

**CHARACTERIZATION OF THE ADIPOGENIC DIFFERENTIATION
POTENTIAL OF BONE MARROW AND ADIPOSE TISSUE-DERIVED
MESENCHYMAL STEM CELLS AND DERMAL FIBROBLAST**

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**KEMİK İLİĞİ VE ADİPOZ DOKU KÖKENLİ MEZENKİMAL KÖK
HÜCRELERİN VE DERMAL FİBROBLASTLARIN ADİPOJENİK
FARKLILAŞMA POTANSİYELLERİNİN KARAKTERİZE EDİLMESİ**

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“Intelligence is not a privilege, it’s a gift, to be used for the good of mankind.”

Doc Ock

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TABLE OF CONTENTS

	Page
TABLE OF CONTENTS.....	xi
ABBREVIATIONS	xiii
LIST OF TABLES	xv
LIST OF FIGURES	xvii
SUMMARY.....	xix
ÖZET.....	xxi
1. INTRODUCTION.....	1
1.1 Aim of the study.....	2
2. ADIPOSE TISSUE AND ADIPOCYTES.....	3
2.1 The importance of Adipose Tissue.....	3
2.1.1 Disorders associated with adipose tissue.....	3
2.1.1.1 Human diseases.....	3
2.1.1.2 Disease models in transgenic animals.....	4
2.2 Adipose Tissue.....	5
2.2.1 Adipose tissue – a specialized form of connective tissues.....	5
2.2.1.1 Human diseases.....	5
2.2.2 Distribution of adipose tissue in the body.....	6
2.2.3 Functions of adipose tissue.....	8
2.2.4 Adipose tissue structure..	9
2.3 Adipocytes..	11
2.3.1 The origin of adipocytes.....	12
2.3.1.1 The contribution of non-adipose tissue resident progenitors to adipogenesis.....	14
2.3.2 A Mysterious Subject – Adipocyte Progenitors.....	15
2.3.2.1 Mesenchymal Stem Cells (MSCs)	16
2.3.2.2 Fibroblast – A Major Member of Skin.....	19
2.4 Adipogenesis.....	22
2.4.1 Cellular Differentiation.....	22
2.4.2 Adipogenic Differentiation	23
2.4.2.1 PPAR γ	25
2.4.2.2 Perilipin	26
2.4.2.3 Adiponectin.....	28
2.4.3 PPAR γ , perilipin and AdipoQ – Early or Late Markers?.....	30
2.5 Extracellular Matrix.....	30
2.5.1 What is Extracellular Matrix?	30
2.5.2 Proteins of Extracellular Matrix.....	31
2.5.2.1 Collagens.....	32
2.5.2.2 Fibronectins.....	33
2.5.2.3 Laminins.....	34

3. MATERIAL AND METHODS	35
3.1 Materials and equipment	35
3.1.1 Equipment	35
3.1.2 Compounds, kits, and buffers	35
3.2 Methods	36
3.2.1 Cell culture and proliferation	36
3.2.1.1 Isolation of MSCs	36
3.2.1.2 Preparation of EMC coated culture vessels	36
3.2.1.3 Cell culture and proliferation	37
3.2.1.4 Cell counting	37
3.2.1.5 In vitro differentiation of MSCs and fibroblasts	38
3.2.2 RNA isolation and reverse transcription reaction	39
3.2.2.1 RNA isolation	39
3.2.2.2 Quantification of the isolated RNAs	40
3.2.2.3 Reverse transcription (rt) reaction	40
3.2.3 Quantitative real time polymerase chain reaction (qRT-PCR)	42
3.2.3.1 A brief overview of qRT-PCR	42
3.2.3.2 Locked Nucleic Acid (LNA) based probes	43
3.2.3.3 qRT-PCR conditions and primers	45
3.2.3.4 Calculation from Ct values	48
3.2.4 Microscopic analysis	50
3.2.4.1 Phase contrast microscopy	50
3.2.4.2 Fluorescence microscopy	51
4. RESULTS & DISCUSSION	55
4.1 Gene expression analyses of adipogenic differentiation	55
4.1.1 Establishment of cell cultures	55
4.1.2 Total RNA isolation results	56
4.1.3 Quantitative RT-PCR results	59
4.1.3.1 BM72 cells	59
4.1.3.2 BM78 cells	61
4.1.3.3 LA47 cells	63
4.1.3.4 Comparison of marker expression in BM72, BM78, and LA47 cells	65
4.1.3.5 DF24 cells	69
4.2 Immunofluorescence staining of DF24 for adipogenic markers	72
4.2.1 Cultivation on coverslips and adipogenic differentiation	72
4.2.2 Following the fibroblasts exposed to adipogenic factors	72
4.2.3 Immunofluorescence staining	75
4.3 Adipogenic differentiation on ECM proteins coated surface	77
4.3.1 Preparation of cell cultures and RNA isolation	77
4.3.2 Phase contrast images of adipogenic induction on ECM coated surfaces	79
4.3.3 Influence of ECM coating on relative gene expression	83
5. CONCLUSION	91
REFERENCES	93
APPENDICES	105
CURRICULUM VITAE	113

