MOLECULAR CHARACTERIZATION OF ADULT STEM CELLS' ADAPTATIONS TO MECHANICAL SIGNALS DURING ADIPOGENIC COMMITMENT

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ABSTRACT

MOLECULAR CHARACTERIZATION OF ADULT STEM CELLS' ADAPTATIONS TO MECHANICAL SIGNALS DURING ADIPOGENIC COMMITMENT

Prevalence of obesity have increased across the years based on technological developments that supported nutritional availability and sedentary lifestyles. Restoring mechanical activity with physical exercise suppresses obesity, and mechanical loading can also be delivered passively with whole body vibrations with high frequency and low magnitude. Anabolic effects of high frequency low magnitude mechanical vibrations on adult stem cells are well identified whereas sensing mechanism of cells and their response to mechanical stimuli is largely unknown. Here, we hypothesed that daily bouts of low intensity vibrations will affect molecular, physical and ultrastructural profile of the cells and the effect will interact with the adipogenic induction. To test this hypothesis mouse bone marrow stem cell line D1 ORL UVA were subjected to mechanical vibrations (0.15g, 90 Hz, 15min/d) for 7 days to both during quiescence and adipogenic commitment. Ultrastructural changes were identified on cellular and molecular levels.

Atomic force microscopy was used to characterize the changes on cell surface and significant increase was observed in cell surface height. Moreover, in order to identify the changes in cytoskeleton structure and physical properties, actin were stained with phalloidin and imaged with inverted microscope. To quantify phalloidin amount, signal intensities and physical features of the cells were measured. It was observed that mechanical stimulation and adipogenic induction affect actin content and the physical structure of the cells significantly. Molecular level analysis of cytoskeleton elements and adipogenic markers were performed with Real time PCR. Dramatic increases in adipogenic markers were detected with adipogenic induction.

These results indicate that mesenchymal stem cells responds to mechanical vibrations by altering their molecular and ultrastructure during both quiescence and adipogenesis.

ÖZET

YAĞ YÖNELİMİNDEKİ ERİŞKİN KÖK HÜCRELERİN MEKANİK SİNYALLERE ADAPTASYONUNUN MOLEKÜLER KARAKTERİZASYONU

Teknolojik gelişmeler, beslenme ve hareketsiz yaşam tarzıyla birlikte son yıllarda obezite yaygınlığında artış görülmektedir. Egzersiz ile mekanik aktiviteyi hayata geçirerek obezite baskılanabilir ve yüksek frekanslı düşük yoğunluklu titreşimlerle tüm vücuda mekanik yükleme uygulamak mümkündür. Yüksek frekanslı düşük yoğunluklu titreşimlerin yetişkin kök hücreler üzerindeki anabolik etkileri bilinirken, mekanik uyarının algılanma ve tepki mekanizması tam olarak bilinmemektedir. Bu çalışmada düşük yoğunluklu, yüksek frekanslı mekanik titreşimlerin, erişkin kök hücrelerine düzenli olarak uygulanmasının, hücre altyapısında, moleküler ve fiziksel profilinde değişikliklere neden olacağı hipotez edilmiştir. Bunu test etmek için fare erişkin kök hücreleri, 7 gün boyunca, günde 15 dk mekanik titreşimlere maruz bırakılmış, bazı deney grupları eş zamanlı olarak yağ doku yönelimine sokulmuş ve hücre altyapılarındaki değişim hücresel ve moleküler düzeyde gözlemlenmiştir.

Hücre yüzeyindeki değişimler için atomik kuvvet mikroskobu kullanılmış ve hücre yüzey yüksekliğinde anlamlı artışlar görülmüştür. Ayrıca hücre iskeleti ve fiziksel özellikleri incelenmek için aktin proteini falloidin boya ile boyanıp florasan mikroskobuyla görsellenmiştir. Sonuç olarak mekanik uyarıların ve yağ doku yöneliminin hücrenin aktin içeriğinde ve fiziksel yapısında önemli değişikliklere sebep olduğu anlaşılmıştır. Hücre iskeletinde ve yağ doku yöneliminde yer alan moleküllerin analizi RT-PCR ile yapılmış, yağ yöneliminde yer alan moleküllerin seviyesinde önemli farklar gözlemlenmiştir.

Bu sonuçlar, erişkin kök hücrelerin moleküler ve altyapısal değişikliklere uğrayarak mekanik titreşimlere yağ doku yönelimi sırasında ve yağ yöneliminden bağımsız olarak tepki verdiklerini göstermiştir

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CHAPTER 1

INTRODUCTION

1.1. Obesity and Adipose Tissue

Obesity is a multifactorial disease that is described as having body mass index (BMI) greater than 30 and it is calculated as division of body mass into square of height (mass/stature^2, kg m^{-2}). The prevelance of morbid obesity increased faster than obesity over the past 20 years, in addition to this, obesity leads to serious health problems including type 2 diabetes, cardiovascular and cerebrovascular diseases, hypertension, digestive disorders and cancer (Kim & Boye, 2009). The World Health Organization (WHO) estimated in 2005 that 400 million people are obese and it is thought to double by 2015 (Withrow & Alter, 2011). Asian populations are more susceptible to risk factor for obesity including type 2 diabetes, heart disease, hypertension, stroke and many cancer types compared to western populations in terms of body fat percentage and these risks can be reduced by even small amount of weight loss. The number of deaths declared by The World Health Report shows the severity of the problem which indicates that more than 2.5 million deaths worldwide per year are weight related (220,000 per year in Europe and more than 300,000 in USA) (Deitel, 2003). Thus, it is important to know the factors that affect obesity; since it is one of the main causes of worldwide deaths.

The causes of obesity involve genetic and environmental factors involving poor eating and exercise behaviors (Wright & Aronne, 2012). Studies done within families, twins and adopted twins showed that heritable factors are responsible for the body mass index variation between individuals. The identified genetic abnormalities are related to impaired satiety affecting the center in brain controlling appetite (Farooqi & O'Rahilly, 2007). On the other hand, environmental factors promote overeating high caloric and fat containing foods that are affordable. Furthermore, the more time spent on sedentary activities like watching television, playing computer games and surfing the internet, as technology developed, decreased the caloric expend (Wright & Aronne, 2012). As a

result of that, decreased physical activity is believed to be a major determinant of the increasing worldwide prevalence of obesity (Gonzalez-Suarez & Grimmer-Somers, 2011).

Obesity can be characterized into two main types, hyperplasic (increase in adipocyte number) and hypertrophic (increase in adipocyte volume). Weight gain in adulthood may result in increase in adipocyte number that is thought to result from the increase in adipocyte size (Moreno-Navarrete & Fernández-Real, 2012). At some extend of hypertrophy is mainly observed characteristic of overweight and obese individuals. On the other hand, hyperplasia is related to the severity of obesity (Hirsch & Han, 1969). Additionally, studies show that hypertrophy may lead to development of type II diabetes (Lönn, Mehlig, Bengtsson, & Lissner, 2010; Weyer, Foley, Bogardus, Tataranni, & Pratley, 2000).

Adipose tissue is composed of heterogeneous cells: adipocytes, preadipocytes fibroblasts, endothelial cells and multipotent stem cells that are able to differentiate into several different cell types. Fat tissue consists of one-third of mature adipocytes and the remaining is combination of MSCs, T regulatory cells, endothelial precursor cells, macrophages and preadipocytes in various stages (Moreno-Navarrete & Fernández-Real, 2012).

