MCE Phase of 3T3-L1 Adipogenesis

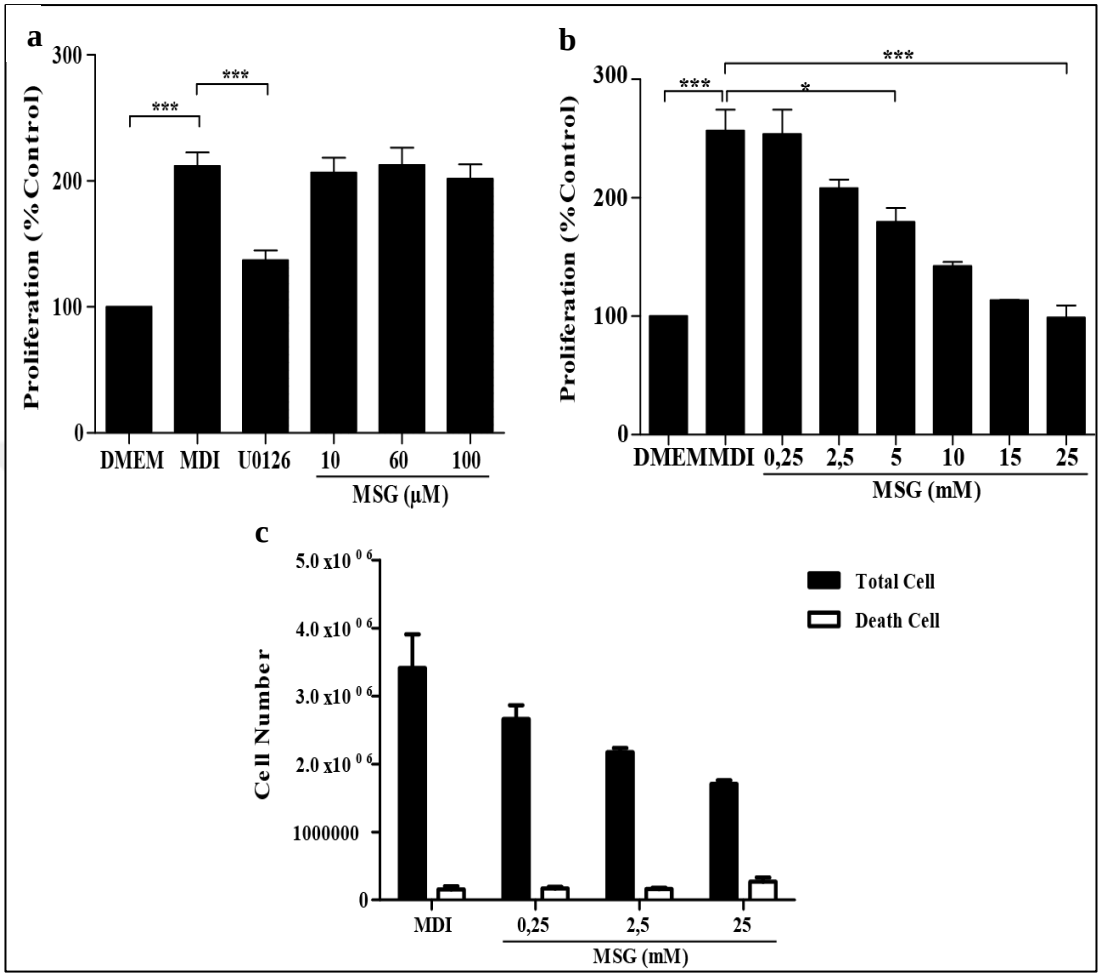

Figure 3.2: Changes in proliferation rate of MCE and cell viability upon low and high dosage of MSG treatment. a) Low dose MSG. b) High dose MSG. c) Viable cell count under high dose MSG via trypan blue staining.

Following, we wanted to investigate whether MSG has any effect on MCE phase that is prerequisite for murine adipogenesis. For this purpose, postconfluent cells were induced to differentiate by using adipogenic medium (MDI) with or without low or high dosages of MSG for 48 hours MCE period. Additionally, a known MAPK kinase inhibitor U0126 was used as positive control to block MCE. Proliferation rates were quantified by WST-1 assay. When compared to control cells treated with only DMEM, a successful MDI induction and expected inhibitor based blocking occurred, but any detectable effect of low dosages of MSG was not observed (Figure 3.2a). However, high MSG concentrations caused attenuation of proliferation in a dose

dependent manner, which is affected firstly at 2,5mM and completely inhibited at 25 mM MSG (Figure 3.2b). Interestingly, we noticed that this inhibitory effect was not resulted with cell death or morphological changes, like in subconfluent cells, during the microscopic examinations (Figure 3.3). To confirm this, we decided to perform viable cell counting with trypan blue. As result of this counting, inhibition of cell proliferation independent of cell death was verified for MCE phase (Figure 3.2c). On the other hand, we found that further increase in the level of MSG (250 mM) was highly toxic to the cells leading to significant morphological abnormalities with severe cell death (data not shown). Because such dose is too high for normal physiologic consumption, it was not used in subsequent experiments.

