THE EFFECT OF MECHANICAL FORCES ON ADIPOGENIC DIFFERENTIATION

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ABSTRACT

THE EFFECT OF MECHANICAL FORCES ON ADIPOGENIC DIFFERENTIATION

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Numerous intra and extra cellular factors take role in differentiation of cell towards a given lineage. These factors have crucial role in cell-cell and cell-environment interactions. In this study, the aim is to investigate the effect of mechanical forces on the adipogenic differentiation of preadipocytes and mesenchymal stem cells in an in vitro model.

Human preadipocytes and mesenchymal stem cells were embedded in 2 % agarose discs. According to the stress-relaxation test results it was observed that initial mechanical properties of agarose-mesenchymal stem cell (MSC) discs did not change compared to acellular agarose whereas those of preadipocytes decreased significantly.

The discs with cells were exposed to compression under different weights (1.4 ± 0.2 g, 7.5 ± 0.2 g, and 14.6 ± 0.3 g.) continuously in differentiation medium for 21 days. The control discs were treated with differentiation medium without any compressive weight on top of them. After 21 days, total ribonucleic acids (RNA) have been isolated. Adipogenic differentiation was investigated via reverse transcription coupled quantitative polymerase chain reaction (PCR). The expression of peroxisome proliferators-activated receptors (PPAR-gamma), CCAAT-enhancer binding protein (C/EBP-Beta), leptin, adiponectin, adipophilin

and human stearoyl-CoA desaturase (hSCD) have been assessed as adipogenic markers. Differentiation to adipocytes has been further investigated by histochemical Sudan IV staining and immunochemistry and compared to control group.

Decrease in the expression of adipogenic factors, size and number of lipid droplets were observed for both MSCs and preadipocytes subjected to compression in agarose discs. The decreases were correlated with the level of mechanical stress. The highest depletion of gene expression was observed in leptin and C/EBPβ. From our results, it was shown for the first time that mechanical stress impaired the adipogenic differentiation of MSCs and preadipocytes in agarose discs. However, the differentiation pathways should be further investigated.

Keywords: Preadipocytes, Mesenchymal stem cells, Mechanical forces

ÖZ

ADİPOJENİK FARKLILAŞMA ÜZERİNE MEKANİK KUVVETLERİN ETKİSİ

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Çeşitli hücre içi ve dışı faktörler değişik doku ve hücre tiplerinin farklılaşmasını etkilemektedir. Bu faktörlerin hücre-hücre ve hücre-çevre etkileşimlerinde önemli rolleri bulunmaktadır. Bu çalışmada agaroz disklere eklenen yağ öncü hücrelerin (preadiposit) ve mezenkimal hök hücrelerinin (MKH) adipojenik (yağ dokusuna) farklılaşmasında mekanik basıncın etkilerinin incelenmesi amaçlanmaktadır.

İnsan MKH ve preadiposit hücreleri agaroz taşıyıcı (% 2) içerisine yüklenmiştir. Disklere uygulanan gerilme (veya yük) boşalması (stress relaxation) testi, başlangıç mekanik özelliklerinde mezenkimal kök hücresi (MKH) taşıyan disklerle boş diskler arasında farklılık olmadığını, ancak preadiposit yüklenen disklerinde aynı özelliklerde anlamlı düşmeler olduğunu göstermiştir.

Mekanik basıncın farklılaşmaya etkisini incelemek amacıyla farklılaşma ortamındaki hücre içeren agaroz disklere 21 gün süreyle farklı ağırlıklar altında (1.4 ± 0.2 g, 7.5 ± 0.2 g, 14.6 ± 0.3 g) tek eksenli basınç uygulanmıştır. Kontrol grubundaki diskler ağırlık uygulanmadan farklılaşma besiyerine tutulmuştur. Bu süreç sonunda hücrelerin toplam RNA'ları izole edilmiştir. Adipojenik farklılaşma kantitatif PCR eşliğinde ters transkripsiyon yöntemi ile incelenmiştir. Adipojenik farklılaşma faktörlerinin (PPAR-gamma, C/EBP-Beta, leptin,

adiponektin, adipopilin ve hSCD) ifade miktarları farklılaşmanın değerlendirilmesinde kullanılmıştır. Ayrıca, Sudan IV boyaması ile de histokimyasal olarak adipojenik farklılaşma araştırılmış ve bütün sonuçlar kontrol grubu ile karşılaştırılmıştır.

Mekanik basınç uygulanan agaroz disklerdeki MKHlerde ve preadipositlerde adipojenik faktörlerin ifadesinde ve hücrelerdeki yağ damlacıklarının sayı-boyutlarında azalma gözlenmiştir. Bu azalmaların mekanik basıncın miktarı ile doğru orantılı olduğu bulunmuştur. Gen ifadelerindeki en çok düşüşün leptin ve C/EBPβ faktörlerinde olduğu görülmüştür. Bu sonuçlarla, mekanik basıncın agaroz disklerdeki MKH ve preadipositlerin adipojenik farklılaşmayı engellediği ilk kez ortaya konmaktadır. Bununla birlikte, farklılaşmanın metabolik yolakları yönünden daha ayrıntılı incelenmesi gerekmektedir.

Anahtar kelimeler: Yağ öncülü hücreler (preadiposit), Mezenkimal kök hücreleri, Mekanik basınç

To My Family

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LIST OF ABBREVIATIONS

3D	Three dimensional
ADD1/ SREB-P-IC	I/sterol responsive element binding protein-IC
AdipoQ	Adiponectin
ADFP	Adipophilin
BAT	Brown adipose tissue
BSA	Bovine serum albumin
cAMP	Cyclic adenin monophosphate
C/EBPβ	CCAAT-enhancer binding protein
DAPI	4',6-Diamidino-2-phenyindole
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EHS	Murine engelbreth-holm-swarm
ES	Embryonic stem
FBS	Fetal bovine serum
GPGH	Glycerophosphate dehydrogenase
hSCD	Human stearoyl-CoA desaturase
IBMX	3-isobutyl-methylxanthine
IGF1	Insulin like growth factor
MAP	Mitogenic-activated protein
MCSF	Macrophage colony-stimulating factor
MOPS	3-[N-morpholine] propane sulfonic acid
MSC	Mesenchymal stem cells
OD	Optical densities
PBS	Phosphate buffered saline
PGA	Poly-glycolic acid
PLA	Poly (lactic acid)

PLGA	Poly (lactic-co-glycolic acid)
PPARγ	Peroxisome proliferators-activated receptors
rt-PCR	Real time PCR
SCD	Stearoyl-CoA desaturase
SRE	Sterol-regulatory element
TBP	Tata-box binding protein
WAT	White adipose tissue

CHAPTER 1

INTRODUCTION

1.1. Cell Sources

1.1.1. Stem Cells

Stem cells, found in multi-cellular organisms, have the ability to renew themselves by mitotic division. They can differentiate into miscellaneous specialized cell types when sufficient and necessary stimulation for a specific cell type is given (Figure 1.1). Stem cells can be categorized in 3 groups as embryonic stem cells, adult stem cells and cord blood stem cells. Embryonic stem (ES) cells which are derived from blastocysts are pluripotent. Three primary germ layers (ectoderm, mesoderm, and endoderm) are derived from these embryonic stem cells. Adult stem cells are found in both children and adults (Jiang et al., 2002). They are multipotent which means they are lineage-restricted. Adult stem cells have two properties. They can divide and make a similar cell by asymmetric cell division. Also, they can divide and make a more differentiated cell (Barrilleaux et al., 2006).

1.1.1.1. Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSC) are multipotent cells that reside in bone marrow and have stromal support for the bone marrow stem cells and haemetopoiesis. They can differentiate into different cell types like osteoblasts, chondrocytes, myocytes and adipocytes in vitro (Ciapetti et al., 2006; Curran et al., 2005; Wang et al., 2006; Vashi et al.,2008) and in vivo (Salasznyk et al., 2004; Chamberlain G. et al., 2007; Ohnishi et al., 2007; Boquest et al., 2006). These cells have the ability to renew themselves.

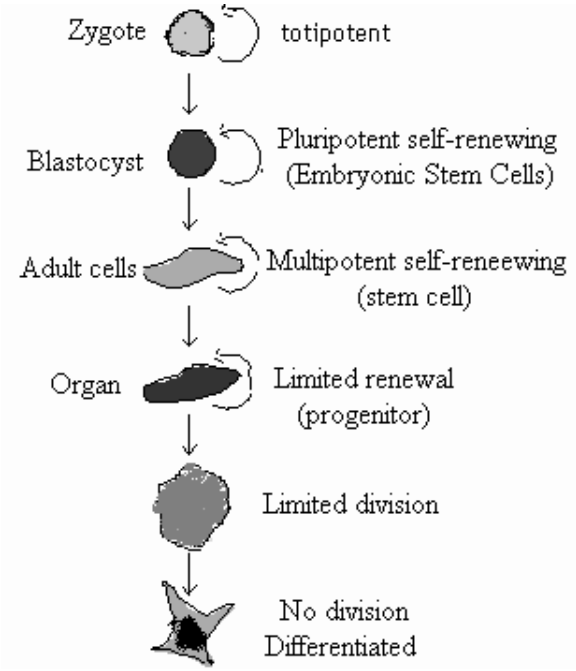

Figure 1.1. Schematic representation of potency of different cell types

1.1.2. Fat Tissues

Adipose tissue or fat tissue is a loose connective tissue which is composed mainly of adipocytes and fibroblasts. Adipose tissues differ from many other tissues in that they occur in multiple dispersed sites around the body. Adipose tissue has several functions. It is the main reserve of energy source for body. It acts as an insulator and thermogenesis (Rosen and Spiegelman, 2000). Adipose tissue can be accepted as an endocrine organ due to producing hormones like leptin, resistin and cytokine TNFα (Kershaw and Flier, 2004). Adipose tissue contains several types of cells including fibroblasts, macrophages, endothelial cells, and mainly adipocytes (Lanza et al., 2000). Fat tissue can be divided into two groups: Brown adipose tissue (BAT) and White adipose tissue (WAT) according to their color (Figure 1.2).

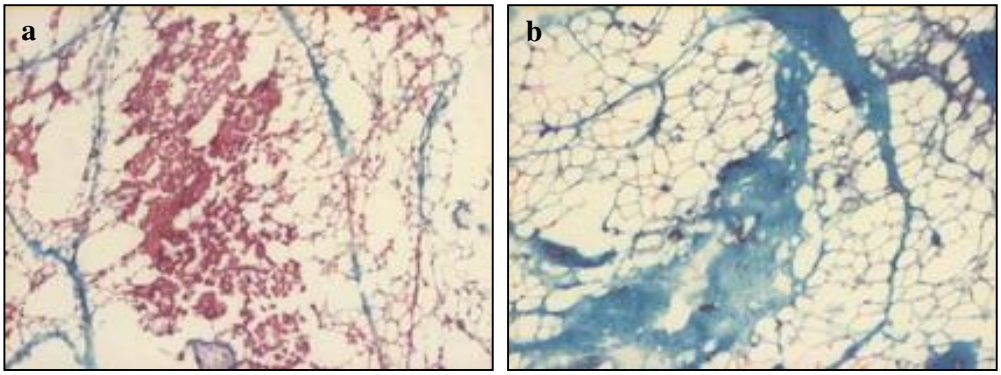

Figure 1.2. Morphological images of a) brown adipose tissue (BAT), b) white adipose tissue (WAT). Trichrome staining, transmission microscopy (courtesy of Dr. Kocaefe, Hacettepe University).

1.1.2.1. Brown Adipose Tissue (BAT)

Brown adipose tissue is mainly found in newborns, hibernating mammals, rodents and migratory birds. It is mostly encountered around the neck and large blood vessels of thorax. The basic function of brown fat is producing heat by non-shivering thermogenesis and protect against obesity (Rosen and Spiegelman, 2000). Brown fat cells consist of several small vacuoles, to store lipids, and plenty of mitochondria. They contain more capillaries than of the white fat tissue because they need more oxygen compared to other WAT in order to oxidize the fatty acids and produce heat. Patients with pheochromocytoma, a tumor which secretes cathecholamine, develop large brown fat depots.

1.1.2.2. White Adipose Tissue (WAT)

White fat tissue constitutes about 20-25 % of the adult human body weight. Brown fat tissue is replaced by white fat cells gradually starting from birth (Lindsay, 1996). White fat cells contain one large vacuole of fat. Thus, 90 % of cytoplasm in these adipocytes consists of lipids. The rest of the organelles are packed in a small area near the nucleus. Although WAT is less vascularized

compared to BAT, still each adipocyte has connection with one capillary (Patrick, 2000). Why the brown fat tissue turns to white is still a secret.

1.1.2.3. Preadipocytes

Preadipocytes are adipocyte precursor cells. They are undifferentiated fibroblast-like cells that can be differentiated into adipocytes (Patrick, 2004). In fact, these cells are morphologically indistinguishable from fibroblasts, although they are already committed to the adipocytic or adipogenic lineage. They are originally fusiform-shaped but when they are treated with prodifferentiative agents (e.g., cAMP, insulin and glucocorticoids), they undergo differentiation to mature fat cells over a 4-6 day period and become round in shape and begin to accumulate lipid droplets (Patrick, 2000). Preadipocytes can be acquired, cultured and proliferated easily compared to mature adipocytes. While mature adipocytes can not be subjected to enzymatic treatment and mechanical dispersion, generally preadipocytes are the choice of cells for expansion and differentiation studies (Gomillion, 2006).

1.1.3. Stem Cell Niche

A stem cell niche can be defined as a specific anatomical location that stem cells are housed and can participate in tissue generation, maintenance and repair. The stem cells rest in undifferentiated state with self renewal ability in niche (Curry et al., 1967; Schofield et al., 1978). In many studies it has been indicated that most adult-tissue stem cells can be quiescent for weeks or even months (Fleming et al., 1993; Cotsarelis et al., 1990; Zhang et al., 2003; Tumbar et al., 2004). However, this is not valid for all stem cells, one exception is the embryonic stem cells (Chambers and Smith, 2004; Uchidan et al., 2004). Two types of niches have been defined. Multi-cellular niche contains two or more parent cell types which are interrelated with each other and stem cells as well. Their function is to build several intercellular adhesion sites and molecular signaling pathways. The second type is acellular niche. It is composed of extracellular matrix elements and glycoproteins (Ohlstein et al., 2004; Micchelli et al., 2006; Jones and Watt, 1993,

Jensen et al., 1999). One of the functions of acellular niche is the expression of certain cell adhesion molecules on stem cell surface in order to interact with specific ECM proteins (Can, 2007). In multicellular stem cell niche two types of stem cells are present; self-renewing stem cells and quiescent stem cells. Self renewing stem cells divide asymmetrically in which one maintains the stem cell fate while the other produces differentiating progenitors (Murphy et al., 2005). Such self-renewing stem cell niches are essential to maintain normal tissue homeostasis (Wilson and Trump, 2006). In quiescence-storage niche, specialized cells create an environment responsible for differentiation or division. In the case of stress these cells can produce mature cells if required. Then, they may return to niche to be stored or self-renewing (Lechler and Fuchs, 2005).

1.2. Microenvironmental Humoral and Paracrine Factors

Extracellular microenvironment plays a number of essential roles in tissue engineering by providing a scaffold for cells to attach and migrate, as a microenvironment of differentiation and a preserver of space (Stillaert et al. 2005). The environment of cell in the culture can affect the differentiation and behavior of the cell and thus the tissue formation. For example, adipogenesis can be influenced by growth factors (IGF1), hormones (insulin), adipokines (leptin, Adipsin) and oxygenation (Feve, 2005) which can affect the differentiation and viability of the tissue. Along with that, other microenvironmental factors such as cell density and cell-cell interactions also play a role in adipogenesis.

Availability of the polyunsaturated fatty acids, glucose concentration, hormones particularly growth hormones, and tissue pH are the major factors that modulate the differentiation and basal metabolic activity of the fat tissue (Rosen and Spiegelman, 2000).