Additionally, there are two forms of adipose tissue: white and brown, with different functions. Brown adipose tissue is specialized for the generation of heat to enable the maintenance of body temperature and number of brown adipose cells diminish with age (Pfannenberg et al., 2010). On the other hand, white adipose tissue (WAT) is specialized for fuel storage providing substrate to other tissues such as muscle during fasting and high energy needing (Trayhurn, 2013). WAT also acts as an endocrine organ by secreting several major hormones: leptin and resistin (Trayhurn, 2013). Leptin plays role in anti-obesity, reproduction, angiogenesis, hematopoiesis and T lymphocyte systems as resistin reduces the intake of glucose to the cells and causes insulin resistance by decreasing insulin sensitivity (Baranova et al., 2006; Silha et al., 2003).

Adipose tissue is mainly located in the subcutaneous, dermal and intraperitoneal part of the body. Adipocytes are approximately in 80 μm diameter and they have lipid droplets (LD) in their cytoplasm which contain triglyceride. The extracellular matrix (ECM) of adipose tissue is composed of stromal ECM and the basement membrane (BM) (Figure 1.1). The stromal ECM consists of collagen type I and II and fibronectin

while BM provides surrounding for adipocytes and it is composed of laminin and collagen. BM also mechanically supports adipocytes and plays role in differentiation, migration and adhesion (Shoham & Gefen, 2012).

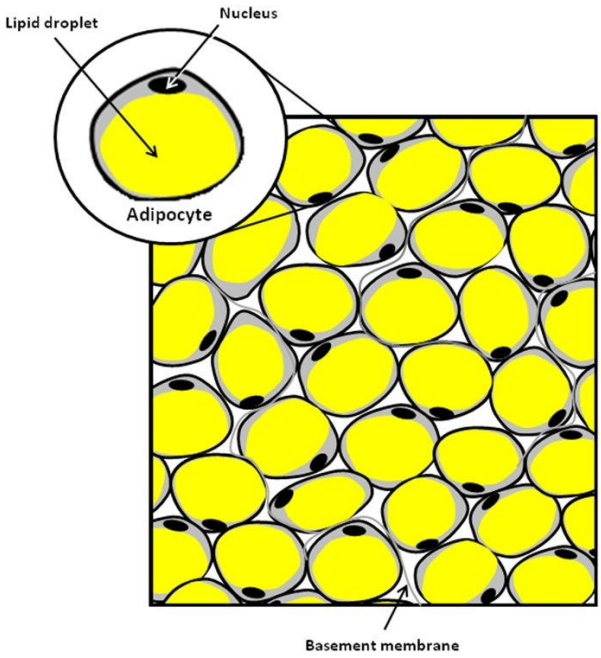


Figure 1.1. General view of adipose tissue
(Source: Shoham & Gefen, 2012)

1.2. Mesenchymal Stem Cells

Stem cells are undifferentiated pluripotent cells that are able to differentiate into different tissues and organs. They have high self-renewal and proliferation capacity providing replacement and cellular turnover during development and adulthood (Yu, Suzuki, & Yanagisawa, 2010).

There are two groups of stem cells that are categorized as embryonic and adult (somatic) stem cells. Embryonic stem cells (ESCs) have capability of differentiating into any cell type belonging to ectoderm, endoderm and mesoderm called as pluripotency (Bosnali, Münst, Thier, & Edenhofer, 2009). They originate from the inner cell mass of blastocysts. Adult stem cells are multipotent meaning they are able to differentiate into a more restricted group of cells. Hematopoietic, neural and mesenchymal stem cells are well known adult stem cell types (Bosnali et al., 2009;

Ghoraishizadeh, Raikar, Ghorishizadeh, Boroojerdi, & Daneshvar, 2014; Yu et al., 2010).

Mesenchymal stem cells (MSCs) are nonhematopoetic adult stem cells that are able to differentiate into mesoderm-type cells such as osteoblast, adipocyte and chondrocytes (Abdallah & Kassem, 2007; Ozcivici et al., 2010) (Figure 1.2). These cells are mainly obtained from bone marrow (Özçivici, 2013) however, they can be isolated from other resources such as liver, umbilical cord blood and amniotic fluid (Ghoraishizadeh et al., 2014).

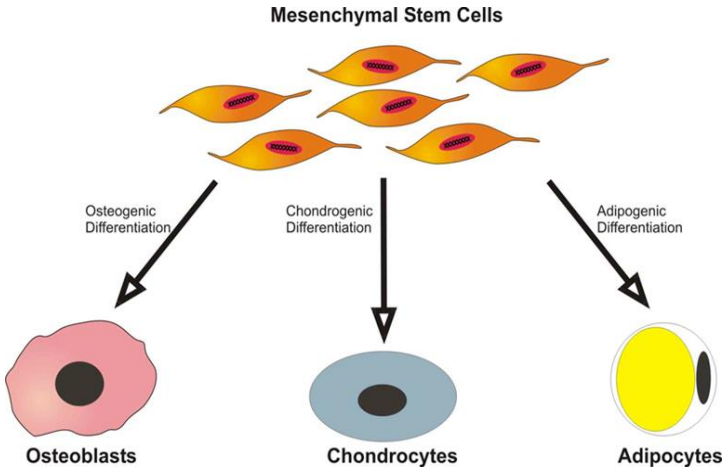


Figure 1.2. Model of mesenchymal stem cell differentiation.
(Source: Penny, Harris, Shakesheff, & Mobasheri, 2012)

1.3. Adipogenesis

Adipocyte formation called as adipogenesis involves two main steps: determination and terminal differentiation phase. In determination phase, multipotent MSCs are alike their original shape but they are committed to adipogenic lineage and they lose their ability to differentiate into other lineages. In terminal differentiation phase, MSCs begin to synthesize lipids, secrete adipocyte-specific proteins and converted to spherical shape by losing their fibroblastic appearance (Figure 1.3). In addition to these two main phases, mitotic clonal expansion involving one or two rounds of cell division is required for murine models (Muruganandan, Roman, & Sinal, 2009).

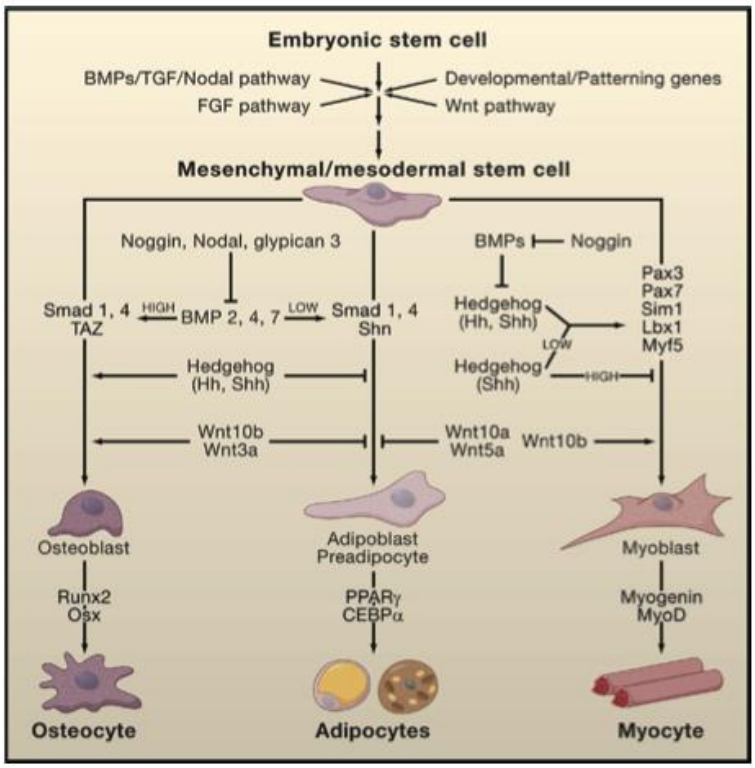


Figure 1.3. Stages in adipogenic differentiation.
(Source: Gesta, Tseng, & Kahn, 2007)