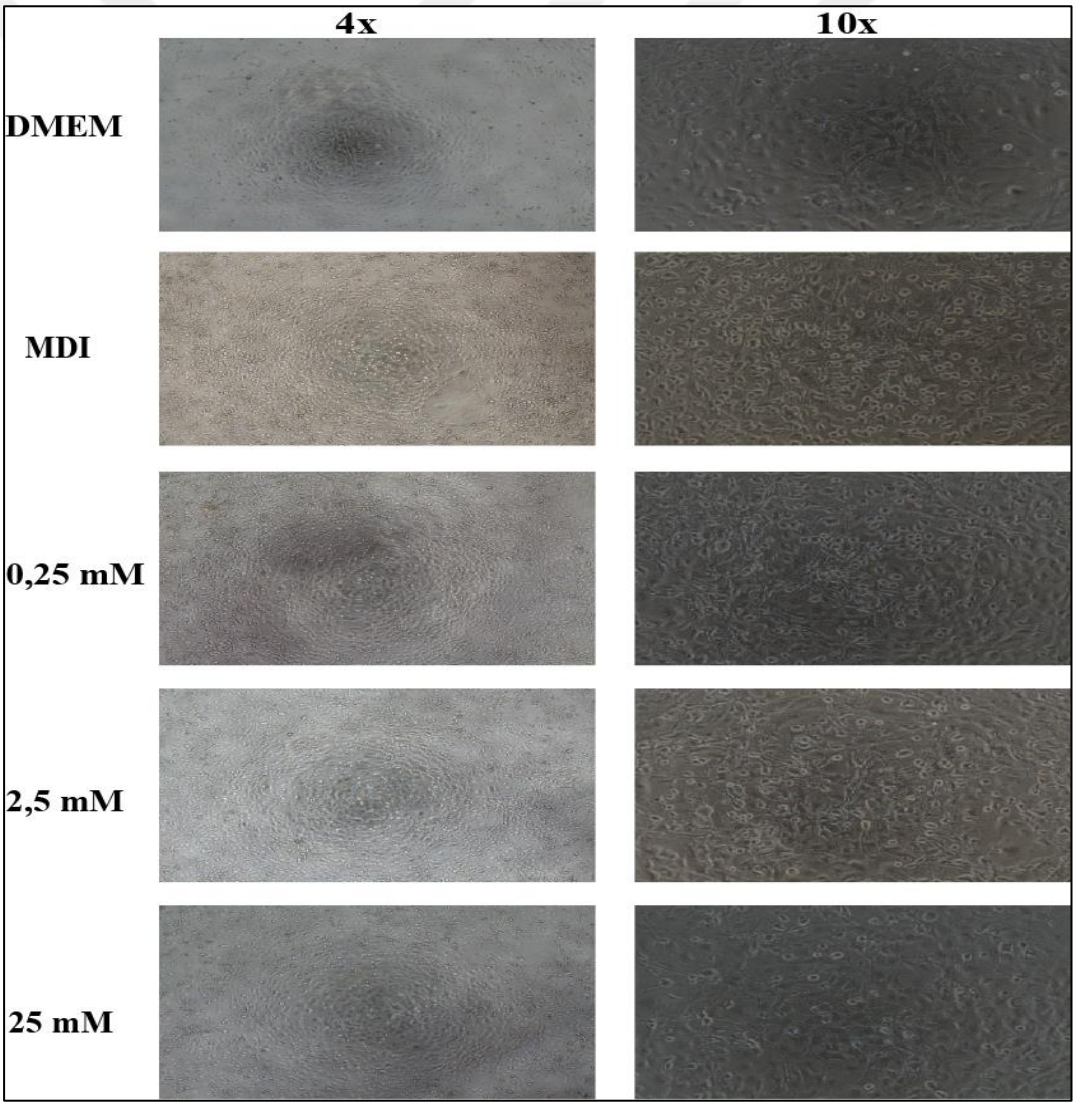

Figure 3.3: Changes in cell morphology upon low and high dosage of MSG treatment during MCE phase.