ABBREVIATIONS

MSC	: Mesenchymal Stem Cell
CFU	: Colony Forming Unit
EMT	: Epithelial-Mesenchymal Transition
MET	: Mesenchymal-Epithelial Transition
AIM	: Adipogenic Induction Medium
AMM	: Adipogenic Maintenance Medium
IBMX	: 3-isobutyl-1-methylxanthine
GM	: Growth Medium
BM	: Bone Marrow
LA	: Lipoaspirates
DF	: Dermal Fibroblast
SF	: Subcutaneous Fat
VF	: Visceral Fat
WAT	: White Adipose Tissue
BAT	: Brown Adipose Tissue
SAT	: Subcutaneous Adipose Tissue
VAT	: Visceral Adipose Tissue
TNF-α	: Tumor Necrosis Factor – alpha
IL-6	: Interleukin 6
GFP	: Green Fluorescence Protein
BMD CPC	: Bone Marrow-Derived Circulating Progenitor Cell
UCP1	: Uncoupling Protein 1
RXR	: Retinoid X Receptor
C/EBP	: CCAAT-Enhancer Binding Protein
ADD	: Adipocyte Determination & Differentiation Factor
SREBP	: Sterol Responsive Element-Binding Protein
CREB	: cAMP Responsive Element Binding Protein
PAT	: Perilipin Super Family
PKA	: Protein Kinase A
HSL	: Hormone Sensitive Lipase
MPR	: Mannose 6-Phosphate Receptor
TBP	: TATA box binding protein (Gene)
GAPDH	: Glycerinaldehyd-3-phosphate Dehydrogenase (Gene)
PPAR	: Peroxisome Proliferator-Activated Receptor (Gene)
ADPQ	: Adiponectin (Gene)
B2M	: β_2 microglobulin (Gene)
PDGFB	: Platelet-Derived Growth Factor Subunit B
NF	: Nuclear Factor
FACIT	: Fibril-Associated Collagens with Interrupted Triple Helices

MACIT	: Membrane-Associated Collagens with Interrupted Triple Helice
PCR	: Polymerase Chain Reaction
qRT-PCR	: Quantitative Real-Time Polymerase Chain Reaction
Ct	: Cycle Threshold
rt	: Reverse Transcription
cDNA	: Complementary Deoxyribonucleic Acid
LNA	: Locked Nucleic Acid
UPL	: Universal Probe Library
FAM	: Fluorescein Molecule
ECM	: Extracellular Matrix
PBS	: Phosphate-Buffered Saline
p	: Passage
U	: Unit
Fig	: Figure
Col I	: Collagen type I
Col IV	: Collagen type IV
FN	: Fibronectin
LN	: Laminin