Several signals take part in the formation of adipocytes from stem cells, including extracellular factors such as the bone morphogenetic proteins (BMPs) transforming growth factor β (TGFβ), insulin- like growth factor 1 (IGF1), interleukin 17 (IL17), fibroblast growth factor 1 (FGF1), FGF2 and activin. Previous studies also show the importance of the supression of Wnt signaling to proceed adipogenesis and the inhibitory effect of hedgehog (HH) signaling on adipocyte differentiation (Lowe, O'Rahilly, & Rochford, 2011).

During adipocyte differentiation, extracellular matrix (ECM) and cytoskeletal components changes influence the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and/or CCAAT/enhancer binding protein α (C/EBP-α). Adipogenic induction rapidly induces expression of key regulators of adipogenesis, C/EBP-β and C/EBP-δ. C/EBP–β and C/EBP-δ are the promoters of the genes encoding the key adipogenic transcription factors C/EBP-α and PPAR-γ and the regulator of lipogenic genes SREBP1 (for sterol-regulatory-element-binding protein 1). PPAR-γ activates the promoter of the gene encoding C/EBP-α and vice versa, creating a positive-feedback loop. In addition, PPAR-γ and C/EBP-α induce the expression of

genes that are involved in insulin sensitivity, lipogenesis and lipolysis, and the secreted factors adiponectin and leptin (Lowe et al., 2011; Muruganandan et al., 2009).

Since lipid accumulation may easily leads to disruption, specific changes in ECM are required for survival of adipocytes and spreading local forces over a larger area of the tissue. Early studies on the protein composition of ECM have demonstrated the presence of type I-IV collagen, laminin and fibronectin in differentiated bovine intramuscular preadipocytes. It is suggested that adipokine secretion is provided by the vesicles and collagens are specifically involved in this event. Another study showed upregulation in various laminin complexes in differentiation of mouse 3T3-L1 preadipocytes. On the other hand, different expression levels of various collagen types were observed during the 12-day period of differentiation and growth of 3T3-L1 indicating that there is a decrease in type I collagen in the early phase of differentiation (Mariman & Wang, 2010).

In preadipocytes, ENC-1, a Drosophila kelch-related actin-binding protein which plays a regulatory role in the early adipocyte differentiation by cytoskeletal reorganization and cell-shape change, colocalizes with actin filament and its expression increases 8-12 fold (Moreno-Navarrete & Fernández-Real, 2012). Actin cytoskeleton remodelling is important to formation of mature adipocytes. Studies on the dynamics of actin cytoskeleton during adipocyte differentiation demonstrated that actin filaments were well organized as regular stress fibres across the cell body in undifferentiated preadipocytes, and at the third day of adipogenic differentiation, actin filaments started to be disrupted from the center region of the cell. As the cells further matured, actin filaments were largely absent in the cell center and organized at the peripheral region of the cell structure indicating the critical role of actin cytoskeleton in the regulation of lipid droplet fusion and expansion (Wulin Yang et al., 2013).

1.4. Mechanotransduction

Although, not too much things are known about how mechanical signals are sensed and cellular components response, there are some identification of mechanosensitive molecules such as stretch-activated ion channels, caveolae, integrins, cadherins, growth factor receptors, myosin motors, cytoskeletal filaments, nuclei,

extracellular matrix, and signaling molecules (Ingber, 2006; Ozcivici et al., 2010) (Figure 1.4).

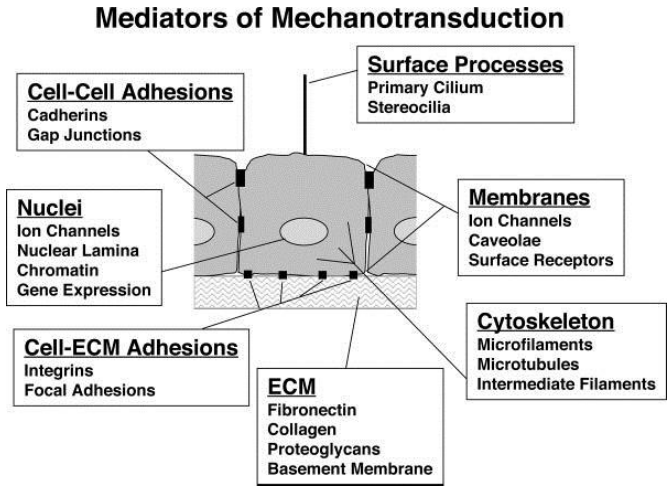


Figure 1.4. Elements taking role in mechanotransduction.
(Source: Ingber, 2006)

The ECM holding cells together reacts immediately when the organ is stressed. Cells sense the rigidity on ECM by transmembrane proteins such as integrin and transmit mechanical signal to cytoskeletal network (Choquet, Felsenfeld, & Sheetz, 1997; Wang, Butler, & Ingber, 1993). Then, the cytoskeleton responses to signal carried by ECM through rearranging actin microfilaments, microtubules and intermediate filaments. It also acts on organelles and nuclei in order to strengthen the cell against to destructive effects of mechanical signals (Matthews et al., 2004; Ralphs, Waggett, & Benjamin, 2002; Wang et al., 1993; Wang et al., 2001). By the transmission of the mechanical force with the transmembrane adhesion receptors and cytoskeletal filament network, cell focus stresses applied at the cell surface and concentrate on focal adhesions as well as at distant sites in the cytoplasm and nucleus. Long distance force transfer over integrins and through the cytoplasm requires an intact cytoskeleton and varies depending on the concentration and the direction of force application. Mechanical interactions between microfilaments, microtubules and cell subtrate adhesions manipulate the shape and stiffness of the cell and ECM. For instance, microtubules carry most of the internal stress in rounded cells whereas the ECM bears most of the load in spread cells on highly adhesive substrates. Integrin-containing focal adhesions are not the only cell membrane sites where mechanochemical conversion

occurs. Mechanical forces also can be transmitted across the cell surface and to the cytoskeleton through cadherins and other transmembrane molecules that form intercellular junctional complexes. However the mechanical linkages formed by cell-cell adhesion molecules are generally not as strong as those mediated by integrins (Hu et al., 2003; Kumar et al., 2006; Maniotis, Chen, & Ingber, 1997; Wang et al., 2001; Wang & Suo, 2005).

Forces transferred through the cytoskeleton to the nucleus can activate stress-sensitive ion channels on the nuclear membrane thus influence gene transcription. They can also change nucleolar function, chromatin folding in DNA, thereby affect access of key proteins to gene regulatory sites. The importance of this mechanical connectivity between the surface, cytoskeleton, and nucleus can reveal why disruption, mutations, or deletions of intermediate filament proteins or nuclear structural proteins (such as lamins) results not only in decreased mechanical stiffness of the whole cell in response to mechanical stress, but also activation of gene transcription (Ingber, 2006; Ozcivici et al., 2010).