3.3. The Effects of MSG on Terminal Differentiation of 3T3-L1 Preadipocytes

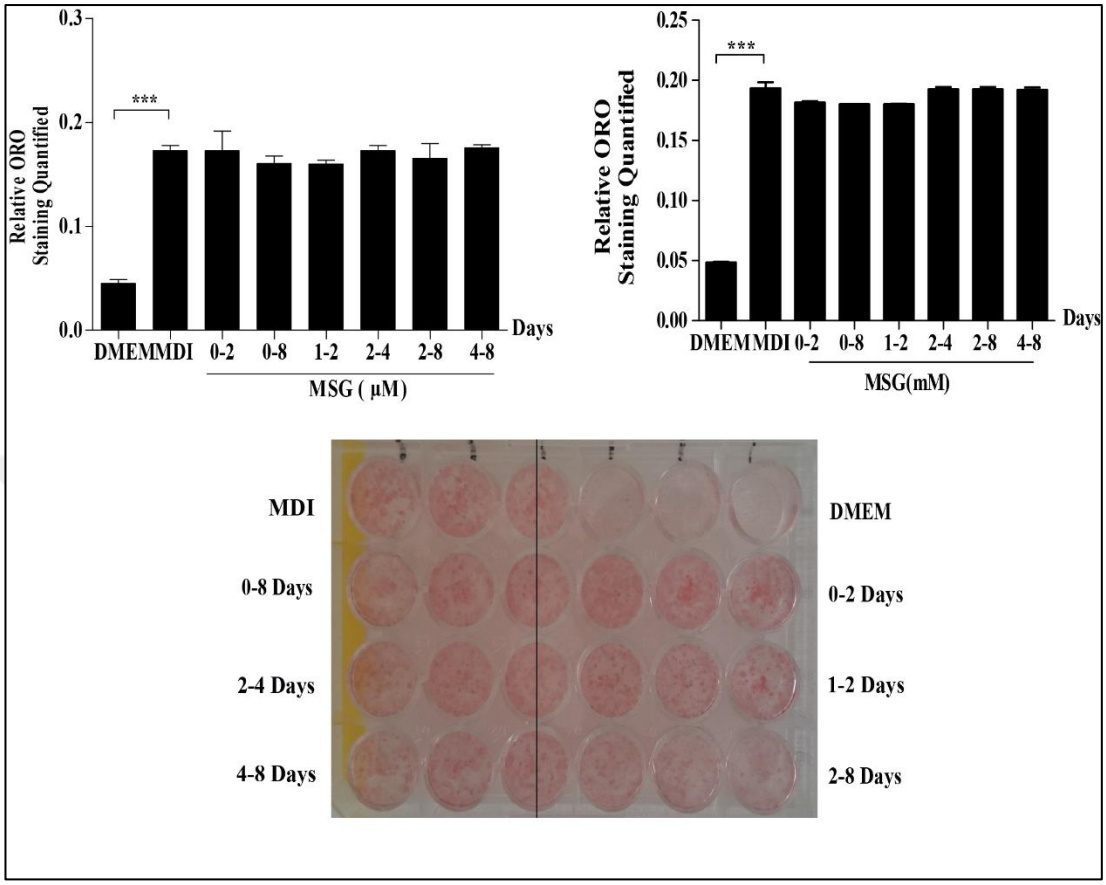

Figure 3.4: Changes in terminal diffentiation upon low and high dosage of MSG treatment.

For investigating the effects of MSG on terminal differentiation phase of adipogenesis, postconfluent cells were induced to differentiate in the presence of either selected low (100uM) or high (2,5 mM) concentrations of MSG for different but overlapping time periods of entire differentiation process. The lipogenic capacity of these cells was measured by Oil Red O staining at day 8 of adipogenesis. Neither low nor high dosages of MSG changed lipogenic capacity of differentiating cells (Figure 3.4). Because of our previous findings on MCE, we decided to also investigate possible effects of MSG on terminal differentiation during MCE period. For this, MCE experiments were repeated with low and high dosages of MSG for entire differentiation process. Treatment of postconfluent cells with low dosages of MSG during MCE had no detectable effect on total adipogenesis (Figure 3.5a). However,

treatment of cells with high dosages of MSG significantly attenuated terminal differentiation dose dependently consistent with MCE experiments (Figure 3.5b).