1.2.1. Extracellular Matrix (ECM)

Extracellular matrix is the supporting structure that surrounds the cellular structures. ECM involves non-cellular materials existing between cells. ECMs are

synthesized and assembled through many steps. Soluble matrix molecules secreted by cells are then modified (proteolytically processed, sulfated, oxidized and crosslinked) and assembled into polymeric complexes. These complexes serve as a scaffold for the tissue and also as a reservoir for small molecules like growth and differentiation factors. ECM proteins coordinate cell migration, proliferation and tissue homeostasis by binding to specific integrin cell surface receptors. Binding to those receptors activates intracellular signaling pathways, cytoskeletal reorganization and alteration of cell morphology (Boudreau and Jones, 1999). The formation of ECM in vivo is unidirectional and irreversible process. The disassembly is not simply reverse of assembly, but involves multiple, highly regulated processes. One of the most important functions of ECM is providing a substratum and a niche for cells to attach.

The differentiation of fat precursor cells depend on temporally controlled expression of multifunctional adhesive glycoproteins and their cellular receptors, and on a tight regulation of different proteolytic enzyme families (Atanassova, 2003). Multifunctional glycoproteins in the ECM regulate adhesive processes, coordinate proteolytic degradation and influence (Salasznyk et al., 2004) cell migration, proliferation and differentiation. Preadipocytes secrete Type-I collagen during their early phases of differentiation. The production and secretion of Type-I collagen ends concomitantly with the maturation of the adipocyte (acquirement of the unilocular phenotype). Fibroblasts and other extracellular matrix components create the mechanical support for the adipocyte niche. Due to the lack of cytoskeletal elements in the adipocyte, without the existence of this extracellular support, fat cells cannot survive (Feve, 2005).

1.2.2. Mechanical Factors

In many studies it has been shown that some cells give response to shear stress and other forms of mechanical pressure. Subjecting cells to mechanical load can cause deformation of cell, fluid displacement, and disruption of molecular transport (Tate, 1999). Various types of cells can behave in different way under variant mechanical pressure. Mechanical pressure is required for the

differentiation and maintenance of some tissue cell types such as cartilage, bone and muscle. Furthermore, shear-stress is also important for the differentiation and maintenance of blood cells and endothelium. Moreover, cellular morphology, adhesion, locomotion and cytoskeletal protein expression can also be affected in response to these internal forces (Geiger B. and Bershasky, 2002)

Several different methods have been reported in literature to apply the mechanical pressure on different types of cells. Fluid flow-induced mechanical pressure (Kobayashi et al. 2004) or fluid shear stress, hydrostatic pressure (Angele et al, 2003), high and low pressure lavage using 30 ml syringe (Bhandari et al, 2002), and elongational stresses such as gravity and impacts with solid subjects (Georges and Janmey, 2005) are some of the methods used recently. Distribution of the mechanical forces on surfaces and within the interior of the cell depends on both the material properties of the cell itself and the properties of the surface or matrix in which cells are embedded (Bischofs and Schwarz, 2003).

Bischofs and his friends (2004) have modeled the cellular reaction to substrate stiffness using some simple assumption about the ECM. They treated the materials of scaffolds as a spring that is stretched by cellular forces (Figure 1.3). The first assumption is that the substrate are accepted to be isotropic that means its properties are the same in all directions. In this model an isotropic ECM was considered as spring with a spring constant K, that exerts a force F, proportional to the distance X, by which it is extended, according to Hooke's law, following the expression:

$$F = KX$$

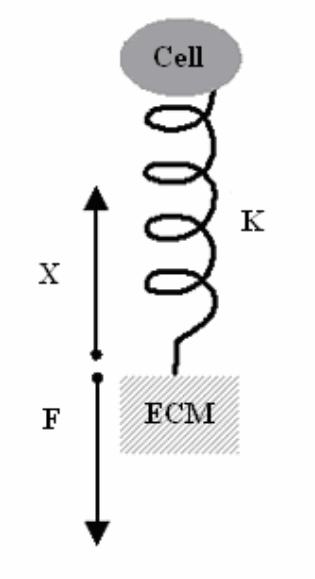

Figure 1.3. Model of an isotropic extracellular matrix as a spring with spring constant, K.

The spring constant, *K*, shows the elastic modulus which is a constant for material that describes its resistance to axial load. Elastic modulus can be calculated as stress over strain. The actin filaments which are anchored at focal adhesion complexes derive the forces on the spring. As indicated in Figure 1.4., in a stiffer ECM with higher *K*, the cell may be targeting a certain displacement of substrate to communicate with neighboring cells, greater values of *F* is required compared to a lower *K* value in softer gels (Georges and Janmey, 2005). This could be the reason that cells embedded in agarose gel shows distinct morphology from those cultured on tissue-culture flasks (Hay, 1982; Grinnell, 2000). It has been shown that fibroblasts loose their F-actin stress fibers that are present when fibroblasts are cultured in tissue flasks (Georges and Janmey, 2005). Its mechanism were related to focal adhesion complex proteins which were down regulated on soft gels but not on tissue culture flasks (Wang et al., 2003).

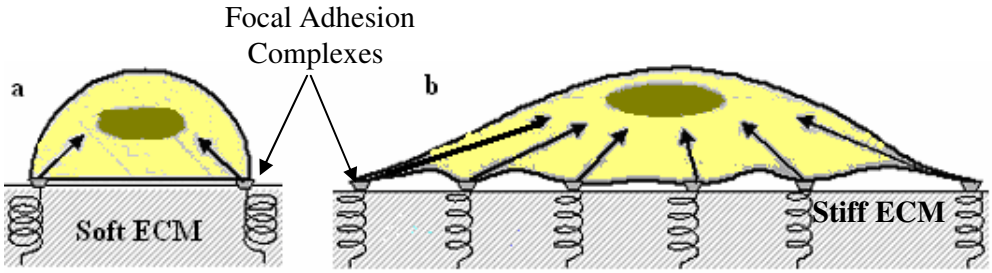

Figure 1.4. Schematic drawing of cell shapes in a) soft ECM and b) stiff ECM.

1.2.2.1. Signal Transduction via Cytoskeletal Elements

Cytoskeleton is a highly dynamic network. It is composed of microfilaments; actin, microtubules; tubulin, and intermediate filaments. Recent advances in the field of adipocyte differentiation have shown that the cytoskeletal elements and especially microtubules are important key elements in adipocyte differentiation and the acquirement of the mature adipocyte morphology (Boström P, 2005).

During adipogenesis in preadipocytes, fibronectin-rich extracellular matrix surrounding the preadipocytes is remodeled to basement membrane of adipocyte (Selvarajan et al., 2001; Smas et al., 1995; Mandrup and Lane, 1997) in which collagen type I and III are downregulated whereas collagen type IV and entactin are up regulated (Gregoire et al., 1998). After this remodeling, the cell-ECM adhesion is distorted. This changes the morphology and volume of the cells which seems to be required for induction of mRNA expression for lipogenic proteins (Lilla et al., 2002). It has been investigated that in physiologic condition, fibronectin-rich matrix inhibits adipogenesis in preadipocytes (Spiegelman and Ginty, 1983) but after confluency, proteases extensively remodel the ECM and degrade the fibronectin (Bouloumie et al., 2001).

Inhibition of microtubule biosynthesis blocks the fusion of lipid vesicles within adipocytes regardless of de novo lipid synthesis. Thus, mechanical pressure is expected to exert a negative effect on acquirement of the mature adipocyte

phenotype via cytoskeletal interactions. Similarly, Rho GTPases that are in tight link with the cytoskeletal elements in mesenchymal stem cells have been linked to the cytoskeletal tension and cell shape which is capable of directing the cell differentiation program in stem cell lineage and commitment (McBeath et al., 2004). Apparently, the RhoA signaling pathway is essential in adipogenic determination of the MSCs. Thus, mechanical tension applied to MSCs may disrupt the actin-myosin tension that is required for the RhoA mediated adipogenic commitment. It is not clear whether if the same pathway is also valid for the differentiation of the preadipocytes.

1.3. In-Vitro Models for Fat Tissue

In cell biology, to study the effect of differentiation, physiological and pathological factors and conditions, different modeling approaches are sought. In vitro modeling of a tissue or an organ is a common approach to mimic the natural conditions. To obtain reliable results, conditions must be set similar to the physiological nature of the original tissue as much as possible. For this purpose, several in vitro models have been designed.

1.3.1. Explant Culture

Explant culture is a technique used for the isolation of cells from a piece or pieces of tissue. Tissue harvested in this manner is called an explant. In this method, the tissue is minced in a sterile manner and pieces placed in a cell culture dish in a cell culture dish containing growth media. As the time passes, progenitor cells migrate out of the tissue onto the surface of the dish and named as primary cells. These primary cells can be expanded and transferred into fresh dishes. In this method the tissue pieces can be cultured directly without harvesting. So, the cells are left in their surrounding extracellular matrix o more accurately mimic the in vivo environment (Orth et al., 2000).

1.3.1.1. Organotypic Fat Tissue Explant Cultures

The main obstacle working with adipocytes is to isolate them alive and to maintain them for a sufficient period of time. Organ culture of adipose tissue has long been accepted as the model of choice for biochemistry experiments where mature adipocytes and their activities can be traced in an isolated environment for a short time (Boone et al., 2000; Vierck et al., 2001). This setup is adequate to observe the impact of short-term modulators or the outcome of environmental changes on the adipocyte metabolism. In our hands, the explanted mature adipocytes can be kept alive for up to three weeks. During the first week, adipocytes tended to loose their fat content rapidly. This is a well known phenomenon which has been inaccurately named “dedifferentiation” (Vierck et al., 2001). There are a significant number of studies using pre-adipocytes and/or adipofibroblasts to investigate adipogenic commitment and differentiation (Gregoire et al., 1998). Although these cells could easily be handled, they naturally harbor a significant plasticity unless they are fully differentiated and are not stable in tissue culture for long term experiments (Vernon, 2000).

One alternative approach of differentiating the primary pre-adipocytes to obtain mature fully differentiated adipocytes. Once the adipogenic differentiation is induced, the precursors undergo commitment and start to accumulate fat vesicles. On the long course of incubation, the average time for these adipocytes to achieve the monolocular mature phenotype is at least 10 to 12 weeks. As the adipocytes grow and acquire more fat in cell culture, they tend to loose their adhesion capabilities and eventually break loose and start floating. This is the main obstacle of this method and generally occurs as the cells achieve the monolocular shape. One approach to overcome this problem was to grow the preadipocytes to confluency before differentiation, fix them with ethanol, wash repeatedly with PBS and achieve a surface for a second round of plating and differentiation. This approach enhanced the adhesion properties and give better results compared to collagen coated tissue culture chambers (Kocaefe et al., 2005).

1.3.1.2. Ceiling Culture

Ceiling culture is another method used for the study of fat tissue. In this method,

the culture flask is filled completely with medium (approximately 70 ml for a T25 cm^2 flask) that contains fat tissue explants floating in the medium. This will allow the cells to adhere to the top inner surface (Sugihara et al., 1986). In this method pre-gassing of culture medium should be applied at least 4-6 hours. One of the advantages of the ceiling method is that it would be able to keep adipocytes alive for a long time, even more than 4 weeks. This method is modified by Zhang et al. (2000). They have cultured adipocytes underneath a floating glass surface. They even refined this method more and cultured preadipocytes on a floating coverslip and placed it in cell culture or dish. They found that this method requires less culture medium (12 ml for a six-well plate) compared to the flask method. Moreover, there is no need to pre-gassing of the culture medium here. It is supposed to be easier to execute and study adipocytes using fluorescence microscopy by this newly modified ceiling method (Zhang et al., 2000).

1.3.2. Polymeric Three Dimensional Scaffolds

Generally, 3 dimensional polymeric scaffolds are used in these models. Among the synthetic and natural polymers used for establishment of such in vitro models are poly (lactic-co-glycolic acid) (PLGA), poly (lactic acid) (PLA), alginate, agarose, matrigel, PGA (poly-glycolic acid) etc (Daya et al., 2007; Hong et al., 2005; John et al., 2002; Fischbach et al., 2004; Patrick et al., 1999).

1.3.2.1. Poly (Lactic-co-Glycolic Acid) (PLGA)

PLGA is a highly biocompatible polymer that degrades into harmless monomer units. Depending on the ratio of the copolymers, a useful range of mechanical properties can be achieved in the scaffolds. Recently, this copolymer and its homopolymer derivatives, poly (lactic acid) (PLA), and poly (glycolic acid) (PGA) have received substantial attention for skeletal repair and regeneration. PLGA has been extensively studied for the in vitro cultivation of adipocytes and

various other cell types (Sung et al., 2004; Lee et al., 2002; Rahman and Tsuchiya, 2001).

1.3.2.2. Matrigel®

Matrigel is a trade name for a gelatinous protein mixture which is secreted from murine Engelbreth-holm-Swarm (EHS) sarcoma. It contains basement membrane proteins such as laminin, collagen IV, and heparin sulfate proteoglycans together with some growth factors (Bioscience product catalog, 2003). It resembles extracellular environments of many tissues. So, it can be used as a substrate for tissue cell culture (Filip et al., 2007). In fact, it is an artificial tissue scaffold that aids the mimicking of the extra-cellular matrix for cell types that require extracellular feed-back for growth and maintenance.

A common procedure for it is to dispense small volume of chilled (4°C) matrigel® onto the plastic tissue culture flask and incubate at 37°C (body temperature). So, a thin film of matrigel® protein self-assembled will cover the surface of the tissue culture flask.

The matrigel® matrix has been extensively used for adipocytes in various studies (Kawaguchi et al., 1998; Kimura et al., 2002). It supports the cross-talk between developing adipocytes and endothelial cells which are not in direct apposition to each other (Stillaert et al., 2007). Besides, it promotes attachment and spreading of preadipocytes whereas it antagonizes spreading of cells other than preadipocytes. Laminin, one of the components of matrigel®, play an important role for such selective proliferation of preadipocytes (Hausman et al., 1996).

1.3.2.4. Low Gelling Temperature Agarose Gel

Agarose gel is a widely used hydrogel, since it permits growing of cells in a three dimensional suspension. The use of hydrogels become popular in biomedical research and tissue engineering (Benya and Shaffer, 1982) especially adipose tissue engineering (Awad et al., 2004) due to their viscoelastic characteristics, biocompatibility, amiability of fabrication into specific shapes, ability to

withstand forces and transferring gases and nutrients (Orwin et al., 2003; Awad et al., 2004). Agarose gel is a linear polysaccharide, extracted from marine red algae (Murphy and Kelly, 2006). When swollen by a solvent, it becomes a porous solid filled with fluid. It is therefore expected to behave as a biphasic substance with viscoelastic properties (Chen et al., 2000). One of the problems with these agarose gels is their poor mechanical properties which restrict their use.

The mechanical properties of agarose gel can be varied by agarose compositions. Measurement of permeability of agarose gels with different concentration (w/v %) of 2 %, 4 %, and 6 % under mechanical forces has showed that the suitable range of concentration for required permeability is 2 % (Mauck et al., 2000).

1.4. Adipogenic Differentiation Pathway

The reprogramming of genes during adipogenesis is controlled tightly by hormones, cytokines, nutrients and signaling molecules.

The first stage in adipogenesis is growth arrest. It is achieved in cultured cell lines after contact inhibition. Initial growth arrest is followed by 1-2 additional rounds of cell division known as cloned expansion. Cloned expansion ceases coincident with the expression of the transcription factors.

The main transcription families which have a key role in regulation of adipogenesis are CCAAT-enhancer binding protein (C/EBP-α, β, and δ), peroxisome proliferators activated receptor (PPARγ, β, and δ), and adipocyte determination and differentiation factor I/sterol responsive element binding protein-IC (ADD1/ SREB-P-IC) families (Holst and Grimaldi, 2002).

Induction of C/EBPα and PPARγ proteins are characterized by a second permanent period of growth arrest which is followed by differentiation of preadipocytes into mature adipose phenotype.