Mechanical forces also important to muscular development. When a mutation occurs in ECM molecules, integrins, adhesion molecules, cytoskeletal proteins or nuclear lamins that mediate force channeling in muscle, it results in severe muscular dystrophy and cardiac myopathy (Spence, Chen, & Winder, 2002).

1.5. Mechanical Regulation of MSCs and Adipogenesis

Exercise and mechanical signals are considered as anabolic to bone and muscle but inhibitory to fat formation. Influence of mechanical signals is not limited only to osteocytes, myocytes and adipocytes but also has impact on differentiation of their progenitor, MSCs. The ability of different mechanical signals to alter the differentiation patterns of MSCs has been used by tissue engineers to promote the growth of both bone and cartilage. (Case et al., 2010; Luu et al., 2009; Sen et al., 2008).

Suppressing the effects of obesity and diabetes with exercise is accomplished by metabolizing caloric intake through work expenditure and high energy demand. This fact brings the idea of more exercise means more metabolized fat. However, it has been shown that rather than long term of high-magnitude mechanical loading, short daily

duration of high-frequency and extremely low-magnitude mechanical signals are more effective on physiological systems. Contrary to metabolic perspective, resulting of reduced adiposity is achieved by influencing the MSC precursors to differentiate into other lineages rather than adipogenic commitment and low-magnitude mechanical signals drive MSCs to form musculoskeletal system (Rubin et al., 2007). For instance, mechanical signals activate Wnt/β-catenin signaling which is correlated with promoting bone formation thus, affecting MSC lineage determination (Case et al., 2010).

Adipose tissue is exposed to mechanical loading in daily life; for instance, peak tensile, compressive and shear stress is subjected to adipose tissue at the buttocks during sitting and the magnitudes of these forces reduce to half in lying position (Linder-Ganz et al., 2008). Furthermore, uniaxial cyclic stretching of mouse preadipocyte 3T3-L1 cells at a frequency of 1 Hz for 15 h or 45 h has inhibitory effect on their differentiation mediated by mitogen-activated protein kinase/extracelluar signal-regulated kinases (MAPK/ERK) system. Similarly, cyclic equibiaxial peak strain at 0.5 Hz for 24 and 60 h inhibits adipogenic differentiation of human umbilical cord perivascular cells (HUCPVCs) by TGFβ1/Smad signaling pathway (Tanabe, Koga, Saito, Matsunaga, & Nakayama, 2004; Turner, Jones, Davies, & Canfield, 2008). On the other hand, constant static stretching of adipocytes promotes lipid accumulation and contributes to obesity (Hara et al., 2011). However, not every static force shows the same effect on adipocytes. For example, when a preadipocyte cell line derived from human adipose tissue is subjected to a static pressure of 226 Pa for 12 h, the compressive loads inhibits adipogenesis, similarly to the effect of cyclic stretching (Hossain et al., 2010) (Figure 1.5).

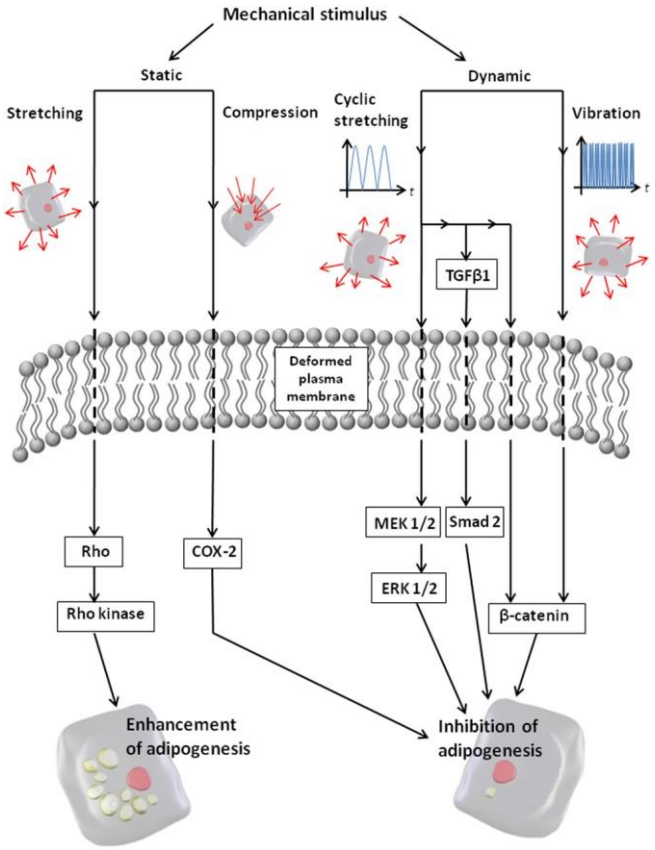


Figure 1.5. Mechanical signals in adipogenic regulation.
(Source: Shoham & Gefen, 2012)

In addition to the type of mechanical signals, dosage dependent manner of signals is also important to regulation. When low intensity and high frequency vibratory signals (0.7g, 90 Hz) with sinusoidal acceleration are applied to embryonic MSCs, adipogenesis can be suppressed and adding more loading session increases the suppressive effect (Sen et al., 2011).

CHAPTER 2

AIM OF THE RESEARCH PROJECT

In view of the fact that mechanical signals play role in adipocyte differentiation by activating different pathways and changing general profile of the cells. In this study, it is aimed to investigate the outcomes of mechanical signals in terms of physical appearance, gene expression and ultrastructure of the bone marrow derived MSCs during adipogenic commitment. It is targeted to demonstrate the differences in expression levels of adipocyte specific genes such as PPAR- γ and C/EBP-α. Apart from this, the changes in expression of several canditate elements taking role in mechanotransduction and MSC differentiation are objected to reveal. Considering physical characteristics of MSCs and adipocytes, the effects of mechanical signals on appearance of the cells and the ultrastructural elements underlying these effects are other questions that are investigated in this study.

CHAPTER 3

MATERIALS AND METHODS

3.1. Maintenance of D-1 ORL UVA Cells

Bone marrow derived mouse mesenchymal stem D-1 ORL UVA cells were grown in high glucose containing DMEM (Hyclone, Cat#SH30243.01) with 10 % fetal bovine serum (FBS) (Biological Industries, Cat#716684) and 1 % penicillin/streptomycin (Invitrogen, Cat#1092595).

Cells were cultured in incubator at 37 °C, 5% CO_2 and lifted off by using 0.25% trypsin/EDTA solution (Biological Industries, Cat# 03-052-1B) at 37 °C, 5% CO_2 for 5 minutes during cell splitting procedure.

3.2. Adipogenic Induction of D-1 ORL UVA Cells

Mesenchymal stem cells were directed to adipogenesis 2 days after cell splitting by adding 5 μg/ml of insulin from bovine pancreas (Sigma, Lot#SLBD3067V), 10 nM of dexamethasone (Sigma, Lot#BCBK1265V) and 50 μM of indomethacine (Sigma, Lot#BCBF9122V) into DMEM growth medium.

3.3. Mechanical Vibration of Cells

90 Hz, 0.1 g vibrations were applied to both induced and uninduced stem cells for 7 days (15 min/day) under room conditions. Signals were generated by a function generator (MLP Lab Electronics) which is connected to an amplifier (Spekon Q1000) and transformed to mechanical signals via high capacity speaker (EVP-X). Mechanical signals were measured with an accelerometer (Kistler) and observed with Lab View Signal Express 2010 software 4.0.0. (National Instruments).