LIST OF TABLES

	<u>Page</u>
Table 3.1: Adipogenic differentiation plan of fibroblasts.....	39
Table 3.2: Conditions of rt reaction for quantitative PCR.....	41
Table 3.3: Conditions of qRT-PCR.....	45
Table 3.4: Genes, primer sequences and UPL probes.....	46
Table 3.5: Plate layout of qRT-PCR.....	47
Table 3.6: The content of reaction in every well.....	47
Table 3.7: Primer efficiency values.....	50
Table 3.8: General features of antibodies.....	51
Table 3.9: Staining protocol.....	52
Table 3.10: Imaging parameters.....	53
Table 4.1: General features of cell cultures.....	55
Table 4.2: Isolated RNA amounts of BM72.....	56
Table 4.3: Isolated RNA amounts of BM78.....	56
Table 4.4: Isolated RNA amounts of LA47.....	57
Table 4.5: Isolated RNA amounts of DF24.....	57
Table 4.6: General features of cell cultures on coated surface.....	77
Table 4.7: Isolated RNA amounts of samples.....	78
Table 4.8: Gene expression results of FN, Col IV and control groups at day 14...	88

LIST OF FIGURES

	<u>Page</u>
Figure 2.1 : Four main classes of human body tissues.....	6
Figure 2.2 : A mature unilocular human adipocyte.	7
Figure 2.3 : Body fat distribution of human.....	8
Figure 2.4 : Important factors and functionsl of adipocytes.	9
Figure 2.5 : Brown adipocyte histology.....	11
Figure 2.6 : Human BM-derived MSC culture	17
Figure 2.7 : Regulatory cascade controlling adipogenesis.....	25
Figure 2.8 : Adiponectin expression profile in human.....	29
Figure 3.1 : Vi-Cell XR cell viability analyzer.....	38
Figure 3.2 : Main steps of the rt reaction.	41
Figure 3.3 : General PCR outline.....	42
Figure 3.4 : Basis of polymerase reaction in qRT-PCR.....	43
Figure 3.5 : Molecular structures of DNA, LNA, and RNA.....	44
Figure 3.6 : Graphic of Ct value.	48
Figure 4.1 : The comparison of RNA amounts isolated from different cells.....	58
Figure 4.2 : Relative adipogenic gene expression profile in BM72 cultures.....	60
Figure 4.3 : Relative adipogenic gene expression profile in BM78 cultures.....	62
Figure 4.4 : Relative adipogenic gene expression profile in LA47 cultures.....	64
Figure 4.5 : PPAR γ expression of MSC samples during adipogenesis.	65
Figure 4.6 : Perilipin expression of MSC samples during adipogenesis	67
Figure 4.7 : ADPQ expression of MSC samples during adipogenesis	67
Figure 4.8 : Relative adipogenic gene expression profile of DF24 culture.	71
Figure 4.9 : Human adipocyte cell culture.....	72
Figure 4.10 : Phase contrast microscope images of DF24 during adipogenesis.....	74
Figure 4.11 : Immunofluorescence staining images of DF24.....	76
Figure 4.12 : Morphological changes of BM72 cells after adipogenic induction cultivated without surface coating.	79
Figure 4.13 : Morphological changes of BM72 cells after adipogenic induction cultivated on collagen type I coated surface.	80
Figure 4.14 : Morphological changes of BM72 cells after adipogenic induction cultivated on collagen type IV coated surface.	81
Figure 4.15 : Morphological changes of BM72 cells after adipogenic induction cultivated on fibronectin coated surface	82
Figure 4.16 : Morphological changes of BM72 cells after adipogenic induction cultivated on laminin coated surface.....	83
Figure 4.17 : Kinetics of PPAR γ expression of BM72 cells after adipogenic induction, grown on different protein coatings.....	84

Figure 4.18 : Kinetics of perilipin expression of BM72 cells after adipogenic induction, grown on different protein coatings	85
Figure 4.19 : Kinetics of ADPQ expression of BM72 cells after adipogenic induction, grown on different protein coatings	86
Figure 4.20 : Comparison of passage 4 and 6 BM72 cells under adipogenic induction due to their relative gene expression results	89

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SUMMARY

Research of especially the past decade has pointed adipose tissue as a highly active endocrine organ rather than being an inert tissue. This also promoted investigations on the origins of adipocytes. In this context, the most important finding was the discovery of the adipose tissue-resident mesenchymal stem cells. However, it has been lately understood that these cells have a limited proliferative capacity, thus the idea occurred that there should be other sources, feeding the tissue from non-adipose tissue resident progenitor cells.