In preadipocytic differentiation, lipoprotein lipase is induced first. It is followed by C/EBPβ and δ which have synergistic role in adipocyte differentiation. These

events are followed by accumulation of C/EBPα and PPARγ. C/EBPα has anti-mitotic properties. When the cells begin to differentiate into adipocytes, they will stop divisions (Rosen and Spiegelman, 2000). C/EBPα is expressed just before the transcription of most adipocyte specific genes is initiated (Mandrup and Lane, 1997). The expression of PPARγ isoforms can be observed in many tissues, but PPARγ2 is expressed preferentially in adipocytes (Feve, 2005). It has been shown that over expression of either of these factors, PPARγ2 and C/EBP in fibroblast is able to convert these originally non-adipogenic cells from fibroblast cells into adipocytes. It has been investigated that the cells can undergo adipogenesis in the absence of C/EBP by over expression of PPARγ2. However, forced expression of C/EBP in the absence of PPARγ will not induce adipogenesis (Rosen and Spiegelman, 2000).

Observation of normal expression of PPARγ in mice with C/EBPβ and γ double knockout reveal another mechanism independent of C/EBP for inducing PPARγ named as salvage pathway. The transcription factor of this pathway is adipocyte determination and differentiation factor I/ sterol responsive element binding protein-IC (ADD1/ SPEB-P-1C). ADD1/SREBP1 is induced during adipogenesis and it is regulated by fasting and re-feeding in vivo. In liver, it binds to sterol-regulatory element (SRE's) in cholesterol regulatory genes. It is a key link between nutritional changes and the lipogenic gene program. Moreover, it can regulate adipogenesis but not as much as PPARγ and C/EBPs. The adipogenic differentiation pathway and role players are summarized in Figure 1.5.

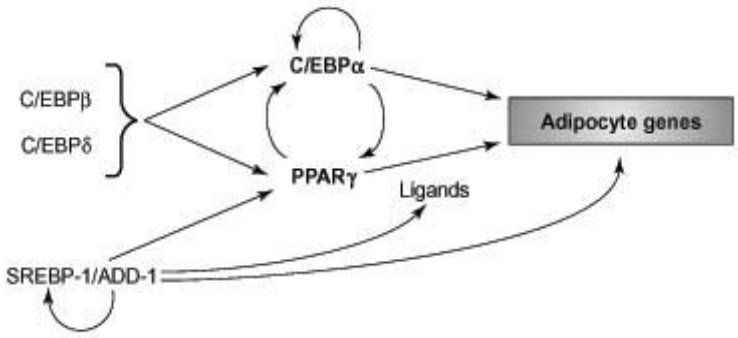

Figure 1.5. Adipogenic differentiation pathway

Several transcription factors other than C/EBP, PPARγ, and SREBP play important roles in adipogenesis. Among these, two members of GATA family of transcription factor, GATA 2 and GATA 3, are expressed in preadipocytes. Their expressions are down regulated after cell maturation (Feve, 2005). Another factor is cyclic adenin monophosphate (cAMP) responsive element binding protein (CREB). It increases C/EBPβ level. Many other factors can either promote or block the adipogenesis. The balance between these opposing signals will determine the fate of preadipocytes to quiescence, division, or differentiation.

1.4.1. Factors That Stimulate Adipogenesis

One of these factors is insulin. Some of the preadipocytes have few insulin receptors. Insulin increases amount of lipid accumulation in each fat cell and percentage of differentiated cells. It has anti-apoptotic activity. Moreover, insulin stimulate insulin like growth factor I receptor (IGF-IR) (MacDougald and Mandrup, 2002).

Another inducing adipogenic factor for in-vitro is glucocorticoids (GC) (Joyner et al., 2000). It is canonically used in the form of dexamethasone (Dex) in studies (Rosen and Spiegelman, 2000). GC induces expression of C/EBPγ (Cao et al., 1991) and PPARγ (Wu et al., 1996) by inducing cAMP secretion (Yeh et al., 1995).

High fat diet also affects the adipogenesis. Saturated fatty acids induce hypertrophy and hyperplasia in adipose tissue. Poly unsaturated fatty acids, on the other hand, are more effective compared to saturated fatty acids in preadipocyte differentiation in culture. It is proposed that poly unsaturated fatty acids act as precursor of ligands for PPARγ.

The other factors are prostoglandins, adiponectins (Fu et al., 2005), thyroid hormone, retinoic acids (MacDougald and Mandrup, 2002) and macrophage colony-stimulating factor (MCSF) (Levine and Jensen, 1998). Adiponectins exhibits more prolonged gene expression of important transcription factors for adipogenesis (Fu et al., 2005).

1.4.2. Factors That Inhibit Adipogenesis

Signals that repress adipogenesis are inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin 1, 6, 11 and leukemia inhibitory factors. Exposure of preadipocytes to inflammatory cytokines inhibits adipogenesis by down regulating PPARγ and C/EBPβ expression (Feve, 2005). Some growth hormones are potent inhibitors of adipogenesis. These inhibitory effects are mediated by activation of classic mitogenic-activated protein (MAP) kinase.

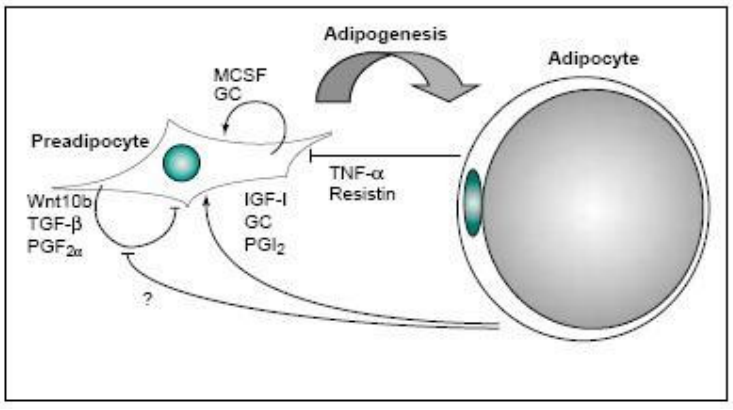

Figure 1.6. Schematic representation of factors other than C/EBP and PPARγ affecting adipogenesis.

1.4.3. Adipogenic Differentiation Markers

Adipogenesis is initiated in response to insulin, insulin like growth factor I, growth hormone, corticosteroids, thyroid hormone (Guller et al., 1987). The early and late markers showing the conversion of cells to adipocytes are lipoprotein lipase (Guller et al., 1987) and glycerophosphate dehydrogenase (GPDH) (Pairault and Green, 1979) respectively. The expression of these markers is followed by appearance of fat droplets in differentiated cells. This will continue until the characteristic morphology of the mature adipocyte is acquired (Shillabeer et al., 1989). The differentiation of preadipocyte is initiated only when the cells are confluent (Wiederer and Löffler, 1987). Preadipocyte differentiation can also be detected by observing expression of some functional genes such as PPARγ, hSCD, leptin, and CEBPβ.

1.4.3.1. Peroxisome Proliferators-Activated Receptors (PPARγ)

Peroxisome proliferators-activated receptors (PPAR) are ligand-inducible nuclear transcription factors. It is the first enhancer element for fat cells which is located at 5 kb upstream of the start of transcription. It is prime mover of fat cell differentiation. PPARγ and C/EBP are also co-expressed in myoblasts, same as fibroblast cells and make adipose tissue (Rosen and Spiegelman, 2000). PPARγ can be stimulated by xenobiotics (such as steroids, insulin and 3-isobutyl-methylxanthine (IBMX) that transactivate itself as well as other master modulators of adipogenic transcription pathway (C/EBPα and β, SREbp1). PPARγ has pivotal role in adipocyte differentiation, fatty acid and lipid metabolism, insulin sensitivity and ovarian steroidogenesis. PPARγ also influences glucose uptake and metabolism in liver, adipose tissue and muscle tissues (Berdiaki et al., 2004). PPARγ is responsible for the transcription of numerous genes that reside on adipogenesis pathway.

1.4.3.2. CCAAT-Enhancer Binding Protein (C/EBPβ)

C/EBP family is a member of basic leucin zipper class of transcription factor. One major function of C/EBPα and δ is to induce expression of PPARγ. The protein encoded by this intronless gene is a transcription factor which can bind as a homodimer to certain DNA regulatory regions. It can also form heterodimers with the related proteins CEBPα, CEBPδ, and CEBPγ. CEBPβ is important in the regulation of genes involved in adipose differentiation, immune and inflammatory responses. It has been shown to bind to the IL-1 response element in the IL-6 gene, as well as to regulatory regions of several acute-phase and cytokine genes. In addition, the encoded protein can bind the promoter and upstream element and stimulate the expression of the collagen type I gene, thus may be responsible for the synthesis of Collagen-I during early differentiation of pre-adipocytes (Otto and Lane, 2005).

1.4.3.3. Human Stearoyl-CoA Desaturase (hSCD)

Human Stearoyl-CoA desaturase (hSCD) is an iron containing enzyme that catalyses the oxidation of palmotoyl-CoA and stearoyl-CoA (Zhang et al., 1999). This enzyme catalyzes the rate-limiting step in synthesis of poly-unsaturated fatty acids, thus has pivotal role in adipocyte metabolism and expressed uniquely in adipocytes (Kim and Ntambi, 1999).

1.4.3.4. Leptin

Leptin is the main marker of body fat index and body mass index. The utmost role of leptin is to organize food intake, energy metabolism and to prohibit obesity by negative feedback effect on appetite by specifically acting on hypothalamus. Leptin is also a key regulator of peripheral glucose uptake and insulin action. Leptin peptide is found in blood circulation as proportional to body fat mass (Aslan et al., 2004). Under physiological conditions, white fat tissue synthesizes and secretes leptin.

1.4.3.5. Adipophilin (ADFP)

Adipophilin (ADFP) is a structural protein surrounding the lipid vesicles within the adipocytes. ADFP is one of the earliest markers of adipocyte differentiation (Targett-Adams et al., 2003). It is also designated as "adipose differentiation-related protein". Together with its partner perilipin, adipophilin forms a protein complex around the cytoplasmic fat vesicles and also modulates the interaction of the lipoprotein lipases and lipid biosynthesis enzymes. The expression of ADFP is tightly controlled by PPARγ and α on the course of adipogenesis (Brasaemle et al., 1997).

1.4.3.6. Adiponectin (AdipoQ)

Adiponectin is expressed in white adipose tissue exclusively. It encodes a protein with similarity to collagens X and VIII and complement factor C1q. The encoded protein circulates in the plasma and is involved with metabolic and hormonal processes. Adiponectin is also known as the adipocyte complement related protein of 30 kDa (Acrp30). PPARγ agonists such as thiazolidinediones (TZD) have been shown to increase plasma adiponectin levels and also induce adiponectin expression up to 50 fold in 48 hours in preadipocytes (Combs et al., 2002). Adiponectin has a physiological role of promoting adipocyte differentiation, insulin sensitivity and lipid accumulation (Fu et al., 2005).

1.5. Aim of the Study

Naturally, the cells are subjected to mechanical forces like shear stress over the endothelial cells lining arteries due to blood flow, or compression on chondrocytes in knee that are impacted when a person walks or runs. Mechanical factors are one of the important determinants of cell fate both in vivo and in vitro.

The aim of this study is to examine the effect of mechanical forces on adipocyte differentiation program by designing a 3-D cell culture model that can mimic the fat tissue and can create differential environment in terms of mechanical forces.

The evaluation of the effects of this environmental factor on adipogenesis was studied by embedding preadipocytes or mesenchymal stem cells into low melting agarose discs and subjecting to static mechanical forces with the application of different weights. The preadipocytes or mesenchymal stem cells embedded in agarose discs in the free swelling condition (under no weight) served as controls. Differentiation of the cells to adipocytes was investigated at the mRNA level through reverse transcription coupled quantitative real time PCR by measuring the gene expression of adipogenesis indicators such as PPARγ, C/EBPβ, Leptin and hSCD and histochemical staining for intracellular lipids using oil red and immunostaining against perilipin.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Agarose type VII, dexamethasone (97 %, powder), ethylene diamine tetraacetic acid disodium salt dehydrate (EDTA) (99 %), Ficol, 3-isobutyl-methylxanthine (IBMX), Insulin (powder), phosphate buffered saline (PBS) (0.01 M, pH 7.4), SYBR Green JumpStart Tag Ready Mix, and trypan blue solution (0.4 %) were obtained from Sigma Chemical Corporation (Saint Louis, USA). Sodium chloride, isopropyl alcohol, ethanol (95 %) and formaldehyde were purchased from Promega Corporation (Madison, USA). Triton X-100 and Sudan IV (25 g) were the products of Sigma-Aldrich GmbH (Germany). Dulbecco's Modified Eagle's Medium (DMEM) (high glucose), DMEM (low glucose), trypsin (0.25 %), bovine serum albumin (BSA) (pH 7.0) were purchased from PAA Laboratories GmbH (Austria). Fetal bovine serum (FBS) and ampicilin/streptomycin (5000Units/5000 µg/ml) were supplied by Biochrom AG (Berlin). Dispase II and collagenase B were the products of Roche, Molecular (Basal, Switzerland). Secondary antibody (Goat anti-Guinea Pig monoclonal antibody) and trizol (100 ml) were obtained from Invitrogen (USA). 3-[N-morpholine] propane sulfonic acid (MOPS) was purchased by Ambion (USA). Dimethyl sulfoxide (DMSO) was supplied by Applichem GmbH (Germany). 4',6-Diamidino-2-phenyindole (DAPI) was obtained from Biochemica-Fluka chem.GmbH (Buchs, Switzerland). Mounting solution was the product of Dako (Denmark). Perilipin, guinea pig polyclonal primary antibody, was purchased by Progen Biotechnik GmbH, (Heidelberg, Germany). Improm II reverse transcriptase was supplied by Promega Corporation (USA). Hematoxyline was obtained from Santa Cruz Biotechnology (Santa Cruz, USA).

2.2. Methods

2.2.1. Cell Culture

Isolated cells were fed with DMEM (low glucose), 10 % fetal bovine serum (FCS), 1 % antibiotic-antimycotic (Penicillin + Streptomycin + Amphotericin B), and 20 μM L-glutamine and cultured at 37 °C, in 5 % CO_2 and 95 % air atmosphere in a carbon dioxide incubator. The cells were grown to 70-80 % confluency and splitted in a ratio of 1:3 with 0.1 % trypsin-EDTA by incubating at 37°C for 5 minutes. Cells up to 10^{th} passage were used in the culture studies.

2.2.1.1. Mesenchymal Stem Cell Culture

2.2.1.1.1 Isolation and Expansion of MSC from Bone Marrow of Donors

Approximately 1-3 ml of bone marrow was supplied by Hacettepe University Bone Marrow Transplantation Unit. The bone marrow of healthy bone marrow donors was taken under operating room condition following their written informed consent. The mononuclear cells were separated from bone marrow using density gradient centrifuge method. Bone marrow was diluted by phosphate buffered saline (PBS) (0.01 M, pH 7.2) and subjected to Ficol gradient with density of 1,077 g/ml and centrifuged at 2000 rpm for 15 minutes in a centrifuge (Hettich Zentrifugen, EBA 20, Germany). Accumulated cells were washed with PBS twice and viable cells were counted using trypan blue dye (0.4 %). About $2x10^6$ of these accumulated cells were seeded in $25cm^2$ flasks and fed with DMEM-LG, 10 % FBS, 1 % Penicillin-stereptomycin, and 1 % L-glutamine. Culture medium was changed every 3-4 days.

2.2.1.2 Preadipocyte Cell Culture

2.2.1.2.1. Isolation and Expansion of Preadipocytes from Subcutaneous Adipose Tissue

Subcutaneous fat tissue was supplied by Hacettepe University Department of Medical Biology DNA / Tissue and Cell bank. Tissue was washed in PBS containing 1 % FBS and 3 % antibiotic-antimycotic solutions and minced on ice to 1-2 mm^3 sized particles. These particles were incubated in PBS (0.01 M, pH 7.4) containing 1 % final w/v of Collagenase B and Dispase II and filtered through 70 nanometer and 30 nanometer nylon mesh filters to remove debris and undigested connective tissue. The rest were plated on 25 cm^2 flask and cultured with growth medium (DMEM, 10 % FBS, 1 % antibiotic-antimycotic, 20 μM L-glutamine). For the pilot experiment the preadipocytes were isolated from the rat inguinal fat pad tissues by the same method.