3.4. Oil-red-O Staining

Outcome of adipogenic induction was visualized by Oil-red-O staining at the end of 7 days of treatment. Cells were washed with 1X PBS twice and fixed with 10% Neutral Buffered Formaline (NBF) for 15 min at room temperature. Then, cells were rinsed with distilled water and stained for 45 min at 37 °C with 60% diluted 0.5% oil-red-O in isopropanol (Amresco, Lot#1291C081). Cells were again rinsed (x3) with distilled water. Lipid droplets were monitored by the light field of an inverted microscope (Olympus, IX71).

3.5. MTT Assay

At the end of one-week induction and/or vibration (at day 9), cells were incubated with 10% MTT solution in DMEM for 4 hours at 37 °C, 5% CO_2. At the end of 4 hours, cells were lifted by DMSO and transferred to 96-well plate for spectrometric measurements. OD measurements were taken at 570 and 650 nm with VarioScan.

3.6. Immunostaining and Fluorescence Microscopy

In order to investigate the changes on actin content and structure, cells were stained with Phalloidin and nuclei were visualized by DAPI staining. At day 9, cells were washed with 1X PBS (x2) and fixed 4% paraformaldehyde (PFA) for 20 min at room temperature. After rinsing PFA three times, cells were permeabilized with 0.1% TritonX-100/1X PBS for 15 min at room temperature, followed by blocking with 3% BSA (Amresco, Cat#0332-1006) in 0.1% TritonX-100/1% PBS (blocking solution) for 30 min at room temperature. Then, cells were incubated with Phalloidin Alexa 488 (Invitrogen, Cat#A22287) stain (1:200) in blocking solution for 30 min in the dark at room temperature. Cells were washed three times with 1%PBS in the dark by incubating 10 min per each wash. Later, DAPI (Sigma, Cat#D9542-10MG) (1:240) in 1X PBS staining was done for 10 min in the dark at room temperature. Finally, cells were washed with ultra pure water and kept in 1X PBS solution. Images were taken

under fluorescent microscope with 40X magnification. Image analysis was done with ImageJ (NIH) software.

3.7. Gene Expression Analysis

Total RNA isolation was performed by using PureLink RNA Mini Kit following the manufacturer's protocol (Invitrogen, Cat#12183018). Purity and concentration of isolated RNA was determined by nanodrop (ND-1000 Spectrophotometer). Two step RT-PCR was performed. cDNA synthesis was done by Fermentas First Strand cDNA Synthesis Kit (Thermo, Cat#K1622) with 1000ng/µl template RNA. RT-PCR was done in BioRad IQ5 Thermal Cycler and results were analysed with Delta-Delta Ct Method. For control mouse GAPDH primers were used.

Table 3.1. Forward and reverse primer sequences used for qRT-PCR.

Gene	**Forward primer**	**Reverse Primer**
Actin	5'-ttctttgcagctccttcgtt-3'	5'-ttctgacccattcccacca-3'
Desmin	5'-gtgaagatggccttggatgt-3'	5'-gtagcctcgctgacaacctc-3'
Lamin	5'-atcaactccactggagaagaagt-3'	5'-cagacaggaggtggcatgt-3'
Vimentin	5'-acggttgagaccagagatgg-3'	5'-cgtcttttggggtgtcagtt-3'
Collagen	5'-caccctcaagagcctgagtc-3'	5'-agacggctgagtagggaaca-3'
ENC-1	5'-aagcttcggcata-3'	5'-aagct$_{11}$a-3'
β-catenin	5'-aaggaagcttccagacatgc-3'	5'-gcttgctctcttgattgcc-3'
PPARγ	5'-gccttgctgtggggatgtc-3'	5'-tccttggccctctgagatgag-3'
Adipsin	5'-gctatcccagaatgcctcgtt-3'	5'-ccacttctttgtcctcgtattgc-3'
C/EBP-α	5'-tggacaagaacagcaacgagtac-3'	5'-gcagttgcccatggccttgac-3'
PTK2	5'-ttggacctggcatctttgat-3'	5'-agaacattccgagcagcaat-3'
GAPDH	5'-gacatgccgcctggagaaac-3'	5'-agcccaggatgccctttagt-3'

3.8. Atomic Force Microscopy

Digital Instruments-MMSPM Nanoscope IV (Bruker Multimode) was used to take AFM images. Cells were washed with ultrapure water and dried in ambient conditions for 15 minutes. Cells were probed with a soft silicon cantilever with semi angle of 35° and 8 N/m spring constant. Locations of cells were detected using an optical microscope (Nikon 10x), and cantilever tip was conveniently adjusted above observed cells. In tapping mode, 50x50 μm^2 area was scanned with rate of 1001 Hz. Cells were analyzed for average surface height and roughness over cytoplasmic regions and physical characteristics of nucleus using AFM image processing software Gwyddion 2.31.

Nanosurf flexafm was used to get AFM images in medium. Cells were probed with NCLR tip with 190 kHz resonance frequency and 48 N/m force constant. Cells were scanned with 71,4143 kHz vibration frequency and 3,07mV vibration amplitude. Cells were analyzed for average surface roughness (Ra), root mean squared roughness (Rq), maximum height (Ry), maximum peak height (Rp), and mazimum valley depth (Rv) using AFM image processing software NanoSurf Scan.

3.9. Statistical Analysis

Unpaired t-test was performed for all results, control and experiment groups for growth and osteogenic medium were compared with each other.

CHAPTER 4

RESULTS

4.1. Cell viability

In order to determine the effect of mechanical signals on cell viability, MTT assay was performed after 7-days mechanical stimulation. Mechanical signals increased cell viability of MSCs in growth media by 25% ($p<0.01$). Adipogenic media induced an apparent 28% decrease in MSC viability compared to growth controls. Mechanical signals normalized cell viability of MSCs under adipogenic condition, observed by a 45% ($p<0.01$) increase in MTT signal compared to adipogenic controls (Figure 4.1).