According to this statement, the principal aim of this master's thesis was to understand the adipogenic differentiation potentials of two bone marrow (BM72 & BM78) and one adipose tissue (LA47) - derived mesenchymal stem cell strains and a dermal fibroblast cell strain (DF24). For this purpose the relative expression levels of the adipogenic marker genes proliferator-activated receptor γ (PPAR γ), perilipin, and adiponectin (ADPQ) were analyzed with qRT-PCR. Upon adipogenic induction, the expression profiles of marker genes were largely intensified in all MSCs from bone marrow or fat tissue, while expression was only rudimentary in dermal fibroblasts. The surprising finding at this part was gained by the comparison of the gene expression kinetics of the MSCs. Accordingly, the differences in expression between these cell strains mirrored an initial adaptation to the different environments or distinct inherent traits. This may point to a difference in the commitment stages of MSCs from bone marrow and adipose tissue, also allowing the discrimination of early progenitors and their progeny, the pre-adipocytes.

For better understanding of the adipogenic differentiation potential of DF24 cells, they were analyzed by immunofluorescence staining in parallel to gene expression assays. Although the observed oil droplet formation or cell shape changes were indicative of adipogenesis, there was no definite proof to say that dermal fibroblasts initiate and proceed in true adipogenic differentiation.

As other connective tissues, adipose tissue is structured by a scaffold of extracellular matrix (ECM) which exerts signalling functions, too. At the last part of the thesis, different types of ECM proteins were examined for their influences on adipogenesis. Herein, promoting and maintaining effects on adipogenic differentiation were found for collagen type I and fibronectin whereas collagen type IV and laminin proteins did not reveal any measurable effect.

KEMİK İLİĞİ VE ADİPOZ DOKU KÖKENLİ MEZENKİMAL KÖK HÜCRELERİN VE DERMAL FİBROBLASTLARIN ADİPOJENİK FARKLILAŞMA POTANSİYELLERİNİN KARAKTERİZE EDİLMESİ

ÖZET

Özellikle son on yılda yapılan çalışmalar, adipoz dokunun inert bir doku olmaktan çok, aktif bir endokrin organ olduğunu göstermektedir. Bu gelişmeler ise bilimcileri adipoz hücreleri olan adipositlerin kökenlerini araştırmaya yöneltmiştir. Bu bağlamda yapılan en önemli keşif, adipoz dokuda yerleşik olarak bulunan mezenkimal kök hücrelerin bulunmasıdır. Ancak bu hücrelerin kısıtlı çoğalma kapasiteleri sonradan anlaşılmıştır, dolayısıyla adipoz dokuyu dışarıdan besleyen, adipoz dokuda yerleşik olmayan ata hücrelerin bulunduğu hücresel kaynaklar fikri ortaya çıkmıştır.

Belirtilen açıklamaya göre bu yüksek lisans tezinin birincil önceliği, kemik iliğinden (BM72 & BM78) ve adipoz dokudan (LA47) elde edilen mezenkimal kök hücrelerin ve dermal fibroblast (DF24) hücrelerinin adipojenik farklılaşma kapasitelerinin anlaşılmasıdır. Bu amaç için 3 tane marker gen (proliferator-activated receptor- γ (PPAR γ), perilipin, and adiponectin (ADPQ)) ekspresyon dereceleri açısından karşılaştırmalı qRT-PCR tekniği ile analiz edilmişlerdir. Adipojenik uyarıma bağlı olarak mezenkimal kök hücrelerde, marker genlerin ekspresyonları yüksek oranda artmış, fibroblastlarda ise tam etkinleşmemiştir. Şaşırtıcı olan sonuç ise mezenkimal kök hücrelerin gen ekspresyon kinetiklerinin karşılaştırılmalarıyla bulunmuştur. Buna göre, ekspresyondaki değişiklikler bu hücre soylarının yeni çevrelere adaptasyon geliştirdikleri veya doğalarında farklı özellikler taşıdıklarını göstermektedir. Bu yüzden, yapılan buluş bu kök hücrelerin farklı adanmışlık seviyelerine sahip oldukları anlamına gelebilir ki, bu ise adipositlere farklılaşan hücrelerin ve onların atalarının ayırımı açısından anlamlı olabilir.