2.2.1.3. In-Vitro Three-Dimensional (3-D) Fat Tissue Model

Low gelling temperature agarose (type VII) was used for embedding the cells in a three-dimensional structure. 4 % (w/v) agarose in phosphate buffered saline (PBS) (0.01 M, pH 7.4) was prepared and autoclaved (ALP CL-40M, Labomoar, Turkey) at 121 °C for 20 min. The cells of our interest were detached from the tissue flasks by incubating with 0.1 % trypsin-EDTA for 5 minutes at 37°C. The cell suspension was centrifuged for 5 min at 1500 rpm, and pellet was resuspended in growth medium consisting of DMEM with 20 mM L-glutamine supplemented with 10 % FBS and 1 % antibiotics (ampicilin and streptomycin). Trypan blue dye was used to count the viable cells. Approximately 5 x 10^6 cells/ml was mixed with an equal volume of 4 % agarose at 38°C in a laminar flow cabinet and pipetted into the mold in blocks as shown in Figure 2.1. Agarose was gelled after 5-10 minutes of waiting at room temperature. 24 agarose discs with 3 mm in height and 5 mm in diameter were prepared for two different cell types; preadipocytes and mesenchymal stem cells. Agarose-cell discs were placed

in 24-well plates and cultured in the growth media for 7 days. Discs were switched to the adipogenic differentiation medium and kept for 21 days.

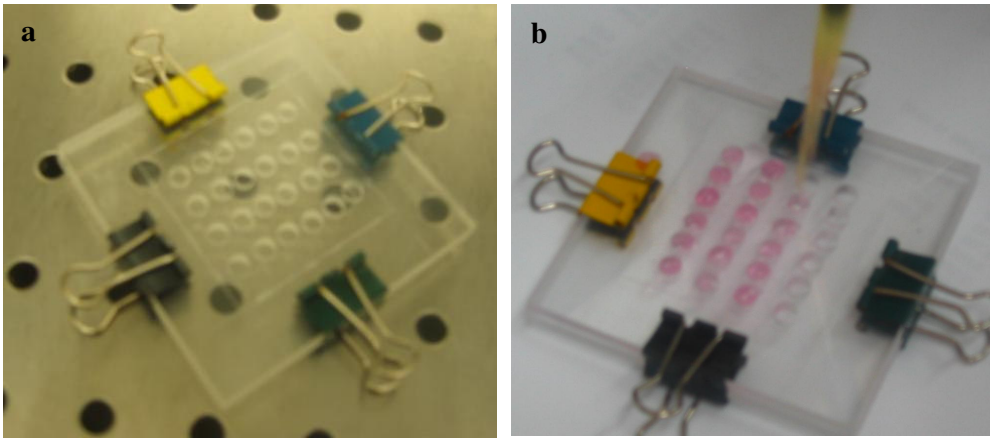

Figure 2.1. Stages in the preparation of agarose discs with preadipocytes and mesenchymal stem cells, a) concatenation of two template plates b) pouring the agarose at 38°C by the help of a micropipette into the moulds between blocks.

The discs were subjected to mechanical forces using weights made of Teflon. The weight of these cylindrical structures varied according to their dimensions. The weight of samples with dimensions 15 mm (diameter) x 1 mm (height), and 15 mm x 18 mm were 1.5 g and 7.5 g, respectively. Each agarose- cell disc was placed in a single well and filled with differentiation medium. Six discs were selected as control group and no external force was applied. Second group of six discs was subjected to 1.4 ± 0.2 g weight. 6 discs of the third group, weights of 7.5 ± 0.2 g were applied. The last six discs were exposed to a weight of 14.6 ± 0.3 g. The agarose discs were fed with adipogenic differentiation medium consisting of DMEM low glucose with L-glutamine, 4 % FBS, 1 % antibiotics, 0.33 % IBMX, 1 % insulin, and 0.01 % dexamethasone every day. After 21 days of differentiation, agarose discs were harvested. From each group, two discs were

placed in 500 µl TRIZOL® for RNA isolation, and two discs were reserved for staining.

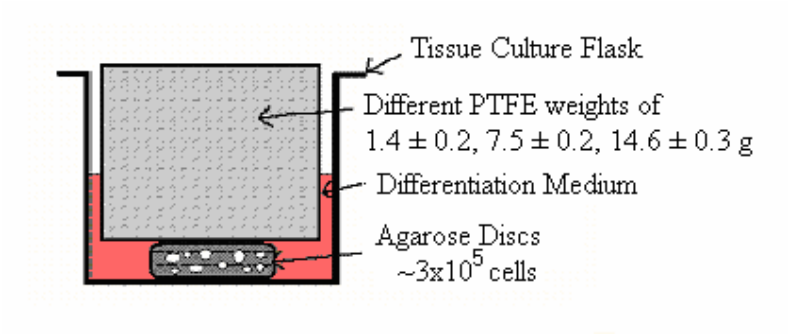

Figure 2.2. In vitro model used for studyng the effect of mechanical forces on adipogenic differentiation of preaipocytes and mesenchymal stem cells using PTFE cylinders withdifferent weights

2.2.1.4. Investigation of Initial Mechanical Properties of Agarose and Agarose-Cell Discs

Initial mechanical properties of the agarose discs (with and without cells) were studied by a computer-controlled testing device (LLOYD LS 500, UK) equipped with Nexygen MT Software Version 4.5 (Ametek Inc.,UK). A 10 N load cell was used to perform unconfined compression tests on 3 groups of agarose discs; empty, agarose-preadipocytes, agarose-MSCs. The discs were prepared as described in section 2.2.1.3 just before application of the stress-relaxation test. All tests were carried out between two rigid-impermeable plates at room temperature. Before each test, free swelling disc thickness and diameters were measured via digital caliper (TJ1006, China). Discs in PBS were first subjected to unconfined stress-relaxation test with a ramp speed of 3 mm/min until reaching 10 % of the free swelling thickness of the discs. Tests were completed after the discs relaxed to equilibrium (600 sec). The equilibrium unconfined compression (Young's) modulus (E_Y) (equilibrium aggregate modulus) was calculated from the equilibrium stress divided by applied strain.

Figure 2.3. The set up used for stress-relaxation tests.

2.2.2. Investigation of the Effect of Mechanical Forces on MSC and Preadipocytes

2.2.2.1. Staining

Agarose discs were frozen in liquid nitrogen and cryosections of 15 µm thickness were taken using a cryostat (Leica 1900, Germany). The cryosections were mounted on SuperFrost microscope slides. Two methods were used for staining the slides; histochemical staining and immunostaining.

2.2.2.1.1. Histochemical Staining

The sections were fixed with 4 % paraformaldehyde solution for 30 minutes after air drying in room temperature. The intracellular unilocular and multilocular lipids were stained with Red Oil O (Sudan IV) with a counterstaining of hemotoxylene (100 ml). Same protocol was applied to identical agarose discs without cryosections to get a three dimensional (3D) image. The nuclei of cells in agarose discs were fluorescently stained with 4', 6-Diamidino-2-phenyindole, dilactate (DAPI).

2.2.2.1.2. Immunostaining

The sections were fixed by 4 % paraformaldehyde, washed with phosphate buffered saline (PBS) containing 0.1 % Triton X-100 blocking buffer containing PBS with 0.1 % Triton X-100 and 10 % BSA (Bovine serum Albumin) was used to block unoccupied protein binding sites. Guinea pig polyclonal antibody was used as a primary antibody against perilipin. The antibody was diluted in blocking buffer in 1:150 ratios. The sections were incubated within primary antibody at 4°C overnight. After washing with PBS (0.1 % Triton X-100) for three times, cryosections were stained with secondary antibody, Goat anti-Guinea Pig monoclonal antibody for 1 hour at room temperature. Nuclei of the cells were stained with 1:10,000 diluted DAPI. After final washing with PBS (0.1 % Triton X-100) the sections were mounted by Dako fluorescent mounting medium and the coverslips were sealed. The slides were stored in 4°C until analyzed with fluorescent microscopy (Leica DM-IL inverted fluorescent microscope) and images of interest were captured via Leica DFC490 Camera (Leica, Germany) and visualized on Leica Application Suit software.

2.2.2.2. Gene Expression at mRNA Level

2.2.2.2.1. Isolation of RNA from Agarose Gel Discs

Agarose discs in Trizol reagent were homogenized on a bead-beater (FastPrep® System). Homogenized samples were incubated in room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 0.1 ml of chloroform was added to each tube, shaken vigorously by hand and incubated at room temperature for 2-3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. Following the centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase (RNA remains exclusively in the aqueous phase). The aqueous phase was transferred to a fresh tube, precipitated the RNA from the aqueous phase by mixing with equal volume of isopropyl alcohol at room temperature for 30 minutes. Samples were centrifuged at 12,000 x g for 10 minutes at 4 °C. After

removal of the supernatant, the RNA pellet was washed once with 75 % ethanol. The samples were centrifuged at 7,500 x g for 5 minutes at 4 °C. The RNA pellet was dried briefly for 5 to 10 minutes after removing of supernatant. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubated for 10 minutes at 55 to 60 °C.

2.2.2.2.2. RNA Quality Control

The quality of the extracted total RNA has been assessed via measurement of optical densities (OD) at 230, 260 and 280 nm using NanoDrop (ND-1000). RNA concentrations were calculated using absorbance at A_{260} nm with the following equation:

$$[C] = A_{260} \, x \, RNA \; constant \; (40)$$

A_{230} and A_{280} measurements were used to assess the RNA purity and to assure that the samples are devoid of contaminants such as phenol, protein or salts.

The integrity and the quality of the total RNA had been assessed via denaturing gel electrophoresis on 1 % agarose gel containing 37 % formaldehyde and 3-[N-Morpholino] propanesulfonic acid (MOPS). The visualization of 18S and 28S ribosomal RNA provides information on the integrity of the isolated total RNA. The gel electrophoresis has been documented using BioRad Chemidoc XRS system (Hercules, USA).

2.2.2.2.3. Reverse Transcriptase and cDNA Conversion

1 µg of isolated RNA was used for synthesis of cDNA via incubating at 37 ºC using 0.5 µg random hexamer oligonucleotide primers and RNAse H(+) reverse transcriptase enzyme Improm II. The volume of cDNA's were completed to 80 µl with dH_2O and reserved in -20 °C.

2.2.2.2.4. Quantitative PCR

The cDNAs had been subjected to semi-quantitative PCR analysis for the genes PPARγ, hSCD, Leptin and CEBPβ. The gene of interest had been amplified from 2 µl of cDNA using gene specific primers via SYBR-green technique. Serial dilutions from a randomly selected sample were utilized to establish the regression curve to interpolate relative expressions of the samples using ΔCt method. A special hot-start polymerase mix had been utilized to avoid non-specific mispriming at lower temperatures.

The mix for PCR reaction was composed of 5 µl of 2x SYBR green mix (S5193 Sigma, St Louis, USA), 1.2 µl of 25 mM $MgCl_2$, 0.4 µl of both 10 µM reverse and forward primers, 1.5 µl of cDNA and 1.5 µl dH_2O.

The PCR condition was programmed as 2 minutes at 94 °C for initial denaturation, 2 seconds at 94 °C for denaturation, and 15 seconds at 60°C for annealing and extension.

The denaturation and annealing-extension cycles have been repeated 40 times and the SYBR green incorporation at each cycle has been detected on fluorometric quantitative thermal cycler Rotorgene 6000 (Corbett scientific, Brisbane, Australia).

The expressions of the gene of interest had been normalized to the expression of Tata-Box Binding Protein (TBP/TFIID) which was used as the normalizator house keeping gene.

2.2.3 Statistical Analysis

Data were analyzed using One-Way ANOVA and Tukey's Multiple Comparison Tests for the post-hoc pairwise comparisons for compression test results and pair-wise Student's T test for gene expression results using SPSS-9 Software (SPSS Inc., USA). Differences were considered significant at $p \leq 0.05$ level.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cell Culture

Mesenchymal stem cells, isolated from the bone marrow of healthy donors at Hacettepe University Faculty of Medicine Bone Marrow Transplantation Unit were passaged up to 9th passage. MSCs preserved their fibroblast like morphology throughout the 10 passages. However, following the 10th passage they began to divide slower than the primary culture and exhibited a larger morphology. These changes were in accordance with the observations of Bonab et al (2006).

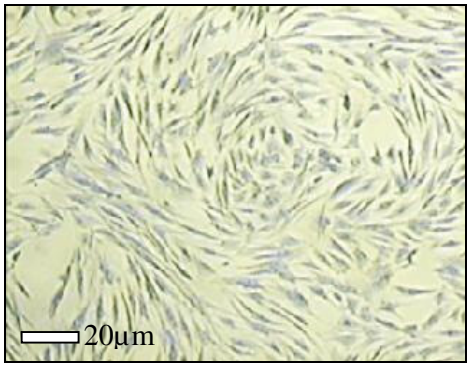

Figure 3.1. Freshly isolated human mesenchymal stem cells in tissue culture flask grown to confluency.

Preadipocyte cells were cultured in growth media and their morphology was observed under the phase contrast microscope (Figures 3.1-3.2) and compared with those presented in the literature. Proliferating preadipocytes exhibited fibroblast-like morphology and grew as a monolayer.

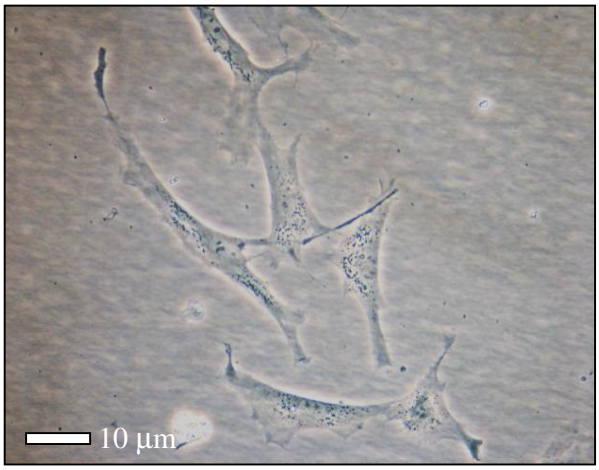

Figure 3.2. Freshly isolated primary human preadipocytes in tissue culture flask displaying fibroblast-like morphology.

The initial preadipocyte morphology and behavior were similar to fibroblasts. However, as the number of passages increased this property has changed. It seemed that preadipocytes eventually lost their contact inhibition characteristic following the 9^{th} passage. Hence, only the cells that are in the early passages (up to 6^{th} passage) were used in the study.

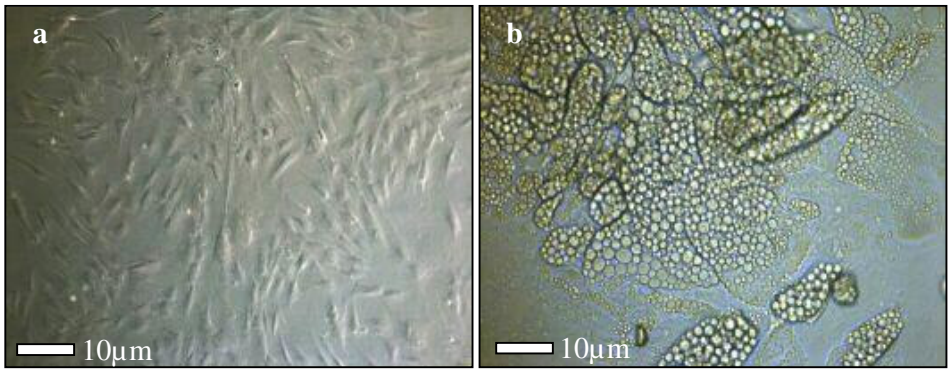

Figure 3.3. Phase-contrast micrographs of (a) undifferentiatied primary preadipocytes, (b) young adipocyte exhibiting phenotype with multilocular lipid vesicles after differentiation for 21 days.