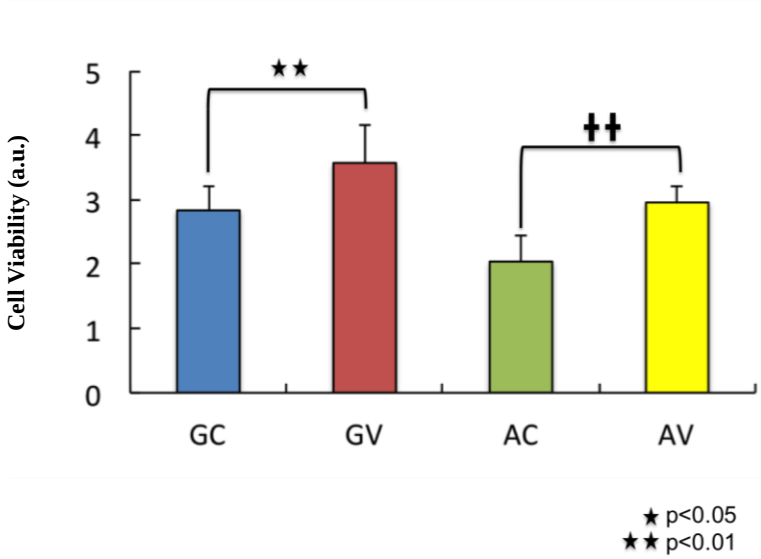
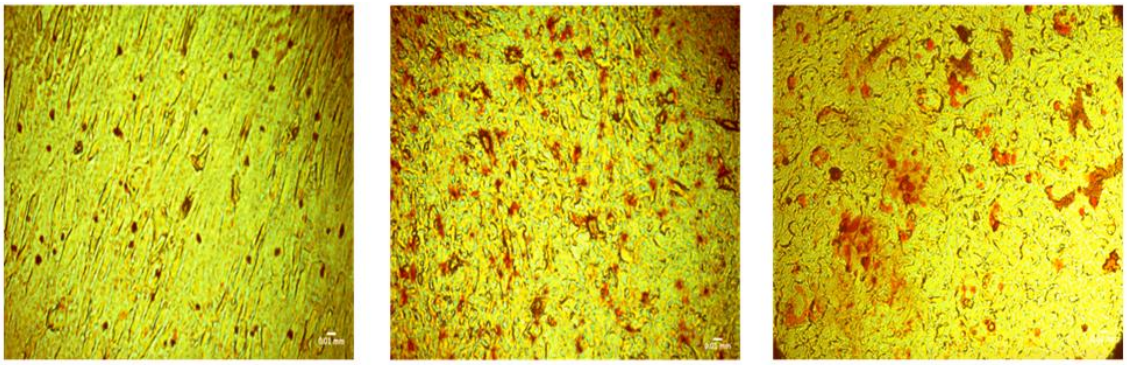


Figure 4.1. Results of cell viability via MTT assay. In both groups, mechanical stimulation increased the cell viability significantly ($p<0.01$).

4.2. Oil-red-O Staining

Oil-red-O staining which was performed to visualize the lipid accumulation within cells proves that MSCs undergo adipogenesis with adipogenic induction. Red droplets show the lipid accumulation in MSCs under adipogenic differentiation (Figure

4.2 A and B). When adipogenic induction is applied for two weeks a major increase in lipid droplet accumulation was observed (Figure 4.2 C).



A) GC after one week B) AC after one week C) AC after two week

Figure 4.2. Oil-red-O stained images at the end of one-week and two-week inductions.

4.3. Immunostaining and Fluorescence Microscopy

4.3.1. Mean Intensity

Cells were stained with Alexa 488 to observe actin content of the cells. Thus, Image J software fluorescence intensity results of Alexa 488 staining indicated that GV cells had a nonsignificant 9% ($p=0.3$) difference compared to GC cells while vibration causes a significant decrease by 22% during adipogenic induction ($p<0.01$). When GC and AC groups were compared to each other, it was observed that adipogenic induction increased Alexa 488 mean intensity per area significantly two fold ($p<0.01$) (Figure 4.3).

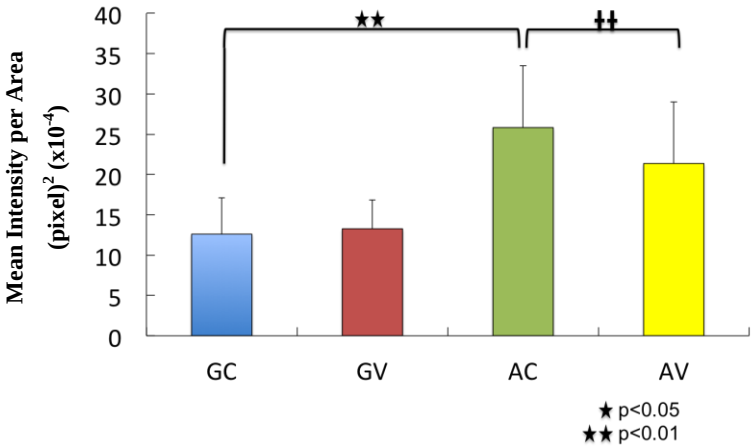


Figure 4.3. Mean intensity values after immunostaining.

4.3.2. Area

Image J software analysis of immunostaining was done in order to compare area values of the cells between groups. Results indicated that GV cells had a nonsignificant 7% (p=0.5) difference compared to GC cells similar to nonsignificant 9% (p=0.4) difference between AC and AV groups. On the other hand, adipogenic induction decreased the area of the cells by 39% significantly (p<0.01) (Figure 4.4).

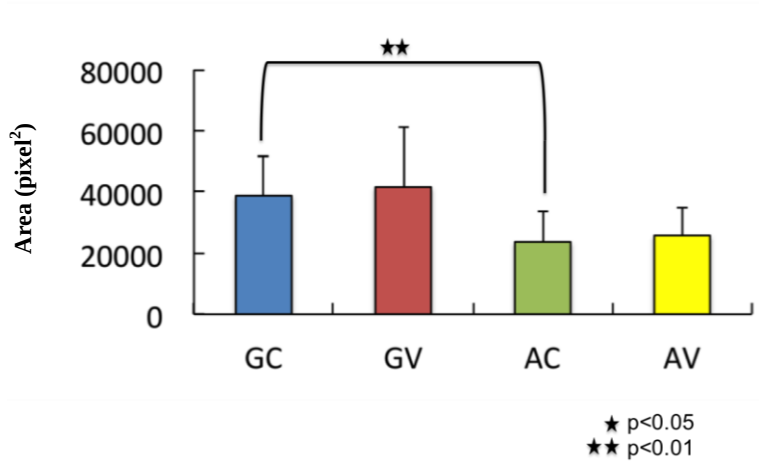


Figure 4.4. Average cell area values of groups.

4.3.3. Perimeter

Perimeter of the cells was calculated via Image J software analysis after immunostaining. While the 10% difference between GC and GV cells were not significant (p=0.2), vibration had a promising decrease by 18% in during adipogenic induction (p=0.05). However, adipogenic induction itself was more effective than vibration in terms of perimeter with decrease by 42% significantly (p<0.01) (Figure 4.5).

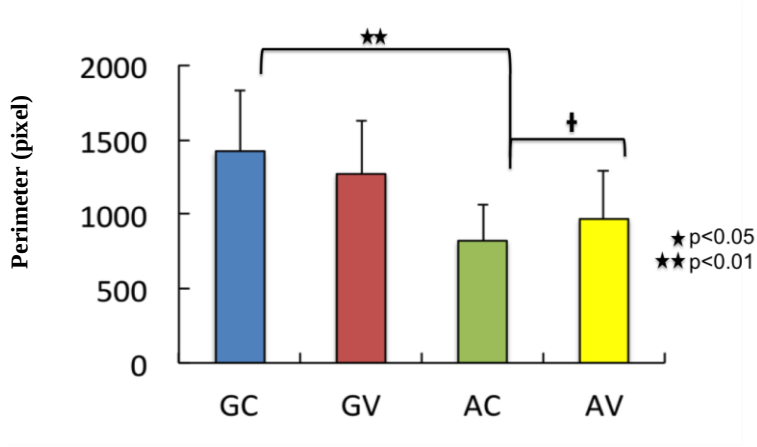


Figure 4.5. Average cell perimeter values after ImageJ analysis.

4.3.4. Circularization

In order to see the effects of adipogenic induction and vibration on the circularization of the cells, Image J analysis was performed after immunostaining. According to the results, the 6% difference between GC and GV was nonsignificant (p=0.5) but vibration resulted in a significant decrease by 20% in adipogenic culture (p<0.01). Whereas, adipogenic conditions increased circularity of the cells significantly by 50% (p<0.01) (Figure 4.6).