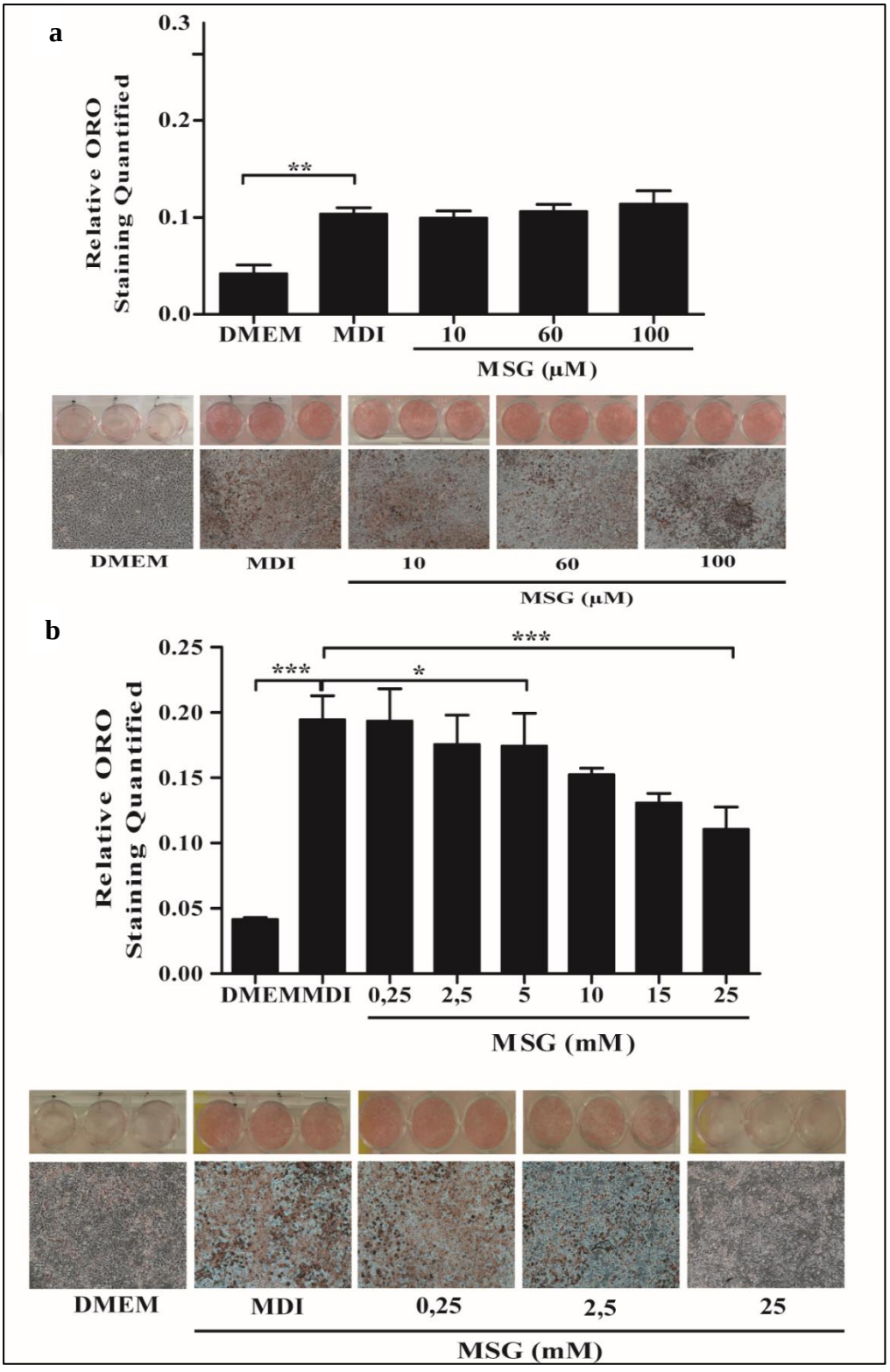

Figure 3.5: Changes in terminal differentiation and lipogenic capacity upon low and high dosage of MSG during MCE. a) Low dose MSG. b) High dose MSG.

3.4. The Effects of MSG on Cell Cycle

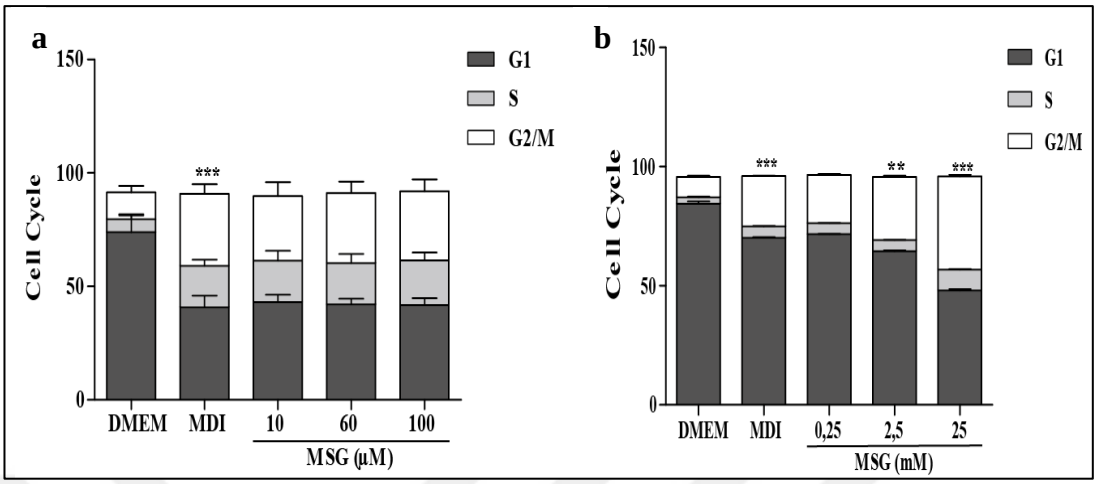

Figure 3.6: Changes in cell cycle progression upon low and high dosage of MSG treatment during MCE period. a) Low dose MSG. b) High dose MSG.

To get further insight into the inhibitory effect of MSG on MCE, we decided to investigate cell cycle progress during the MCE. For this purpose, postconfluent cells were induced to differentiate with or without low or high dosages of MSG for 48 hours MCE period. Following, these cells were fixed and stained with PI for flow cytometer analysis. As expected, MDI induction promoted significant increase in number of cells entering the S phase accompanying with increase in G2/M cell population and decrease in G1 population. Treatment of cells with low dosages of MSG during MCE had no detectable effect on cell cycle (Figure 3.6a). However, treatment of cells with high dosages of MSG significantly changed cell cycle in a dose dependent manner, which contain G2/M and S phases arrests (Figure 3.6b). Consistent with our previous findings, this effect occurred firstly at 2,5mM and maximized at 25 mM MSG (Figure 3.7).