As the sign of adipogenesis, young adipocytes gained round morphology as shown in Figure 3.3. In fact, the change to round morphology during adipogenesis has been shown to be accompanied by change in cytoskeletal organization and contacts with the ECM (Kawaguchi, 2003) in which fibronectin, integrin, actin and other cytoskeletal proteins are downregulated (Fernandez and Ben-Ze'ev, 1989; Spiegelman and Farmer, 1982). Concomitant with the onset of adipogenic differentiation, the fibronectin-rich ECM are degraded and actin cytoskeleton undergoes reorganization and the cell shape change (Cornelius et al., 1994). Moreover, the actin stress fiber networks formed in preadipocytes before differentiation also disappears and reorganizes into cortical actin networks (Kawaguchi, 2003).

Different types of scaffolds (i.e. PLGA, PGA, PLA, fibrin, matrigel and collagen) have been reported in the literature for growing adipocytes (Baehm, E.K. 2003, Mauney, J.R 2007). The polymeric scaffolds such as PLGA and PLA provide a rigid support mimicking the trabecula of the bone, thus stimulating the mesenchymal stem cells (Imbronito et al., 2005, Abukawa et al., 2004). On the other hand, agarose discs were suggested to provide a better supportive model to

mimic the fat tissue stroma which is composed of mainly type-I collagen fibers formed by fibroblasts (Neville M. C, 1998).

In this study, agarose was chosen to mimic the physiological tissue architecture for its viscoelastic nature similar to the fat connective tissue environment. Its compressible nature, biocompatibility and mechanical properties make it possible to study the effects of mechanical stress on cells (Mauck et al, 2000, Awad, 2004).

The behavior of cells in agarose discs was observed for several days before applying mechanical forces (compression under known weight). Also, preadipocytes harvested from rat inguinal fat pads have been tested in the pilot experiments for their faster ability to differentiate into monolocular mature adipocytes (Daya et al., 2007). In the preliminary experiments conducted with rat preadipocytes (Figure 3.4), it was observed that the mature monolocular adipocyte phenotype could easily be achieved in agarose discs (Shillabeer and Lau, 1994). This may be natural consequence of the rodent adipocytes as they also have tendency to accumulate fat even without induction with PPAR agonists such as the IBMX, dexamethasone and insulin cocktail used in this study.

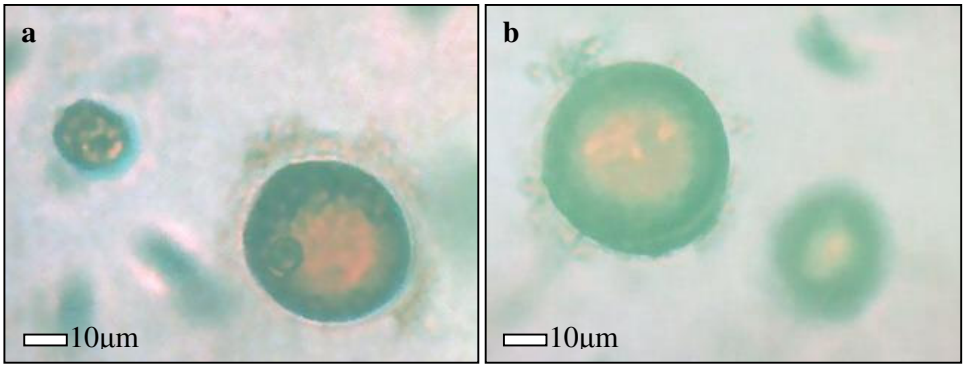

Figure 3.4. Rat preadipocytes embedded in agarose and subjected to adipogenic differentiation for 21 days exhibiting monolocular mature adipocyte phenotype. (Sudan IV staining).

Apparently, cells (both preadipocytes and MSCs) showed tendency to escape from the agarose discs when kept in growth medium. However, after replacing the growth medium with differentiation medium, cells stopped proliferation and mobility. Thus, they stayed in the discs and began to differentiate. The undifferentiated cells in agarose discs were shown in Figure 3.5.

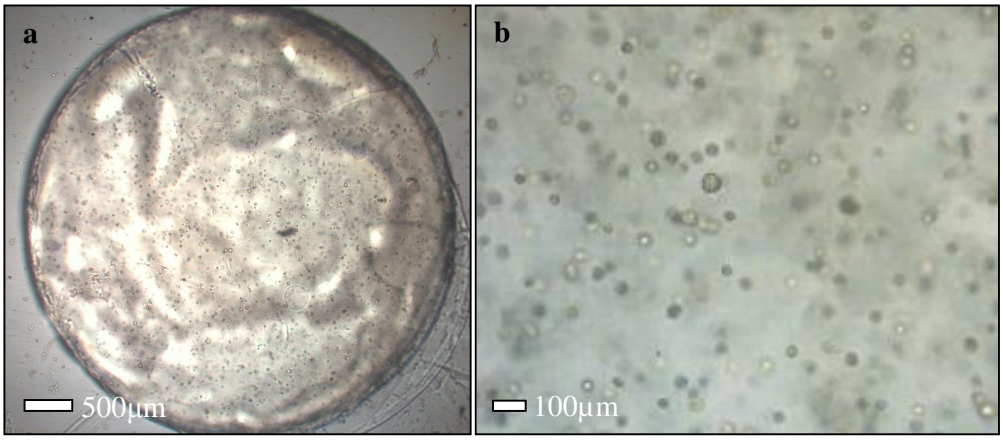

Figure 3.5. Undifferentiated preadipocyes a) in agarose discs and b) in growth medium.

3.2. Differentiation of Preadipocytes and Mesenchymal Stem Cells in Agarose Discs - Proof of Concept

In order to investigate and document that the adipogenic differentiation occurs as expected in agarose discs, preadipocytes and mesenchymal stem cells have been cultured in adipogenic differentiation medium for 21 days without static compression. The differentiation of these cells have been investigated at the morphological level by sudan IV staining and at the gene expression level via RNA isolation and quantitative PCR on adipogenic differentiation related transcripts.

3.2.1. Cellular Morphology

On the course of 21 days of differentiation, adipogenic lipid droplets have started to form after 10 days in tissue culture. These multilocular fat droplets increased in size over days and also exhibited fusion with each other. However, it was observed that the occurrence of these lipid droplets were both delayed and their sizes were smaller than their counterparts grown to confluency in tissue culture flasks. One explanation for such difference would be the necessity of confluency for a successful adipogenic commitment (Rosen and Spiegelman, 2000). Initially, only 10^5 cells were embedded in agarose discs. The cells were lacking confluency and contact stimuli. In order to overcome this, cells embedded in agarose discs were kept in growth medium for 7 days before switching to the differentiation medium. But still many of the cells were observed to be in the resting state (Wilson and Trumpp, 2006) rather than proliferation.

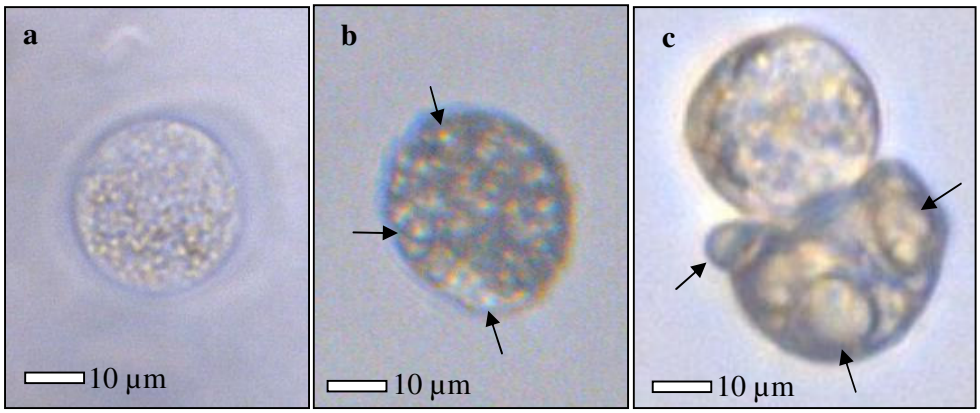

Figure 3.6. Preadipocytes embedded in agarose discs accumulating lipid granules (arrows) on the course of differentiation; a) day 1, b) day 8, c) day 12.

When the preadipocytes and MSC cells were embedded in the agarose gels, they became round in shape (Figures 3.6-3.7). However, this change to round morphology was observed when the cells were embedded in the agarose gel

before the completion of differentiation. These observations pointed out that the agarose polymer scaffold may suppress proliferation of the cells probably evoking a stimulus similar to cell-cell contact inhibition. However, this signaling pathway is not the same as sensing the confluency as the cells do not express the same differentiation kinetics in the culture flask. The gene expression quantitation experiments provide a better insight into this speculation. Preadipocytes embedded in type I collagen or fibronectin gels have been shown to provide a better matrix material and enhance differentiation in vitro (Daya et al., 2007). Thus, the agarose matrix alone may not provide the best environmental signaling for the differentiation of the preadipocytes or MSC, but the gene expression studies pointed out that the induced differentiation program can be quantitated and observed at the mRNA level.

As explained in Section 3.2, change to round morphology occurs during adipogenesis as a result of the degradation and reorganization of the cytoskeleton. Different mechanical signals at the cellular scale can produce different cellular behavior as well as variation in biochemical composition. Thus, these changes result in different cell binding and signaling within the agarose gel matrix (Hunter, 2002). This has been observed in studies on cartilage (Murata et al., 2003) in which static mechanical compression modulates some receptors, perhaps relocating them or influencing their gene expression. So, the postulate may also be valid for the disruption of the adipogenic differentiation observed in this study.

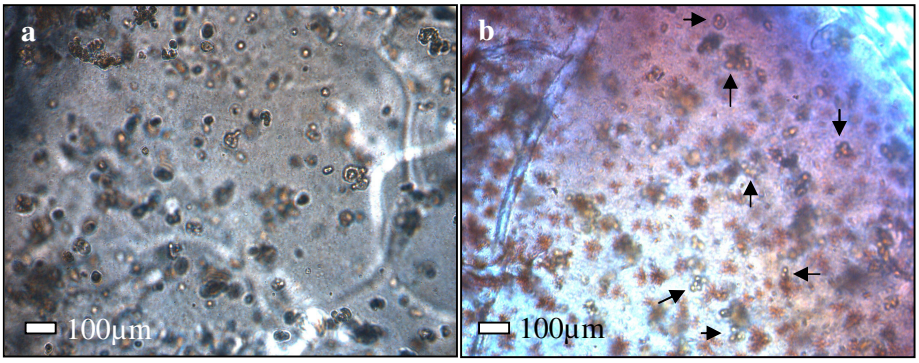

Figure 3.7. MSCs embedded in agarose discs on the course of differentiation a) day 1, b) day 2, arrows show the accumulating lipid granules.

3.2.2. Adipogenic Differentiation at the Gene Expression Level

Six agarose discs, each carrying about 10^5 preadipocytes were prepared. Three discs were subjected to adipogenic differentiation at the end of the 7^{th} day. Same number of cells was plated in 6 well plates and was subjected to adipogenic differentiation following the 7^{th} day. After 21 days of adipogenic differentiation, total RNA was isolated and subjected to reverse transcription. The relative expressions of SCD, PPARγ, ADFP and GPDH have been investigated via quantitative rt-PCR. The results have indicated that for the preadipocytes differentiated in agarose discs, expressions of SCD, PPARγ and GDPH were induced compared to their counterparts that have been kept in proliferation medium for 21 days (Figure 3.8). Quantitative gene expression study revealed that SCD expression was induced 3.41 folds compared to the controls satisfying the Student's T-test with a p value smaller that 0.01. Similarly, PPARγ and GPDH expressions were observed to be upregulated 4.91 and 1.5 folds, respectively, providing statistically significant induction ($p \leq 0.01$ for PPARγ and $p \leq 0.05$ for GPDH). However, ADFP upregulation was only 1.35 folds and not satisfying the T-test cut off.

The observed induction of the above mentioned adipogenic transcript was almost 10 times greater for adipocytes that have been differentiated in cell culture flask.

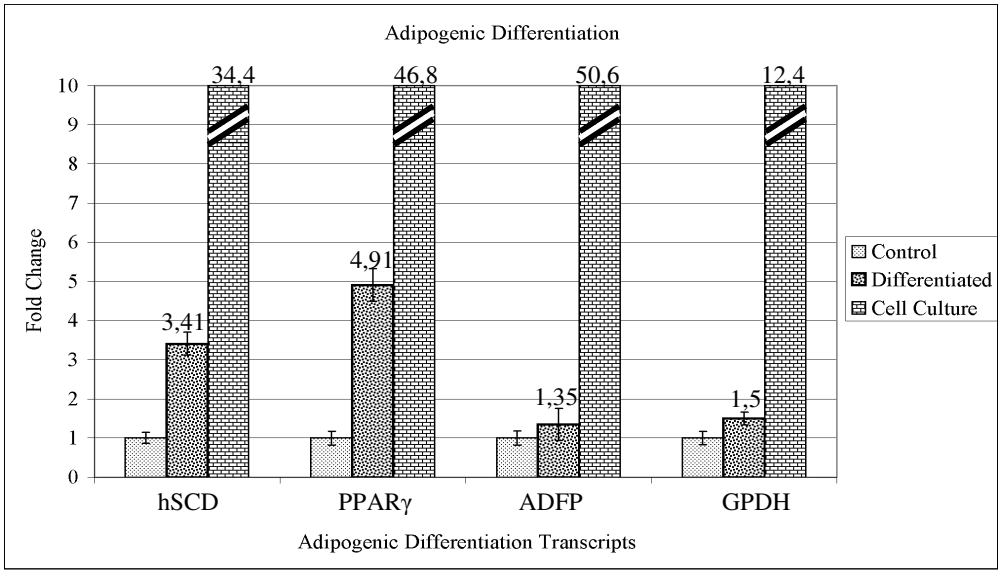

Figure 3.8. The relative expression values of four adipogenic transcripts of preadipocytes differentiated in agarose discs and in cell culture flasks using semi-quantitative rt-PCR and normalized to the undifferentiated controls in agarose discs.

These results indicated that even though the adipogenic differentiation of the preadipocytes was at a quite quantifiable level for the gene expression, these values were much lower than their counterparts that were differentiated in classical cell culture conditions. Thus, it pointed out the fact that the preadipocytes did not like differentiation in agarose discs.

3.3. Evaluation of the Effects of Mechanical Forces on Cell Differentiation

In this study, different weights (1.4 ± 0.2 g, 7.4 ± 0.2 g, and 14.6 ± 0.3 g) were placed on agarose-cell discs. The discs were subjected to continuous static compression for 21 days. The effect of mechanical compressive stress on adipogenic differentiation has been investigated. First, the morphological changes of the differentiating cells have been observed and documented. Then, the major transcripts of the adipogenic differentiation program have been investigated by total RNA isolation and quantitative PCR analysis. Finally, the expression of perilipin protein, the major structural component of the cytoplasmic fat vesicle, has been investigated by cryosectioning of the blocks and immunostaining.

3.3.1. Evaluation of the Effects of Mechanical Forces on Differentiation of Human Mesenchymal Stem Cells Embedded in Agarose Discs

Human mesenchymal stem cells were embedded in agarose discs (10^5 cells each) and kept in growth medium for seven days and subjected to adipogenic differentiation and compression under different weights (1.4 ± 0.2 g, 7.5 ± 0.2 g, and 14.6 ± 0.3 g). Six agarose-MSC discs were cultured in differentiation medium and were not subjected to compression to be used as no-weight (no-compression) control.

After 21 days of differentiation under continuous static compression, three discs were subjected to RNA extraction and quantitative expression analysis while three agarose discs were spared for morphological analysis via sudan IV staining.

3.3.1.1. Morphological Results

The agarose-cell discs under no static compression preserved their original structure and morphology throughout the 21 days of observation period. Likely, the agarose discs to which 1.4 ± 0.2 g weights were applied did not loose their original dimensions. On the contrary, the agarose discs that were compressed with 7.5 ± 0.2 g weights, exhibited a contraction in their vertical dimensions; i.e. a decrease from 3 mm to 1.5 mm was observed in their heights. Similarly, in the

discs treated with 14.6 ± 0.3 g weights, the thickness had regressed to less than 1 mm. The latter group also exhibited cracking and shattering in some of the discs.