Fibroblast hücreler, adipojenik farklılaşma kapasitelerinin daha iyi anlaşılması için, gen ekspresyon çalışmalarına ek olarak immünofloresans boyama ile analiz edilmişlerdir. Bu hücrelerin yağ parçacık yapılarına ve hücre şekillerine dair anlamlı bulgular elde edilmiş olsa da, fibroblastların bu farklılaşma türüne başlayıp devam ettiklerini net olarak söylememize olanak sağlayacak bulgulara rastlanılamıştır.

Diğer bağ doku üyeleri gibi, adipoz dokuda da ekstrasellüler matriks yapısal bir iskele görevi görür. Bu ise dokudaki sinyalizasyon olayları açısından gereklidir. Tezin bu son aşamasında, farklı türdeki ekstrasellüler matriks proteinleri, adipojenik farklılaşma üzerindeki etkileri açısından analiz edilmişlerdir. Bu bağlamda, kollajen tip I'ın ve fibronektinin adipojenik farklılaşmayı arttırıcı ve koruyucu etkilerine rastlanırken, kollajen tip IV ve laminin nötral bir etki göstermişlerdir.

1. INTRODUCTION

Adipose tissue is an important member of connective tissues in humans. It serves as an energy storage in the body, dampens mechanical impacts of outside world and provides thermal insulation. However, studies especially in the last decade have changed our beliefs about this tissue. The regulatory roles of adipocyte cells on the energy metabolism of the body, and adipocytes' immunomodulatory nature are being better understood everyday [1].

Severe obesity, where adipose tissue tends to expand in the organism, is considered as a much more insidious condition or disease than previously believed. It is an important defect which generally decreases the quality of life of an individual. Today, this excessive adipose tissue expansion is recognized as an important risk factor for other common disorders such as diabetes, various cardiovascular diseases, cancer and metabolic syndrome [2].

These two mentioned facts underline the importance of adipose biology as research area for human health care. Adipocytes, the main adipose tissue cell population, play a crucial role for formation of adipose tissue. Although there is vast amount of literature on the molecular details of these cells, the unknowns, still, by far exceed what is known. The origins of adipocytes is currently one of the most controversial issue. Yet which cell types give rise to adipocytes is not completely clear. The overcrowded and intricate nature of these tissues complicates the entire understanding of adipocyte origins.

Connective tissues consist highly of the extracellular matrix (ECM). Some common ECM proteins (such as collagen type I, collagen type IV, fibronectin, laminin that were studied in this thesis) in skin ECM layers were examined for their influence on adipogenic differentiation. Accordingly, bone marrow-derived mesenchymal stem cells were cultured on surfaces coated by distinct ECM proteins and analyzed for their adipogenic gene expressions.

1.1 Aim of the study

The aim of this study was to investigate the adipogenic differentiation potentials of mesenchymal stem cells derived from bone marrow and fat tissue in comparison to dermal fibroblasts. For this purpose, gene expressions with special focus on adipogenic marker genes were analyzed in mesenchymal stem cells and fibroblasts subjected before and after induction to adipogenic differentiation in cell cultures. We examined the adipogenic potential of dermal fibroblasts using fluorescence staining and microscopy, in order to consolidate previously found relative gene expression data of the sample. At the final stage of the project, we aimed to study the influence of ECM protein coatings on adipogenic differentiation through the analysis of relative PPAR γ , perilipin and adiponectin gene expression.

2. ADIPOSE TISSUE AND ADIPOCYTES

2.1 The Importance of Adipose Tissue

During evolution, different cell types have taken associative tasks and consequently they have given rise to structured tissues. Organs and systems are major parts of organisms which are developed in time by tissues that have been working in harmony. Human is a well developed mammalian organism which has come into existence with the relevant associations of organs and systems [3]. Therefore, anatomical and physiological unison of tissues better be considered as a very basic, important factor of a healthy organism.