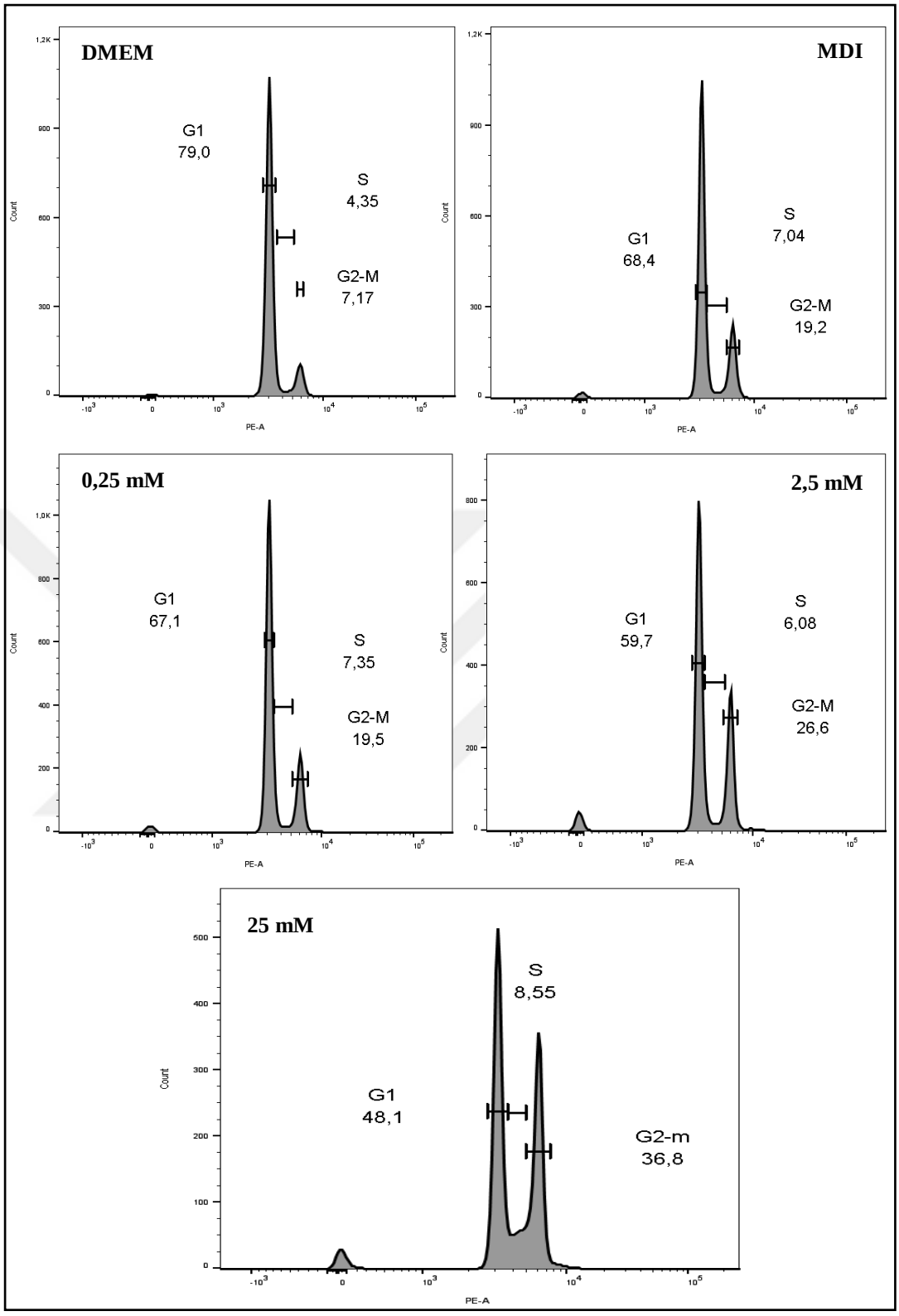

Figure 3.7: FlowJo based graphical illustration of changes in cell cycle progression upon high dosage of MSG treatment during MCE period.

3.5. The Effects of MSG on Key Adipogenic Pathways

According to all of these results, we hypothesized that if increasing concentration of MSG can reduce capacity of cells to undergo MCE and thus adipogenesis, the molecular pathways underlying these processes may also change. Therefore, we selected three major pathways responsible for mediating proliferation and differentiation of adipogenesis; mitogenic signaling pathway (Cyclins, CDKs etc), insulin receptor signaling pathway (IRS1, GAB1 etc) and adipogenic regulation pathway (CEBP-β, PPAR-γ etc). To control possible effects of MSG on these pathways, postconfluent cells were induced to differentiate with or without low (10 uM) or high dosages of MSG for 24 and 48 hours. Following, total proteins were extracted from these cells for immunological detection. In response to low dosage MSG treatments, we detected various changes in the protein levels of some critical regulatory proteins, whereas these changes did not cause any abnormal adipogenic phenotype (Figure 3.8a). For instance, levels of key cell cycle regulatory proteins including CEBP-β, Cyclin A and $P27^{KIP}$ changed especially following 24 hours of adipogenic induction, but these changes did not cause any abnormal cell cycle phenotype within our previous flow cytometer experiment (Figure 3.6a). Consistent with this result, adipogenic master regulator PPAR-γ not changed upon treatment of low MSG concentration.

On the other hand, in response to high dosages of MSG treatment, we detected critical changes in different proteins (Figure 3.8b). Interestingly, major adaptor proteins of insulin receptor signaling pathway, IRS-1 and GAB-1 affected differentially. IRS-1 increased at 48 hours of MCE, whereas GAB1 remained the same. However, this increased IRS1 level was not accompanied by increase in the remaining pathway elements (AKT, ERK etc.) and was compensated in dose dependent manner. We also observed that high dosage of MSG prevented early accumulation of CEBP-β in a dose dependent manner, but had no effect on PPAR-γ during MCE period. We found that among the cell cycle regulatory proteins; CEBP-β, cyclin B1, CyclinD1, CyclinA2 and $P27^{KIP}$ were mostly affected by high dosage of MSG during MCE. Taken together with previous cell cycle results (G2/M and S arrest) we detected that CEBP-β, Cyclin B and $P27^{KIP}$ as attractive canditates for inhibitory effect of MSG on MCE (Figure 3.8b).