MSCs in agarose discs without any weight treatment exhibited differentiation profoundly by deposition of multiple lipid droplets following Sudan IV staining as indicated in Figure 3.9. Compression of the agarose discs with different weights has affected the accumulation of lipid droplets in a negative way. Thus, progressively less and smaller sized lipid droplets were observed in differentiated MSCs that were subjected to compression under1.4 ± 0.2 g and 7.5 ± 0.2 g weights within agarose discs. The most significant loss in lipid droplet amount and size was observed in MSCs differentiated under 14.6 ± 0.3 g weights (Figure 3.9.d). Therefore, a negative correlation between lipid droplet accumulation, droplet size and mechanical pressure exposure might be suggested from the morphological signs of differentiation.

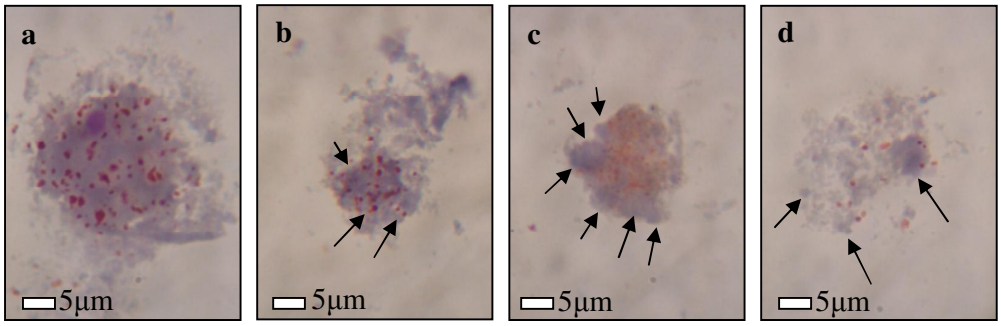

Figure 3.9. Light microscopy images of general morphology and differentiation of MSCs for 21 days in agarose discs compressed under a) no weight b) 1.4 ± 0.2 g weight c) 7.5 ± 0.2 g weight d) 14.6 ± 0.3 g weight as investigated by sudan IV staining and counter-staining with hematoxylin. Red points indicate sudan IV stained lipid droplets and arrows indicate the nuclei.

3.3.1.2. Quantitative Gene Expression Analysis

Total RNA was extracted from three agarose discs of each group which have been subjected to compression under different weights and 2 μg of RNA was reverse transcribed to obtain cDNA. Quantitative expression of adipogenic transcripts was investigated via real-time PCR using the SYBR Green technique. The results were shown in Appendix A. Each expression study has been carried out in triplicates and the average values were calculated.

The quantitative gene expression analysis results showed a remarkable decrease in transcription of all tested adipogenic transcription factors (Figures 3.10-3.12). These results indicated that the adipogenic differentiation was impaired at three different levels; i) the master transcriptional regulators of adipogenic differentiations achieved by the expression of C/EBPβ and PPARγ. Their expression exhibited a negative correlation with the amount of applied mechanical pressure (Figure 3.10 a and b), ii) the expression of the structural component of the lipid granules (ADFP) and biochemical enzyme, hSCD involved in long chain fatty acid biosynthesis were impaired (Figure 3.11 a and b), iii) The two adipokines (leptin and adiponectin) which are expressed as a function of white adipose tissue were also down-regulated as inversely proportional to the amount of the applied pressure (Figure 3.12 a and b).

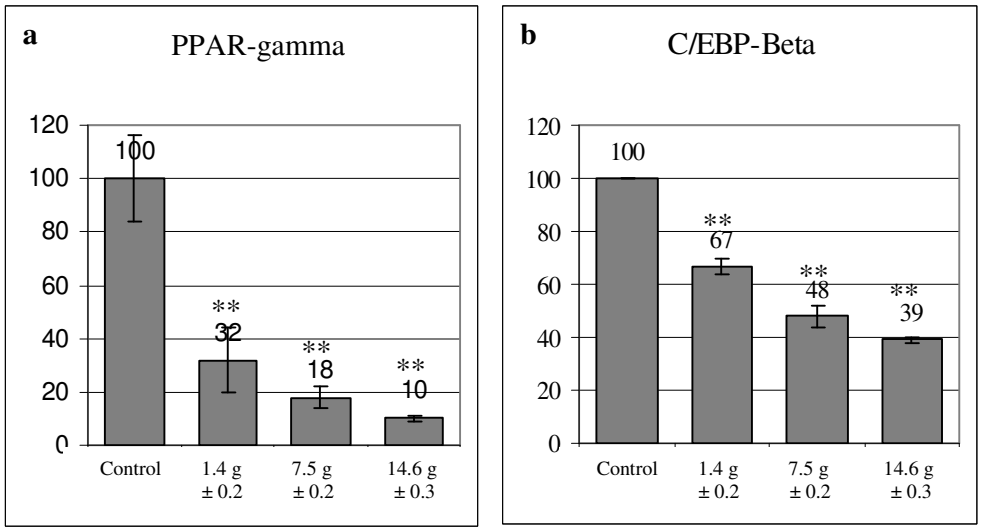

Figure 3.10. Expression of adipogenic transcription factors a) PPARγ, b) C/EBP-β in MSCs embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

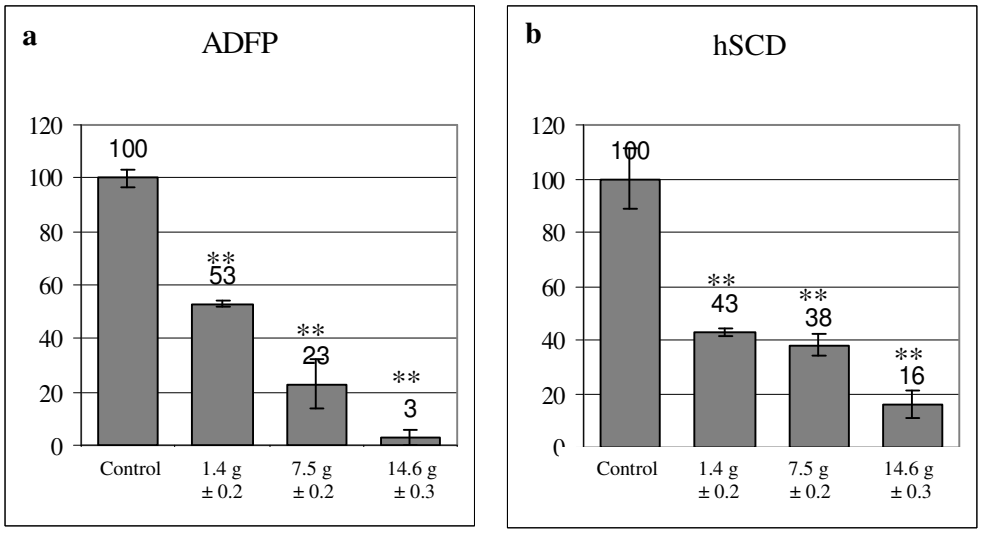

Figure 3.11. Expression of a) adipogenic structural protein, ADFP, b) lipogenic enzyme hSCD in MSCs embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

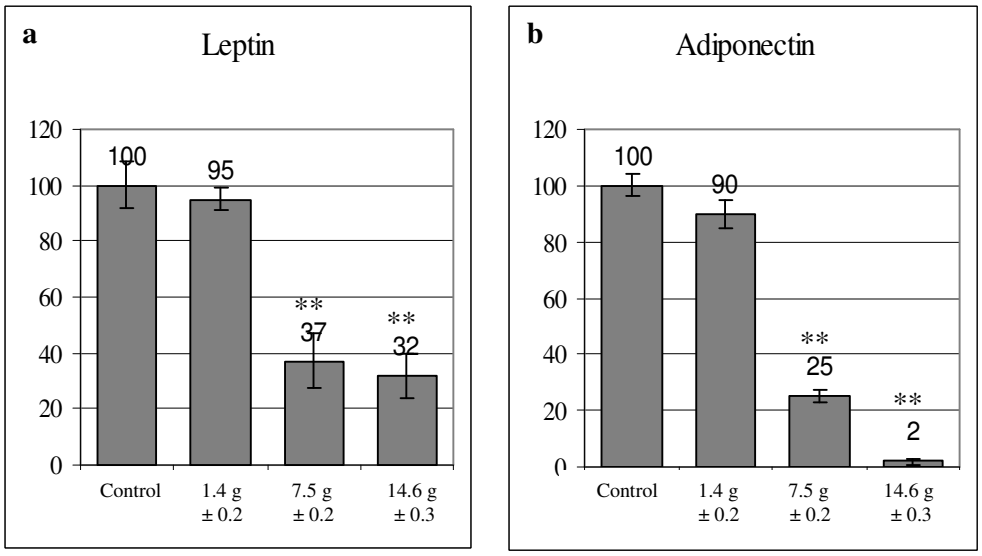

Figure 3.12. Expression of WAT specific adipokines a) leptin, b) adiponectin in MSCs embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

The two differentiation factors PPARγ and C/EBPβ that control adipogenesis displayed inversely proportional expression with regard to the weight applied on the agarose discs with MSCs (Figures 3.10-3.12). The expression of PPARγ was induced only up to 32 % (1.4 ± 0.2 g), 18 % (7.5 ± 0.2 g) and 10 % (14.6 ± 0.3 g) of the controls at the end of 21 days of adipogenic differentiation. Likewise, C/EBPβ expression was reduced to 67 % (1.4 ± 0.2 g), 48 % (7.5 ± 0.2 g) and 39 % (14.6 ± 0.3 g) of the controls. These results clearly indicated that the application of weight (mechanical stress) on preadipocytes impaired the differentiation cascade in a negative way. As a result of the negative effect on the

adipogenic master regulators, other functions of the adipogenic differentiation were also observed to be disrupted. ADFP, the structural component of the lipid vesicle was also downregulated to 53 % (1.4 ± 0.2 g), 23 % (7.5 ± 0.2 g) and 3 % (14.6 ± 0.3 g) of the controls (Figure 3.11a). hSCD, the key rate limiting step enzyme of the fatty acid biosynthesis cascade was also impaired similarly; downregulated to 43 % (1.4 ± 0.2 g), 38 % (7.5 ± 0.2 g), and 16 % (14.6 ± 0.3 g) compared to control (Figure 3.11b).

The endocrine function of the white adipocytes was investigated via the expression of the two main polypeptide adipokines, leptin and adiponectin. The production of these two adipokines is the most sophisticated function of the adipocyte phenotype. The endocrine function of the MSCs were also impaired at the gene expression level via reduction of the leptin expression to 95 % (1.4 ± 0.2 g), 37 % (7.5 ± 0.2 g) and 32 % (14.6 ± 0.3 g) compared to the controls. The expression of the adiponectin was reduced to 90 % (1.4 ± 0.2 g), 25 % (7.5 ± 0.2 g) and 2 % (14.6 ± 0.3 g) compared to the controls (Figure 3.12b). Apparently, the endocrine function of the MSCs was least affected with the application of 1.4 ± 0.2 g of weight but the adiponectin expression was almost shut-off (2 %) under 14.6 ± 0.3 g of weight.

3.3.2. Evaluation of the Effects of Mechanical Forces on Differentiation of Human Preadipocytes Embedded in Agarose Discs

Human preadipocytes obtained from the human subcutaneous tissue were embedded in agarose discs like the MSCs and kept in growth medium for 7 days. Following this, the agarose-preadipocyte cell discs were subjected to static compression under 1.4 ± 0.2 g, 7.5 ± 0.2 g and 14.6 ± 0.3 g weights for 21 days of the adipogenic differentiation period. Six discs were kept in differentiation medium with no compression to serve as control samples. The preadipocytes have been treated equally and handled together with the MSCs that have been discussed earlier. The results of preadipocyte differentiation also provided similar results with those of MSCs.

3.3.2.1. Morphological Results

The agarose discs under free swelling condition (not subjected to static compression under weight) kept their structure and gross morphology throughout the observed differentiation period. No changes in dimensions and general appearance were observed for the agarose-cell discs under 1.4 ± 0.2 g weight. Similar to the results obtained from the MSC embedded agarose discs, the agarose-preadipocyte discs subjected to compression under 7.5 ± 0.2 or 14.6 ± 0.3 g weights have exhibited reduction in their thickness.

By the microscopic examination, preadipocytes differentiated in agarose discs where no weights had been applied, even after 21 days lipid vesicles were small and their morphology was far from the observed lipid droplets of the preadipocytes differentiated in tissue culture flasks. The observed lipid droplets were similar in no-weight and under 1.4 ± 0.2 g weight agarose-preadipocyte cell discs. However, they were smaller in size and less in numbers in agarose-preadipocyte discs compressed with 7.5 ± 0.2 g weight. No lipid droplets were visible in preadipocytes under 14.6 ± 0.3 g weight (Figure 3.13d).

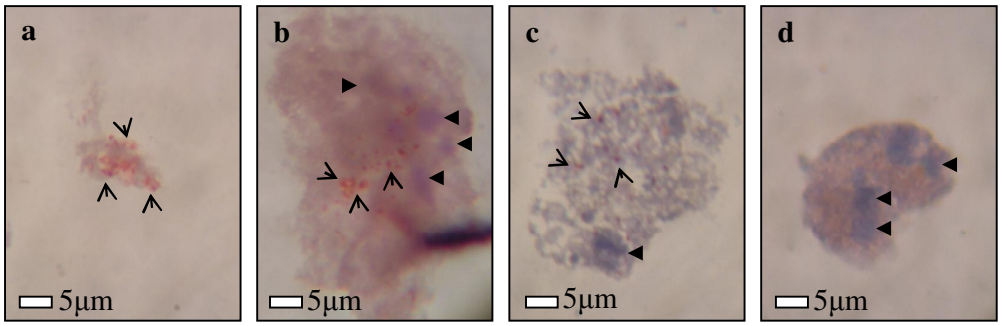

Figure 3.13. Light microscopy images of general morphology and differentiation of preadipocytes for 21 days in agarose discs compressed under a) no weight b) 1.4 ± 0.2 g weight. c) 7.5 ± 0.2 g weight. d) 14.6 ± 0.3 g weight as investigated by Sudan IV staining and counter-staining with hematoxyline. Arrowheads indicate Sudan IV stained lipid droplets and filled arrowheads indicate the nuclei.

3.3.2.2. Quantitative Gene Expression Analysis

Total RNA was extracted from three agarose discs of each group which have been subjected to compression under different weights. 2 μg of RNA was reverse transcribed to obtain cDNA. Quantitative expression of adipogenic transcripts was investigated via real-time PCR using the SYBR Green technique. The results were shown in Appendix B. Each expression study has been triplicated and the average values were calculated as well as the standard deviations of the biological triplicates. The quantitative gene expression values for each group which has been subjected to compression under weight were normalized to the control samples. The variations between each treatment group versus the control group have also been tested for statistical significance using pair-wise Student's T test and indicated where applicable.

The two differentiation factors PPARγ and C/EBPβ that control adipogenesis displayed inversely proportional expression with regard to the weight applied on the agarose discs (Figure 3.14). The expression of PPARγ was induced only up to 78 % (1.4 ± 0.2 g), 68 % (7.5 ± 0.2 g) and 1 % (14.6 ± 0.3 g) of the controls at the end of the 21 days of adipogenic differentiation. Likewise, C/EBPβ expression was reduced to 70 % (1.4 ± 0.2 g), 40 % (7.5 ± 0.2 g) and 3 % (14.6 ± 0.3 g) of the controls. These results clearly indicated that the application of mechanical stress on preadipocytes impaired the differentiation cascade adversely similar to the MSCs but the impairment was the most prominent with 14.6 ± 0.3 g of weight on preadipocytes with the reduction of the expression to less than 3 % of the controls for both adipogenic transcription factors.