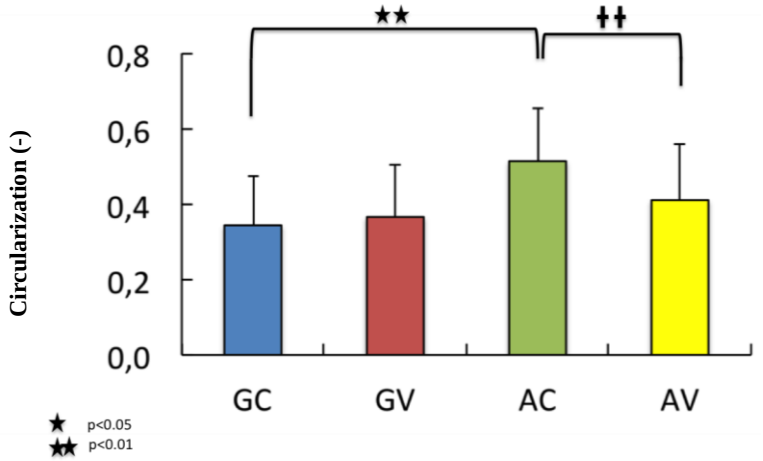


Figure 4.6. Circularization value of groups affected by vibration and induction

4.4. Atomic Force Microscopy

Changes on ultrastructure of the cells were examined via AFM. According to the AFM results, 1% difference between GC and GV groups in cell surface roughness was nonsignificant (p=0.95) likewise 6% difference between AC and AV groups (p=0.67). On the other hand, vibration increased cell surface height by 84% significantly (p=0.05) in growth conditions. Similarly, mechanical signals increased the cell surface height in adipogenic conditions by 78% significantly (p=0.01) (Table.4.1).

Table 4.1. Cell surface height and roughness values (nm)

Group	Average height (nm)		Average roughness (nm)
GC	1.78±0.48	+	8.33±2.93
GV	3.27±0.96		8.42±1.87
AC	3.52±0.89	★★	10.11±4.33
AV	6.29±0.84		9.43±1.36

★ $p<0.05$
★★ $p<0.01$

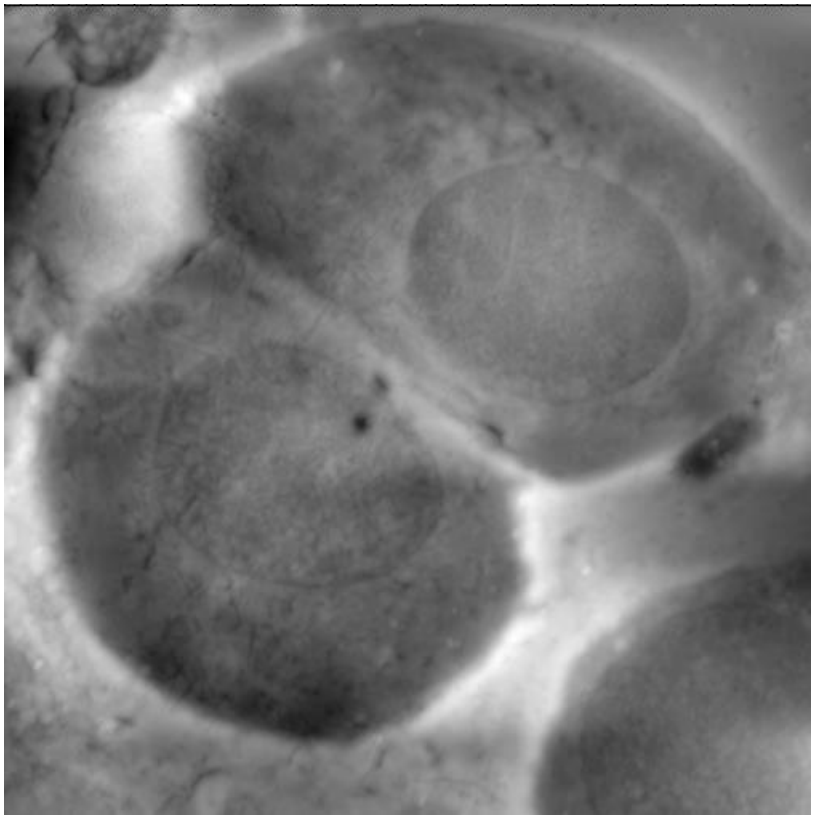


Figure 4.7. AFM image of cells.

In addition to the AFM results performed with air drying and water washing, cell surface roughness values of the alive cells in their medium were obtained by NanoSurf Scan software. According to the AFM results performed in medium, cell surface roughness of the cells was increased by 132% significantly with mechanical signals in growth conditions ($p<0.01$). Any significant deifference was not seen between AC and AV groups (Figure 4.8).

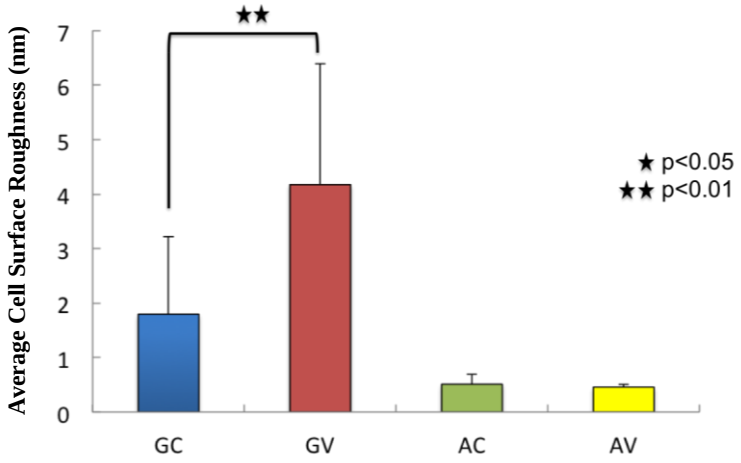


Figure 4.8. Cell surface roughness values of AFM imaging in medium.

4.5. Gene Expression Analysis

The effect of adipogenic induction and vibration on the expression level of several genes related to adiogenesis and mechanotransduction was evaluated after performing quantitative RT-PCR. Results showed that vibration increased actin level 5-fold in growth conditions significantly ($p=0.05$) while it caused a significant decrease in collagen by 64% and 57% in growth and adipogenic conditions respectively ($p=0.05$ and $p<0.05$). On the other hand, vibration had different effects on intermediate filamet proteins desmin and vimentin: while it caused a nonsignificant difference in both conditions for desmin, it increased vimentin level by 58% between GC and GV groups significantly ($p<0.05$) but decreased by 36% during adipogenesis ($p<0.05$). The 4% and 28% differences in β-catenin levels were nonsignificant between GC-GV and AC-AV groups, respectively. While vibration caused nonsignificant 60% difference in adipsin level between GC and GV groups, it supressed the adipogenic effect significantly by 59% which was increased by 19-fold during adipogenesis significanlty ($p<0.05$ and $p<0.01$).

No significant change was observed in ENC-1 and lamin levels within the groups. However, PTK2 level increased by 37% significantly with vibration in growth culture ($p<0.05$). PPARγ expression was affected by vibration during adipogenesis by

40% decrease (p=0.06). Also, mechanical signals decreased the expression of c/EBP-α gene in both conditions by 33% and 52% significantly (p<0.05) (Figure 4.9).