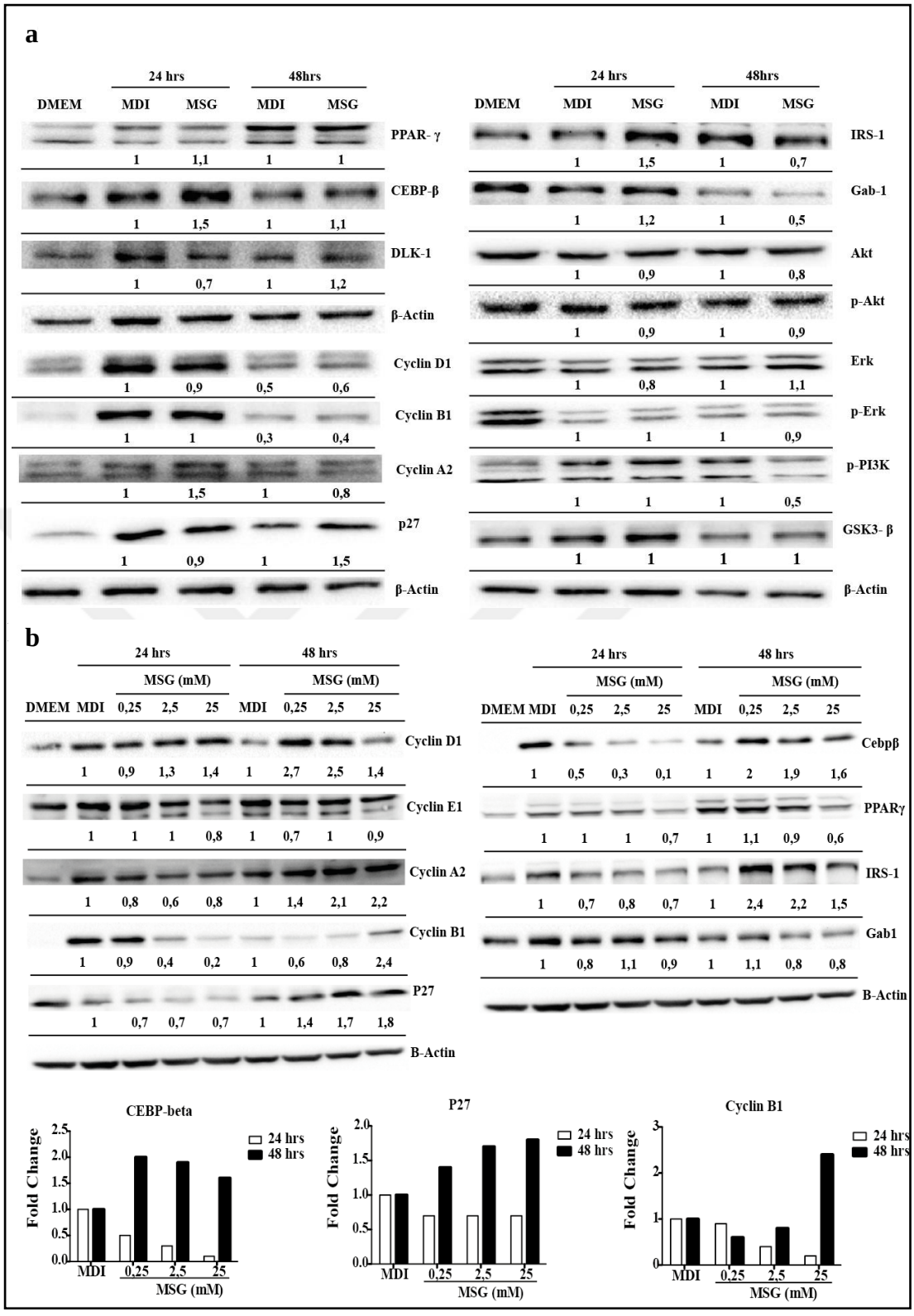

Figure 3.8: Changes in expresion levels of regulatory proteins from major pathways related to adipogenesis upon low and high dosage of MSG treatment during MCE period. a) Low dose MSG. b) High dose MSG.

4. DISCUSSION and CONCLUSION

Obesity is a global epidemic disease caused by long-term positive energy balance. This excess energy is stored in different fat depots by adapting storage capacity of pre or existing adipocytes via increasing hyperplasia and hypertrophy [5], [18], [19]. Under normal feeding conditions these are balanced to maintain energy homeostasis whereas disrupted in the favor of latter in obese individuals. Adipocyte hypertrophy promotes a vicious cycle between ER stress and inflammation leading to development of insulin resistance [20]. Obesity is considered a highly penetrant risk factor for development of different metabolic, cardiovascular and neurodegenerative diseases. Monosodium glutamate is a food additive generally used as a flavor enhancer especially for processed foods and also a favorite ingredient of Asian cusine. Recent epidomiologic studies point out the correlation betweeen dietary use of MSG and increased BMI and obesity especially in Asian countries. Although, intraperitoneally injected high dosages of MSG to newborn rodents has been well established to cause obesity by distrupting HPA axis, the link between the low dosages of dietary MSG and increased BMI and obesity even in the absence of detectable neurodegeneration in the hyphothalamus remains elusive. However, understanding the mechanisms of dietary MSG in the etiopathogenesis of obesity is highly challenging due to and complexity and limit access to human studies as well as its high metabolic turnover. To simplify and circumvent these difficulties we used 3T3-L1 preadipocytes to investigate whether different dosages of MSG have any effect on different phases of adipogenesis.

For this purpose, we analysed the effects of increasing dosages of MSG on early, late, and terminal phases of adipogenic differentiation. Surprisingly, in response to treatment of under confluent preadipocytes with increasing dosages MSG caused significant cell death starting from 5mM and above whereas postconfluent differentiating cells responded by reduced proliferation in a dose dependent manner detectable at 2,5 mM reaching to maximum level at 25 mM similar to level reported for the uninduced cells in the absence of cellular death. Although we are not certain about the different sensitivities of pre and post confluent cells to increasing dosages of MSG it is palusable to suggest that proliferation and MCE may confer different sensitivities to toxic subtances due to distinct nature of these two molecularly distinct proliferative states. Therefore, we speculated that in nutritional excess MCE

contributing hyperplastic expansion of fat tissue might be inhibited by excess dietary MSG in the favor of hypertrophy leading to development of obesity. Our data are in line with the decreased proliferation rate and increased capacity of Adipose Precursor Cells (APCs) from MSG-obese rats [49]. As expected high dosages of MSG added during MCE reduced terminal differentiation capacity of preadipocytes dose dependently reflecting its anti-mitotic effect observed at MCE. According to these results, we suggest that MSG by limiting the MCE capacity of preadipocytes may exert its obesegenic effect at least in murine adipogenesis model.