ADFP, the structural component of the lipid vesicle was also downregulated to 58 % (1.4 ± 0.2 g), 38 % (7.5 ± 0.2 g) and 1 % (14.6 ± 0.3 g) of the controls (Figure 3.15). hSCD, the key rate limiting step enzyme of the fatty acid biosynthesis cascade was also impaired similarly; downregulated to 70 % (1.4 ± 0.2 g), 60 % (7.5 ± 0.2 g), 6 % (14.6 ± 0.3 g) compared to control. Here, it was observed that under 14.6 ± 0.3 g of weight the impairment of the adipogenic

proteins was more pronounced with the preadipocytes (ADFP: 1 %, hSCD: 6 %) compared to the MSCs (ADFP: 3 %, hSCD: 16 %).

The endocrine function of the preadipocytes was investigated via the expression of leptin and adiponectin. They were impaired at the gene expression level via reduction of the leptin expression to 46 % (1.4 ± 0.2 g) 19 % (7.5 ± 0.2 g) and 0 % (14.6 ± 0.3 g) compared to the controls (Figure 3.16a). The expression of the adiponectin was reduced to 66 % (1.4 ± 0.2 g), 37 % (7.5 ± 0.2 g) and 10 % (14.6 ± 0.3 g) compared to the controls (Figure 3.16b). The expression of leptin was more impaired in preadipocytes compared to the MSCs, (0 % vs 32 %, respectively).

The expression of the adipogenic transcripts displayed a downregulation compared to the control samples as inversely proportional to the amount of mechanical pressure applied. These results were very much in accordance with the observations of the mesenchymal stem cells. One important aspect to note is that the adipogenic transcripts displayed very minimal expression under the heaviest weights (14.6 ± 0.3 g). This observation is concordant with the morphological studies where no Sudan IV stained lipid granules were observed in differentiated preadipocytes of this group (Figure 3.13d).

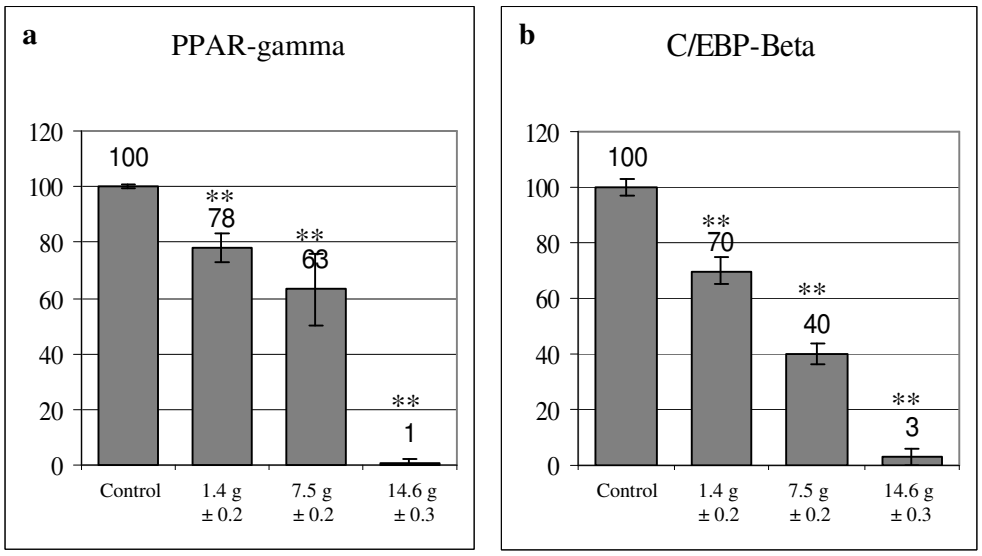

Figure 3.14. Expression of adipogenic transcription factors a) PPARγ, b) C/EBP-β in human preadipocytes embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

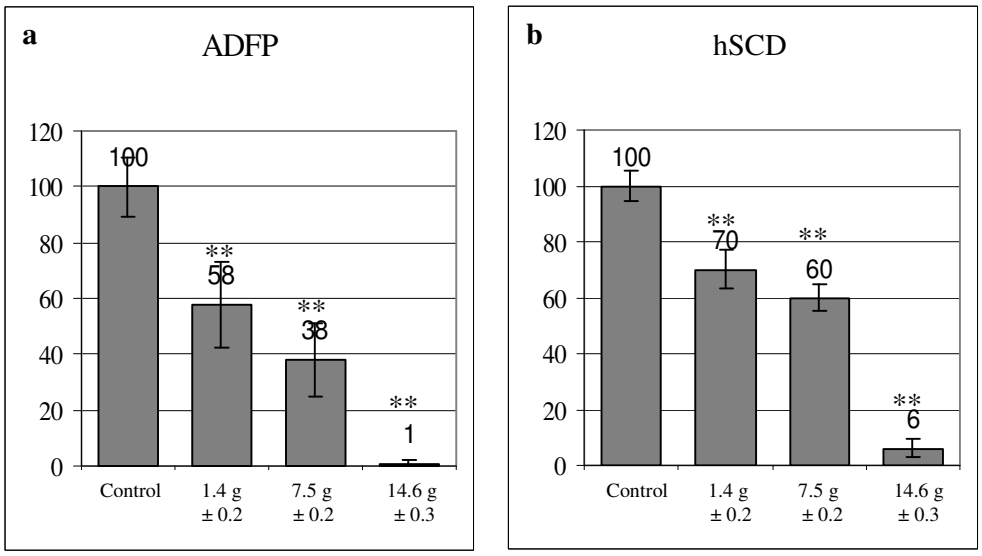

Figure 3.15. Expression of a) adipogenic structural protein ADFP, b) lipogenic enzyme hSCD in human preadipocytes embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

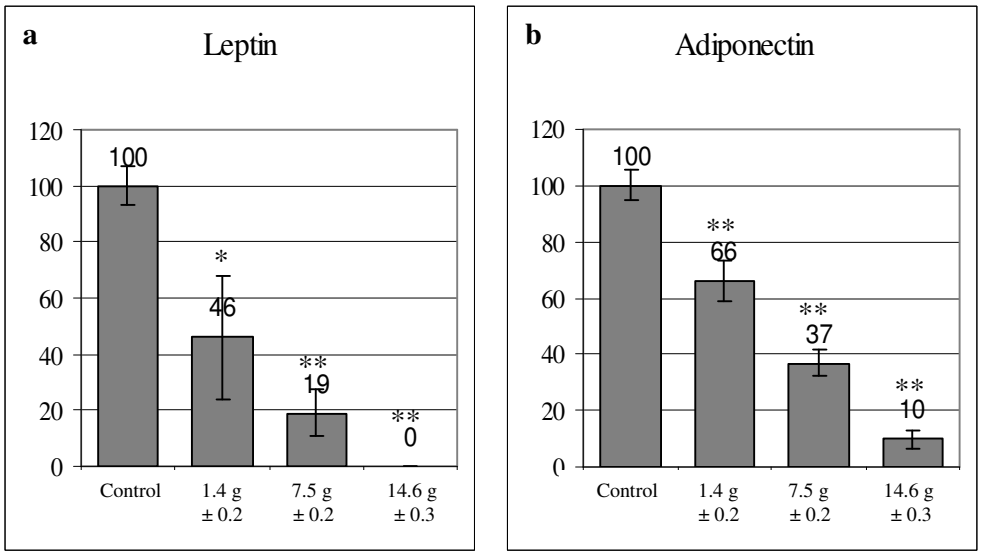

Figure 3.16. Expression of WAT specific adipokines a) leptin, b) adiponectin in human preadipocytes embedded in agarose discs differentiating under compression with different weights for 21 days. Y axis represents relative expression values where control discs have been designated as 100 arbitrary units. Expression values obtained from weight application groups have been normalized and compared to the controls for significance via pair-wise Student's T test. * indicates $p \leq 0.05$ and ** indicates $p \leq 0.01$. Vertical error bars indicate standard deviation in triplicated biological replicates.

These results clearly indicated that the mechanical pressure has a negative effect on the differentiation of both the preadipocytes and mesenchymal stem cells in agarose discs. From the reported data in literature, it is possible to speculate that creating a mechanical force on the cytoskeletal elements may likely to disrupt the RhoA signalling which is essential for the determination to the adipogenic differentiation route of the mesenchymal stem cells. Whether the same pathway (Mcbeath et al., 2004) also counts for the preadipocytes has not been investigated and yet to be determined.

3.3.3. Comparison of Adipogenic Differentiation of MSC and Preadipocytes Under Mechanical Pressure

The human preadipocytes obtained from subcutaneous tissue share the same morphology and differentiation properties as the fibroblasts. In practice, it is not possible to discriminate between a proliferating preadipocyte and a fibroblast. Thus, in numerous studies, subcutaneous fibroblasts or preadipocytes have been substituted for each-other (Spiegelman and Ginty, 1983). Thus, as in many in-vitro studies, observation of various adipogenic differentiation related data on fibroblasts are also valid for preadipocytes (Jeney et al., 2000) and preadipocytes can be accepted as fibroblast cells.

Actin filaments are one of the most important structural components of the cell cytoskeleton having important role in determining cell-shape, mobility and mechanical properties (Tsukita and Yonemura, 1999; Fenerberg et al., 2004). Confocal microscopy images revealed significant differences in the cytoskeleton arrangement in MSCs and fibroblast cells. Mesenchymal stem cells have many thick actin stress-fibers extending through the cytoplasm and ending at focal contacts on the cell basolateral surface. However, in fibroblasts actin filaments are organized into a thin dense meshwork with fewer and smaller stress fibers. Moreover, it has been observed by laser optical tweezers that interaction between the membrane and actin cytoskeleton in fibroblasts are stronger than the same interaction in MSCs. As a result, disintegration of cytoskeleton during the adipogenic differentiation cause a larger effect on fibroblasts or preadipocytes compared to MSCs (Titushkin and Cho, 2006). This could explain the reason for depletion observed in adipogenic transcription factors were higher in preadipocytes compared to MSCs in the present study.

Table 3.1. Comparison of adipogenic differentiation markers between MSCs and preadipocytes differentiated under 14.5±0.3 g weight for 21 days.

	MSCs	**Preadipocytes**
PPARγ	10 % ± 1.22	1 % ± 0.32
C/EBPβ	39 % ± 2.1	3 % ± 2.4
ADFP	3 % ± 0.9	1 % ± 0.45
hSCD	16 % ± 4.2	6 % ± 3.2
Leptin	32 % ± 7.6	0 %
Adiponectin	2 % ± 0.84	10 % ± 1.2

Here, only the adiponectin expression was increased in preadipocytes compared to MSCs. As it explained before, adiponectin is expressed in white adipose tissue (monolocular). Longer incubation of human preadipocytes and MSCs in agarose discs revealed multilocular young adipocyte morphology (Figures 3.17 and 3.18). It was observed that preadipocytes formed monolocular lipid droplet in 60 days while MSCs, although they were enlarged, were still multilocular. This showed that preadipocytes are more prone to form white adipose tissue than MSCs. This could be the reason for higher expression of adiponectin in preadipocytes compared to MSCs.

Gene expressions of different adipogenic factor are different from each other. So, the observation of an unequal depletion in gene expression of these markers in both preadipocytes and MSCs was not an unexpected result. Moreover, mechanical pressures could affect different steps of gene expressions.

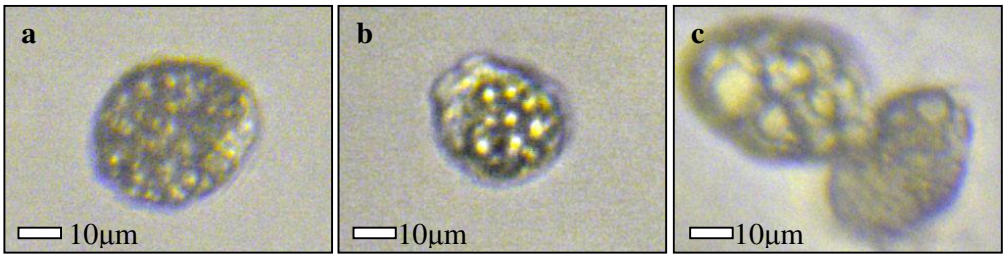

Figure 3.17. Human MSCs embedded in agarose and subjected to adipogenic differentiation. a) 30 days, b) 40 days, c) 60 days.

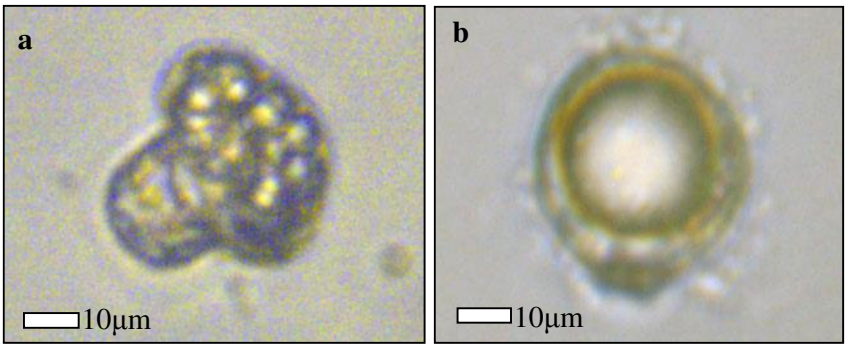

Figure 3.18. Human preadipocytes embedded in agarose and subjected to adipogenic differentiation. a) 40 days, b) 60 days.

The human preadipocytes embedded in agarose discs did not differentiate as their counterparts in tissue culture flasks. As discussed previously, one possible explanation to this might be the lack of cell to cell contact signaling that is required for the commitment and differentiation of these cells (Rosen and Spiegelman, 2000). The same concept is also valid for adipogenic differentiation

of the MSCs. Thus, blending of the agarose discs with type-I collagen, collagen Type IV A, or laminin may enhance the differentiation capacities of these cells.

3.4. Immunostaining

Perilipin, the counterpart of adipophilin (ADFP), is the structural protein that surrounds the intracellular lipid vesicle that resides in the cytoplasm of the adipocytes. Perilipin and adipophilin (ADFP) are globular protein monomers that solely exist in adipocytes. Perilipin is more confined to lipid droplets that are enlarging and accumulating fat droplets. Adipophilin exists together with lipoprotein lipases that are undergoing lipolysis but also exists in resting-state lipid vesicles (Kocaefe, 2005). In this study, the existence of perilipin vesicles within adipocytes that were differentiated in agarose discs has been investigated by the immunostaining technique. Large, multilocular perilipin vesicles could only be observed in adipocytes that were differentiated without compression (control group). Some perilipin vesicles have been observed in agarose discs differentiated under 1.4 ± 0.2 g weights. No perilipin surrounded vesicles could be observed in agarose discs with 7.5 ± 0.2 g or 14.6 ± 0.3 g weights (Figure 3.19).

The perilipin localization study further strenghtened the fact that preadipocytes differentiation was impaired under mechanical pressure.

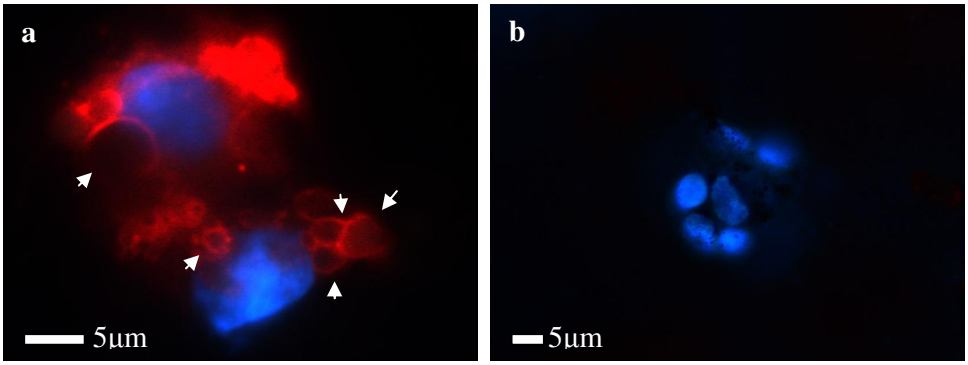

Figure 3.19. Fluorescent microscopy image of perilipin immunostaining on preadipocytes differentiated in agarose discs under a) no compression, b) 14.6 ± 0.3 g weight. Arrowheads point the perilipin positive lipid vesicles. Blue color for DAPI nuclear staining and red color for perilipin.