Diseases impair normal functioning of tissues. Today, we are able to fight against the diseases with the contributions of a more profound understanding of physiological and anatomical basics of organs and systems. And this manner is achieved by the better understandings of the tissues which are structured by specialized cells and their products. Adipose tissue is an important tissue for the survival of the organism [5]. It associates with almost all units of the body. It contributes to the structures of many organs including the surrounding of intestines, muscles, and liver. It works together with endocrine system and digestive system. It provides insulation for the body and a cushion against mechanical impacts of the environment. Besides that, adipose tissue is the most important energy reservoir for the organism [6].

2.1.1 Disorders associated with adipose tissue

2.1.1.1 Human diseases

Obesity is a condition which recently was gaining importance as a generalized disease with large impact on human health. It is described as higher amount of weight exceeding the normal standards for the human body. But this overweight situation is accounted by the total amount of fat tissue, not the body mass per se.

Though obesity reduces the life quality, most importantly it mediates the occurrence of various other disorders.

An obese individual exhibits an excessive increase in the number of adipose cells [8]. The reason of obesity can be either environmental and genetic. Today many mutations are known to be related with obesity throughout the human genome [9]. Adverse effects of obesity on health are generally related to metabolic irregularities which create cardiovascular risk factors [10]. Impaired glucose tolerance is one of the mainly considered metabolic defects in this respect [11]. Today, the sum of these risk factors is called “metabolic syndrome”. This syndrome includes disabilities such as insulin resistance, abdominal obesity and hypertension [12].

Some clinical trials have reported that in obese individuals incidence of malignancy and metastasis rates are higher than in normal individuals [16-18]. One explanation is that adipocytes reaching a certain size slows down their metabolism to store more lipids inside. Especially the decrease of adiponectin causes regional neovascularization which is needed for expansion of adipose mass. However, this incident unwittingly creates the relevant status for tumor neovascularization [19]. Thus plenty of cases have been reported where certain cancers correlated with overexpression of a variety of adipose tissue-derived molecules [20].

2.1.1.2 Disease models in transgenic animals

Problems associated with adipose tissue should not be only considered just as a result of over production of adipocytes. There are intriguing results from transgenic animal studies done in several laboratories. Adiponectin for example is an important regulatory molecule which is expressed only by white adipose tissue [13]. This molecule is involved in energy metabolism of the organism. A research has reported that knock-out mice fed with a normal diet do not show significant differences compared to wild-type, however, they developed severe insulin resistance when fed with sucrose and oil based diet. This insulin resistance has been observed either in obese mice or lipotrophic mice. Deterioration of insulin mediated mechanisms lead to plenty of disorders such as diabetes, hyperglycemia and liver enlargement (hepatomegaly) [15].

2.2 Adipose Tissue

2.2.1 Adipose tissue - a specialized form of connective tissues

In histology there are four classified tissue types for mammals: Epithelial, muscular, nervous, and connective tissue (Fig. 2.1). Connective tissue provides shape and structure of the body and keeps its units and organs together. It consists of a comprehensive part of extracellular matrix. The most abundant protein in connective tissues is type-I collagen which generates 25% of the total protein mass in mammals.