Next, we step forward to understand the molecular nature of the observed inhibitory effect of MSG on adipogenesis. Although, treatment of preadipocytes with low dosages of MSG potentiated some marginal changes in the level of some critical cell cycle regulatory proteins including CEBP-β, Cyclin A2 and $P27^{KIP}$, this was not reflected by corresponding changes in the cell cycle as analyzed by flow cytometer. It is possible to suggest that the early increases detected in CEBP-β and Cyclin A2 levels might be functionally neutralized by a counterbalancing increase in cell cycle inhibitor $P27^{KIP}$ at the end of MCE. On the other hand, accounting for the reduced MCE capacity and hence adipogenesis treatment with high dosages of MSG caused cell cyle arrest both at S and G2/M phases of the cell cycle. Parallel increase were also detected in the level of cyclins Cyclin D and B. Taken together increased Cyclin A2 and D levels are compatible with enhanced G1/S transition reflected by reduced G1 population in cells treated with high dosages of MSG during MCE. Intriguingly, both CEBP-β and Cyclin B levels were either downregulated or did not respond to MDI induction during the first round of cell cycle division but demonstrated a compensantory increase at the end of the MCE in response to treatment with increasing dosages of MSG. This may be responsible for the S and G2/M reterdations detected at MCE in MSG treated preadipocytes. Furthermore, late induction of $P27^{KIP}$ might also contribute to the cell cycle reterdation at S phase. Surprisingly, we detected, increased level of IRS1 during MCE in response to treatment with both low and high dosages of MSG. However, this increase is not accompanied by a corresponding activation of its major effector pathways AKT and ERK excluding the possible involvement of insulin signaling in the observed cell cycle phenotype. As expected, the level of PPAR-γ the master regulator of adipogenesis remained unchanged in response to increasing dosages of MSG treatment except 25 mM. This result shows that anti proliferative effect of MSG

during MCE is probably mediated independent of PPAR-γ which defines the completion and appropriate execution of MCE required for adipogenesis.

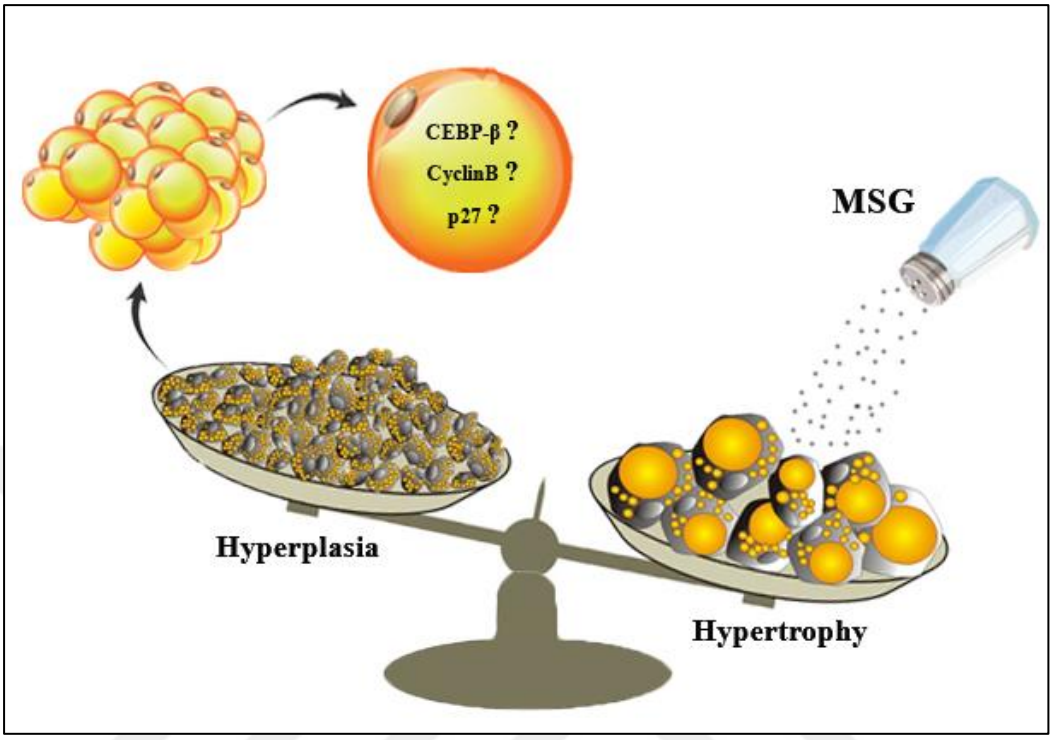

Figure 4.1: Model for possible effects of MSG on adipose tissue homeostasis.

In conclusion, our data showed for the first time that CEBP-β and presumably the other cyclins (B, A and D) may mediated the inhibitory effect of MSG on MCE at elavated its concentrations. Based on the data presented we suggest that dietary MSG in human may alter the adipose tissue homeostasis by abrogating the balance between hyperplasia and and hypertrophy in the favor of latter to mediate its obesogenic affect [50]. However, further epidemiologic and animal studies are needed to decipher direct targets of dietary MSG that might change the level and activity of CEBP-β and Cyclins depicted above (Figure 4.1).