3.5. Comparison of Initial Mechanical Properties of Discs

In order to evaluate the initial mechanical properties of the agarose discs with or without cells, stress relaxation tests were carried out. A typical stress-relaxation curve of agarose discs is shown in Figure 3.20. It was observed that stress-relaxation curve obtained in all groups were similar in general appearance to theoretical and experimental curves observed in literature for unconfined compression-stress-relaxation studies for viscoelastic materials (Mauck et al., 2000).

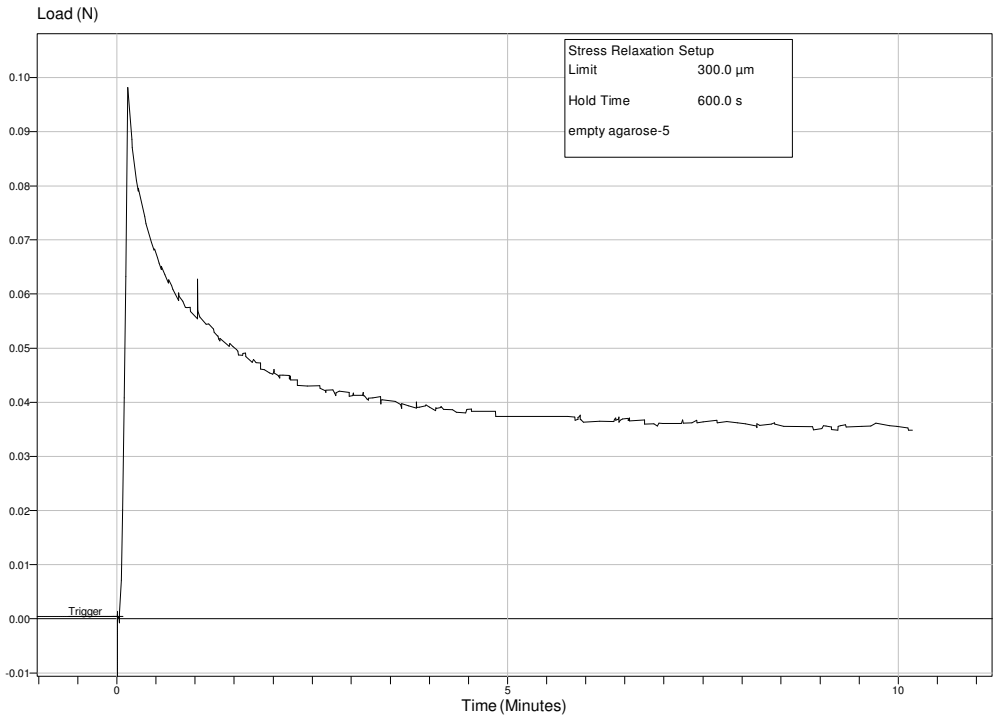

Figure 3.20. Typical unconfined compression-stress relaxation curve obtained by 10 % strain of the agarose discs.

During 21 days of weight application on agarose discs containing cells, it was observed that the discs under 1.4 ± 0.2 g weights preserved their initial dimensions. However, the thickness of agarose discs under 7.5 ± 0.2 g and 14.6 ± 0.3 g weights decreased dramatically. Especially under 14.6 ± 0.3 g of weight some of the agarose discs were smashed to bits. The loads applied to the agarose discs in culture condition were calculated by using the equation:

$$F = m.\ g$$

where F (force) is the load applied on the agarose discs, m is the mass of the weights and g is the gravitational acceleration.

As a result of this calculation, the weights of 1.4 ± 0.2 g, 7.5 ± 0.2 g, and 14.6 ± 0.3 g applied loads of 0.0137 ± 0.0020 N, 0.0736 ± 0.0020 N, and 0.1432 ± 0.0029 N on the agarose discs, respectively. By looking at the compression relaxation curve and comparing to the peak load, it can be observed that the load applied by 1.4 ± 0.2 g weight was far below the peak load whereas the loads applied by 7.5 ± 0.2 g was close to the peak load for 10 % strain. On the other hand, the load applied by 14.6 ± 0.3 g was above the peak load. Despite the fact that the loads applied by weights were nondestructive, some of the agarose discs under 14.6 ± 0.3 g of weight were damaged after 21 days of incubation. This could be due to changes in the composition of discs and also continuous constant static loading.

From the load-displacement data obtained for 10 % strain of the agarose discs peak stress values were calculated by normalization with the area of each disc (Figure 3.21). Comparison of peak stresses for each group showed an insignificant difference ($p \leq 0.927$) in agarose-MSC and acellular discs. However, a significant difference between agarose-preadipocyte and the other two groups of discs have been observed indicating the loss of mechanical properties of agarose-preadipocyte discs.

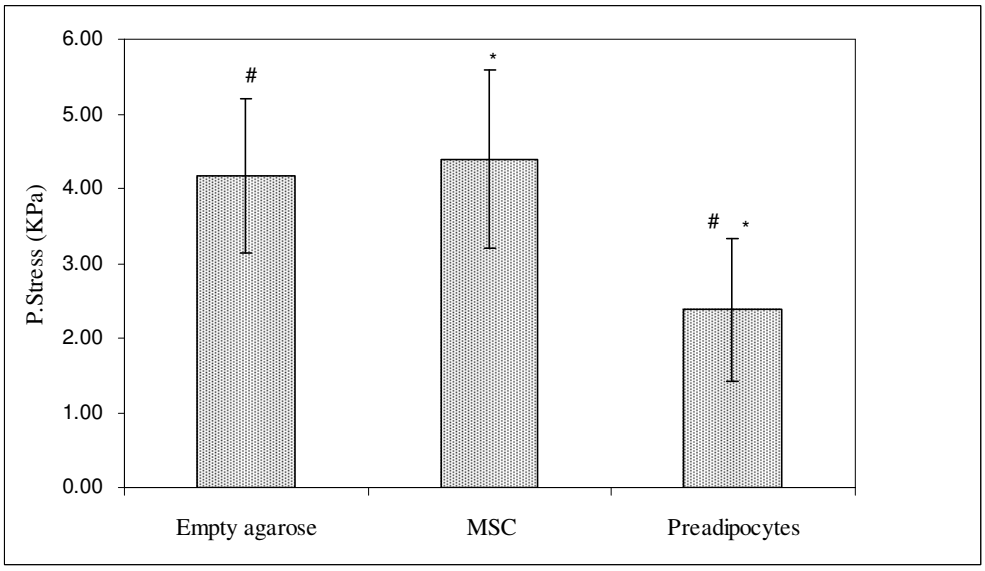

Figure 3.21. Comparison of peak stress values reached during 10 % strain in stress-relaxation tests for agarose-MSC, agarose-preadipocytes and control (acellular agarose discs) (Significant difference was obtained between agarose-Preadipocyte and both agarose-MSC (#:$p \leq 0.007$) and control (*: $p \leq 0.02$) discs.

Comparison of equilibrium aggregate moduli of groups yielded similar results with peak stress values as shown in Figure 3.22. Although the agarose-MSC and acellular discs had statistically indifferent equilibrium aggregate moduli, the agarose-preadipocyte discs showed significant difference from the other two groups. This means that the viscoelastic property of the agarose gels has been modified with embedding preadipocytes. On the other hand, embedding of the MSCs, in spite of numerical increase in equilibrium aggregate modulus, did not show any significant change in viscoelasticity of the agarose discs. Such numerical difference could be explained by the pericellular matrix carried along with the MSCs into the agarose discs.

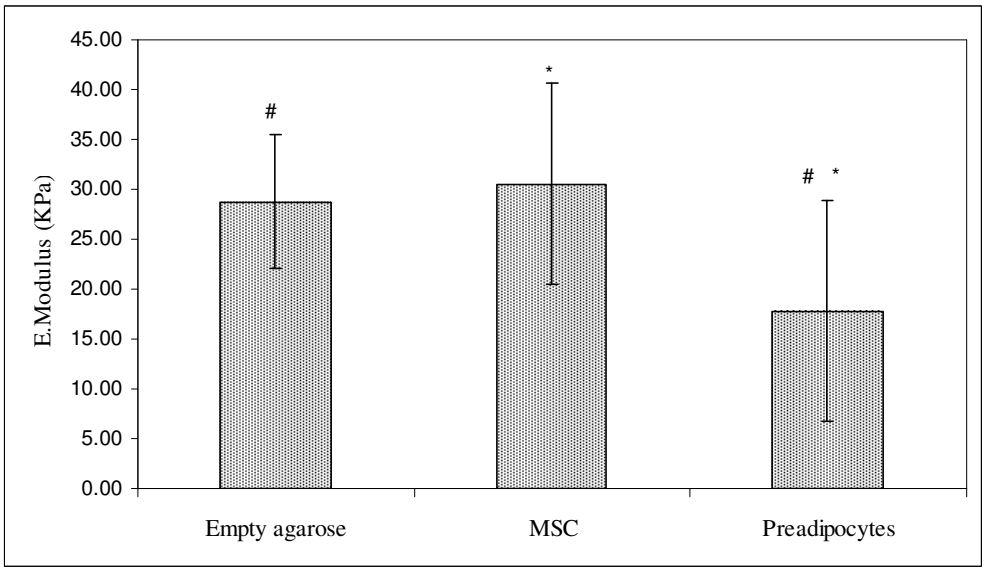

Figure 3.22. Comparison of equilibrium aggregate moduli of agarose-MSC, agarose-preadipocytes, and acellular agarose discs (Significant differences between groups: *: $p \leq 0.006$; #:$p \leq 0.018$).

For preadipocytes, stress relaxation experiment was repeated by increasing the cell number embedded in the agarose discs. However, similar results have been obtained. In fact, according to Darling and his co-workers (2007) the measurement of viscoelasticity of MSC and preadipocytes exhibited similar properties but mechanically distinct from primary cells. An explanation for such observation could be related to the ECM excreted by cells before cell seeding in discs. The cells were cultured 10 days before seeding. So, although preadipocytes were not differentiated, they might be started to differentiate due to a long period of culturing. Thus, these secretions may alter the factors that affect the stiffness of the gel by modifying the crosslinks between the fibers as observed in alginate (Awad, 2004) or by changing the pH - ionic contents (Miyata et al., 2004), or by causing a phase separation between hydrophilic agarose environment and

insoluble secretions (extracellular matrix) of the adipocytes grown to confluency in the medium.

As a concluding remark, numerical (although statistically insignificant) increase in both peak stress and equilibrium aggregate modulus was observed when MSCs-agarose discs were compared with acellular agarose discs. Besides, a significant decrease in mechanical properties of agarose-preadipocyte discs were observed in respect to both agarose-MSC and acellular agarose discs.

CHAPTER 4

CONCLUSION

Naturally, the cells are subjected to mechanical forces like shear stress over the endothelial cells lining arteries due to blood flow, or compression on chondrocytes in knee during locomotion, etc depending on their physiological and anatomical aspects. Therefore, mechanical stress is one of the important determinants of their health and functionality and fate.

In this study, the effect of mechanical stresses on the differentiation of MSCs and preadipocytes was studied in the agarose discs. The results showed that both types of cells could be differentiated inside the agarose discs. On the other hand, gene expression of adipogenic transcripts, which are PPARγ, C/EBPβ, hSCD, and Leptin, of both preadipocytes and MSCs embedded in agarose discs was about 10 fold lower than their counterparts that were proliferated and differentiated in tissue culture flasks. From these two observations, although agarose discs seemed unsuitable as a scaffold for human preadipocytes and mesenchymal stem cell differentiation, the effect of the mechanical stresses on cell differentiation was quantifiable at mRNA level and also observable by microscopy.

The effect of static mechanical compression on adipogenic differentiation of preadipocytes and MSCs embedded in agarose discs was investigated for the first time, to the author's knowledge. Agarose discs were exposed to weights of 1.4 ± 0.2 g, 7.5 ± 0.2 g, and 14.6 ± 0.3 g. The expressions of adipogenic marker genes were shown to be impaired with increasing mechanical stresses. The uppermost decrease in adipogenic differentiation was observed in cells under 14.6 ± 0.3 g of weight, the heaviest used in this study. Moreover, decrease in size and number of lipid droplets with increase in mechanical forces was observed. As a result, it may

be concluded that mechanical forces can affect the adipogenic differentiation of preadipocytes and mesenchymal stem cells adversely. The immunostaining for an adipocyte specific structural protein "perilipin" supported these observations.

The initial mechanical properties of the agarose discs embedded with preadipocytes cells were significantly lower than those of acellular and MSCs embedded discs. However, a numerical (although statistically insignificant) increase in both mechanical properties of agarose-MSC was observed compared to empty agarose discs. This observed difference in the mechanical properties among groups could be explained in terms of the pericellular matrix carried along with the embedded cells.

Engineered adipose tissue can be used in plastic and reconstructive surgery to augment soft tissue defects due to surgery of mastectomy or lumpectomy or trauma injury. This study brings new insights for tissue regeneration in such conditions where mechanical pressure may be an argument. Furthermore, the results of this study may partially elucidate the adipogenic differentiation defects in certain diseases.

Further studies on preadipocytes and mesenchymal stem cells need to be conducted. The molecular mechanisms that lead to the impairment of adipogenic differentiation under mechanical pressure needs further elucidated. Genome wide transcriptome analysis may provide answers to this question. Besides, it needs further investigation to elucidate the reversibility of the impairment of the adipogenic differentiation under mechanical forces. The cells can be extracted from the agarose discs following weight application and then re-assayed for adipogenic differentiation. Moreover, the contribution of the cytoskeletal elements to the blockade of adipogenic differentiation cascade needs to be clarified. Further expression studies on cytoskeletal elements may provide an answer.

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APPENDIX A

Table A.1. Results of quantitative rt-PCR for MSCs

MSC-LEPTIN				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
MSC-k	1557.190	16.433	3	1
MSC 1-2 g	2992.924	34.128	3	0.93
MSC 7-8g	489.674	14.437	3	0.36
MSC 14-15g	413.872	15.132	2	0.29

MSC-PPARg				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
MSC-k	16.572	16.219	3	1
MSC 1-2 g	6.810	20.622	1	0.32
MSC 7-8g	2.415	13.205	3	0.18
MSC 14-15g	1.247	12.541	3	0.1

MSC-CEBP				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
MSC-k	7.453	4.930	3	1
MSC 1-2 g	20.608	20.461	3	0.67
MSC 7-8g	2.243	3.096	2	0.48
MSC 14-15g	2.682	4.548	1	0.39

MSC-hSCD				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
MSC-k	162	16.684	3	1
MSC 1-2 g	130	31.269	2	0.43
MSC 7-8g	71	19.066	1	0.38
MSC 14-15g	24	14.873	3	0.16

APPENDIX B

Table B.1. Results of quantitative rt-PCR for preadipocytes

Preadipocyte-LEPTIN				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
Preadipocyte-K	2084.234	13.672	3	1
Preadipocyte 1-2g	15941.282	29.222	1	0.58
Preadipocyte 7-8g	403.870	13.869	3	0.19
Preadipocyte 14-15g	1218.028	114.550	2	0.07

Preadipocyte-PPARgamma				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
Preadipocyte-K	1.315	14.194	3	1
Preadipocyte 1-2g	1.242	17.089	3	0.78
Preadipocyte 7-8g	877	14.326	3	0.66
Preadipocyte 14-15g	1.246	1297.382	3	0.01

Preadipocyte-CEBP				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
Preadipocyte-K	10.028	3.486	3	1
Preadipocyte 1-2g	6.960	3.453	2	0.7
Preadipocyte 7-8g	6.027	5.197	1	0.4
Preadipocyte 14-15g	3.669	41.630	1	0.03

Preadipocyte-hSCD				
Replicate Name	**Gol Conc.**	**Norm. Conc.**	**Norm. Count**	**relative Conc.**
Preadipocyte-K	36	13.887	3	1
Preadipocyte 1-2g	47	25.474	2	0.7
Preadipocyte 7-8g	23	14.709	3	0.6
Preadipocyte 14-15g	258	1539.798	3	0.06