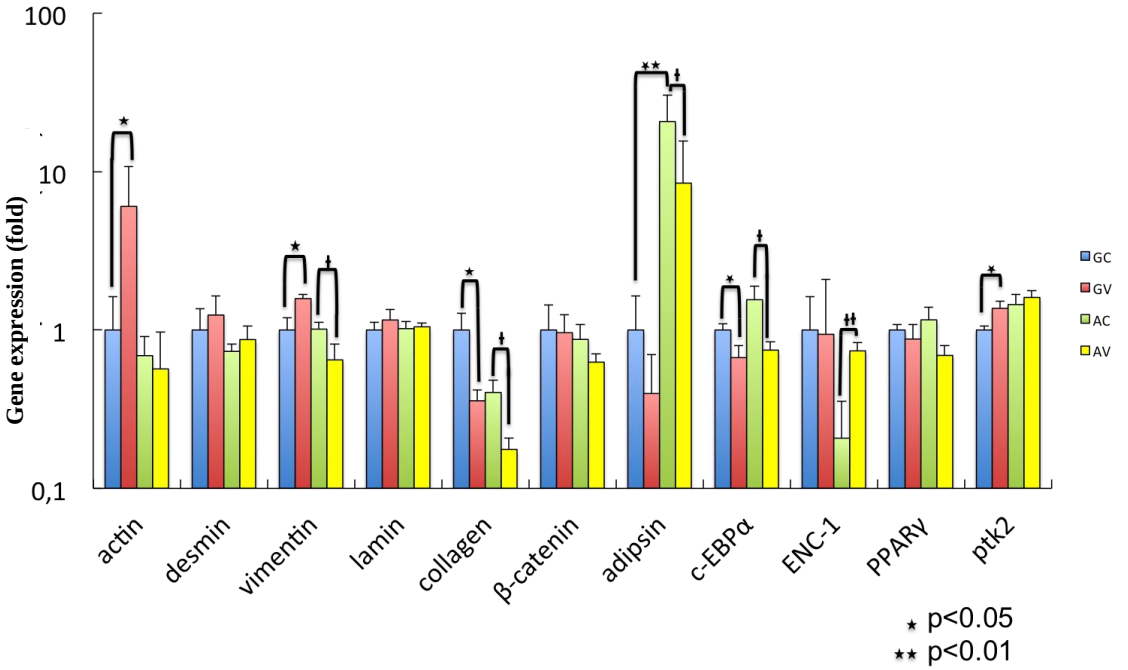


Figure 4.9. Quantitative RT-PCR Results confirmed the adipogenesis of stem cells by increase in adipogenic marker adipsin.

CHAPTER 5

CONCLUSION

In this study, it was aimed to identify the effects of mechanical signals on MSCs during adipogenic commitment. 9-day cell culture was performed by growth medium and adipogenic medium. During this period, 15 min/day mechanical vibrations was applied. Cells were imaged with AFM and fluorescence microscopy and then analyzed via Gwyddion 2.3.1 and ImageJ softwares, respectively. Also, effect of mechanical signals on cell growth and particular genes were investigated as well as physical changes.

Actin network, microtubules and intermediate filaments are the main determinants of the mechanical structure of the cells. Especially actin cytoskeleton maintains the shape changes during cell cyle and motility. AFM is the preferred technique for the examination of cytoskeleton structure with its high resolution quality (Berdyyeva, Woodworth, & Sokolov, 2005; Cai et al., 2010; Rotsch & Radmacher, 2000). According to this study, low magnitude and high frequency mechanical signals, increased the average cell surface height of MSCs while causing a nonsignificant change in cell surface roughness. However, AFM imaging was performed without fixation and cells were dried after washing with ultra pure water. When AFM was performed in medium with their culture conditions, a significant increase in cell surface roughness was observed between GC and GV groups.

Actin also plays role in adipocyte differentiation and in this study, fluorescence microscopy results indicate that actin content increases by adipogenic induction. The rearrengement and increase in actin are thought to provide stability and maintenance of the cell shape which can be easily disrupted due to lipid accumulation (W. Yang et al., 2014). Vibration decreased actin content and normalized the effect of adipogenic induction. Also, according to the previous studies, non-differentiating 3T3-C2 cells did not reorganize their cytockeletal structure resulting decreased biosynthesis of actin (Antras, Hilliou, Redziniak, & Pairault, 1989). In addition to actin, ENC-1, an actin binding protein, plays mediatory role iin diiferentiation of fibroblastic preadipocytes to mature adipocytes during cytoskeletal reorganization. It is also found in very low level

in mature adipocytes (Zhao, Gregoire, & Sul, 2000). The reason for decreased level of ENC-1 in this study may stem from the chemical induction used in culture that can accelerate the maturation of adipocytes. ENC-1 level increased by mechanical signals and reached the close value with GC group at the end of vibration application.

Mechanical signals and adipogenic induction resulted in physical changes in the cells. While cells becoming circular during adipogenic differentiation (Muruganandan et al., 2009) mechanical signals suppressed the effect of adipogenesis by decreasing circularity value of the cells. They also became smaller in perimeter as they lost their fibroblastic appearance and appendices during differentiation. Mechanical vibration removed the effect of adipogenesis and increased the size of the cells. Furthermore, cell viability increased by the mechanical signals in MSCs likewise the effect of uniaxial strains on MSCs (Kurpinski, Chu, Hashi, & Li, 2006).

Vimentin is an intermediate filament protein belonging to cytoskeletal elements and it is spesific for mesenchymal tissue (Quax, Khan, Quax-Jeuken, & Bloemendar, 1985). Its expression level increased significantly by vibration in growth culture in order to reinforce MSC against the effect of mechanical signals. On the other hand, vimentin expression decreased and normalized by vibration during adipogenesis. Actin protein is another cytoskeleton element supporting and giving mechanical property to the cell. It also regulates cell proliferation and differentiation (Hüttelmaier et al., 2005). The results obtained by MTT assay indicating increase in cell viability by vibration must be correlated with the 5-fold increase in actin level.

Adipsin is an adipocyte specific gene that is secreted by adipocytes (B. S. Rosen et al., 1989) and its expression increased 19-fold by adipogenic induction. It was highly downregulated by mechanical signals supporting the idea of normalization effect of vibration on adipocytes. PPARγ regulates the adipogenic differentiation so that its expression increases during adipogenesis (Lowe et al., 2011; Muruganandan et al., 2009). In this study, PPARγ level decreased with mechanical vibration showing the inhibitory effect of mechanical signals during adipogenesis. c/EBP-α is the promoter of leptin which is an adipose tissue specific factor (E. D. Rosen & MacDougald, 2006) playing role in body weight homeostasis. Thus, c/EBP-α is suggested for treatment in human obesity (Miller et al., 1996). Its decreased expression due to the vibration in adipogenic culture proves the suppressive mechanism of mechanical signals during adipocyte differentiation.

Increase in PTK2 level between GC and GV groups stem from the response of MSCs to mechanical signals. PTK2 concentrates the focal adhesion between cells and its activation might be important to early steps in intracellular signal transduction induced by mechanical stress (Schaller et al., 1992; Torsoni, Constancio, Nadruz, Hanks, & Franchini, 2003).

Suppressive and neutralizing effects of mechanical signals during adipogenic commitment that were indicated in this study can be a promising treatment for the obese individuals. Mechanical signals may replace the physical activity passively by changing the differentiation direction of MSCs. Disabled, old and bed rest people can benefit from the rehabilitative action of low magnitude high frequency mechanical signals.

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