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BIOGRAPHY

Mehmet Soner TÜRKÜNER was born in Istanbul, Turkey, in 1993. He graduated from Hacı Hatice Bayraktar high school with a first degree in 2011. He recieved bachelor's degree in Molecular Biology and Genetics from Gebze Technical University with a first degree in 2015. At the same year, he joined the Department of Molecular Biology and Genetics, Gebze Technical University and graduated in 2017 with master of science degree.

APPENDICES

Appendix A: List of antibodies using in this project

Table A1.1: List of using antibodies

List of Antibodies	
CST #3407	IRS1
CST #3232	GAB1
CST #4691	AKT
CST #4060	p-AKT
CST #4695	ERK
CST #4370	p-ERK
CST #4228	p-PI3K
CST #9832	GSK3-β
CST #2430	PPARγ
CST #3087	CEBP- β
CST #2069	DLK1
CST #4656	Cyclin A2
CST #2926	Cyclin D1
CST #4138	Cyclin B1
CST #4129	Cyclin E1
CST #2546	CDK2
CST #12790	CDK4
CST #3136	CDK6
CST #2947	P21
CST #3686	P27
SC-47778	β-Actin

*CST: Cell Signaling Tehnologies

*SC: Santa Cruz

Appendix B: Full western images for adipogenic pathways.

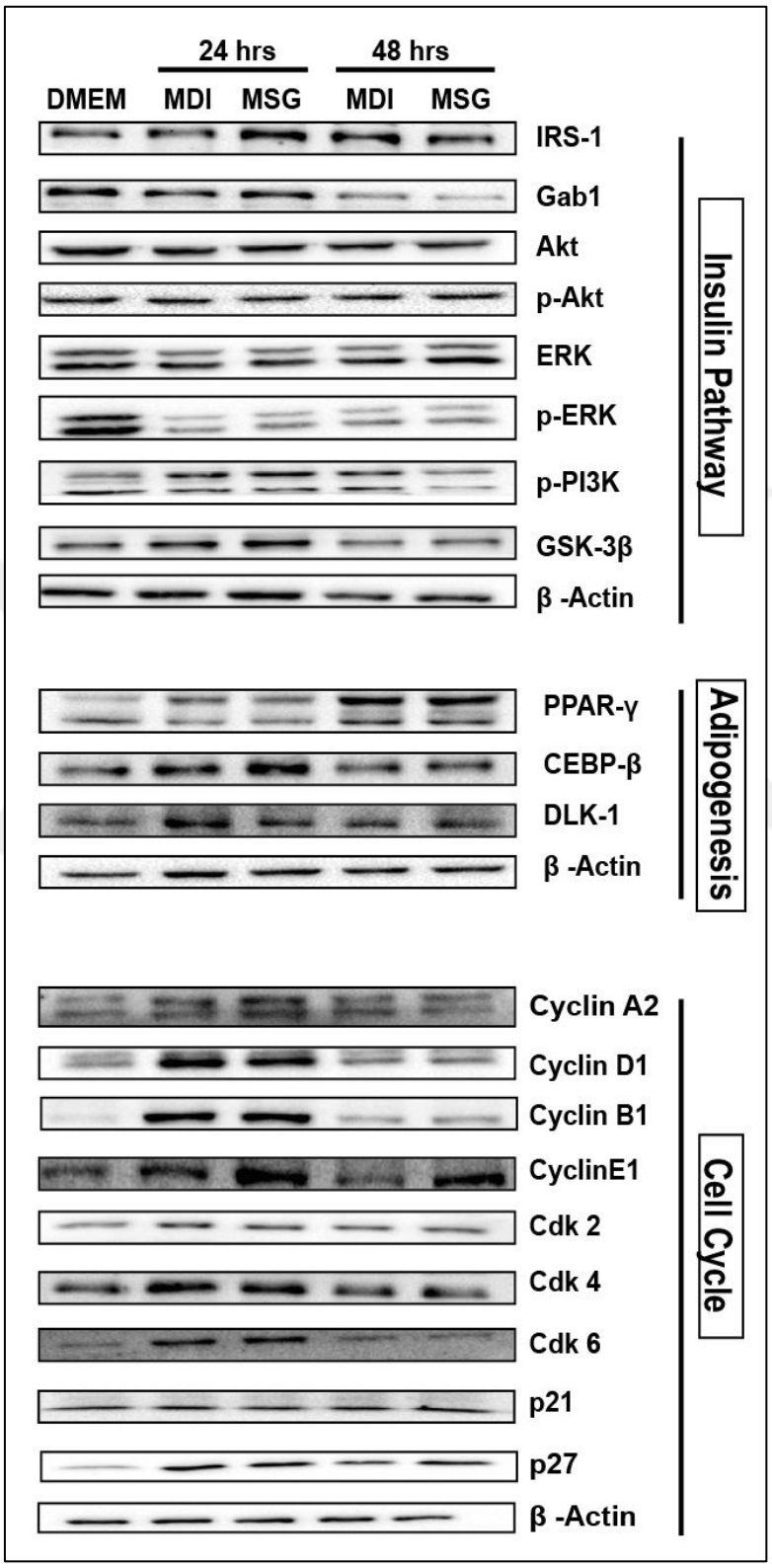

Figure B1.1: Changes on key adipogenic pathways upon low dosage (10 μM) MSG treatment during mitotic clonal exansion period.