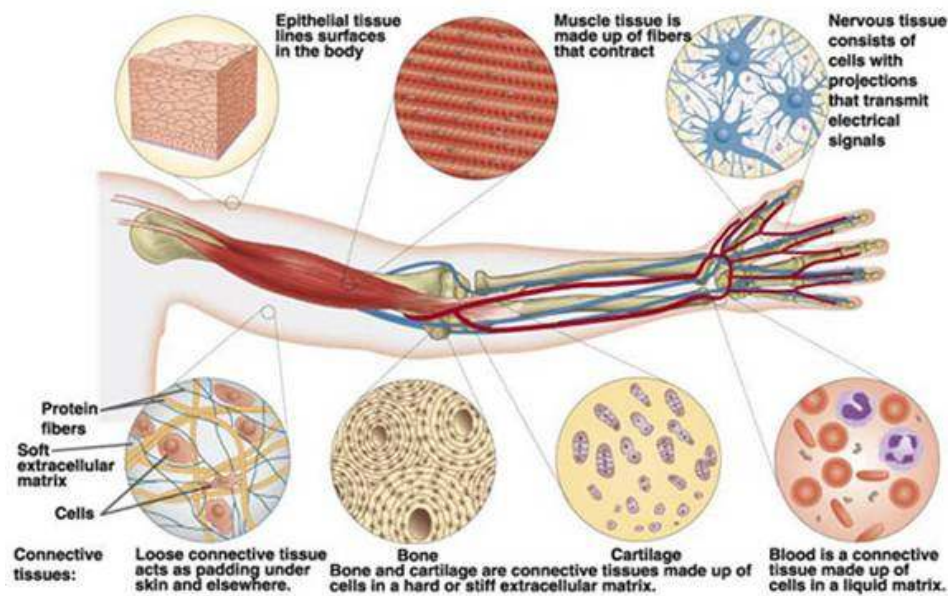


Figure 2.1: Four main classes of human body tissues (connective tissues - www.hawaiianshirttray.com).

Today, still the term “connective” is used to address this tissue. However, current knowledge over these body units tells us that they have additional significant roles for the body far beyond providing scaffolds or merging structures. Adipose tissue is a quite distinct non-fibrous example of connective tissue, where its most important regulatory role concerns endocrinological tasks [22].

Adipose tissue is generally formed by the cells that have the capability to store high amounts of triglycerides (fats) [23]. Even today, despite the recognition of adipose tissue as such centuries ago, its secrets need to be fully elucidated yet. In humans, it is generally located beneath the skin and also around the organs, yellow bone marrow in long bones, breasts and hips [24]. The adipose tissue under the skin is called “subcutaneous fat” (SF), whereas surrounding the organs it is called “visceral fat” (VF) [25]. There are some similarities but also differences between SF and VF.

About 80% of the total adipose tissue molecules are triglycerides (oil). The resident adipocytes are specialized for storing oil and the mature cell usually carries a single large oil drop in its cytoplasm [26]. This oil drop pushes all cell organelles outwards the plasma membrane causing overhang structures around the cell (Fig. 2.2). A significant layer of extracellular matrix surrounds the cells. The adipose tissues which contain one oil drop adipocytes, appear in white color to our eyes. Thus, these are called “white adipose tissues” (WATs) in histology [27]. Brown adipose tissues (BATs) are the only alternatives of WATs.

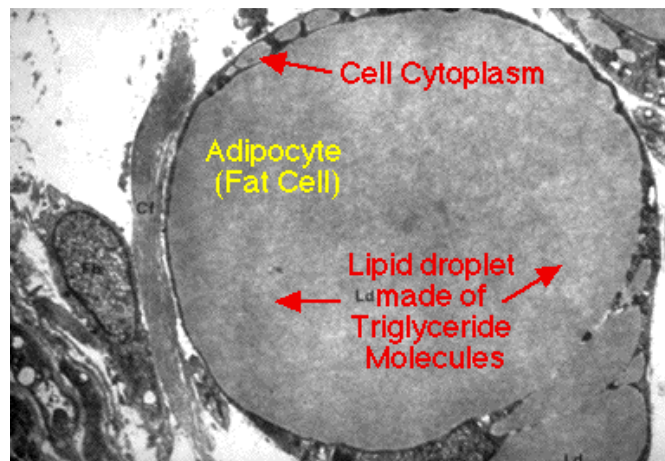


Figure 2.2: Mature unilocular human adipocyte (bio107, University of Connecticut).

2.2.2 Distribution of adipose tissue in the body

Adipose tissues are further classified as VF or SF according to their locations. VFs are found within the abdominal cavity and they usually surround the organs. Epicardial fat which surrounding the heart is such an example of VF. Accumulation of VF is known as central obesity and it has a direct relation with cardiovascular diseases [28]. Moreover, relationships are found to other diseases such as diabetes and insulin resistance [29]. SF is settled under the skin in the hypodermis layer. These fat deposits are thought to have protective function against obesity-linked disorders in contrast to VFs [28].

Considering general human anatomy, male body shape is evident with the accumulation of total body mass at the upper-segment. In women, total body mass trends to accumulate downwards in the body. This difference occurs due to the endocrinological disparities between men and women (Fig. 2.3). Furthermore, hormonal changes in women after menopause can have an effect on the way how a woman is gaining weight [29].

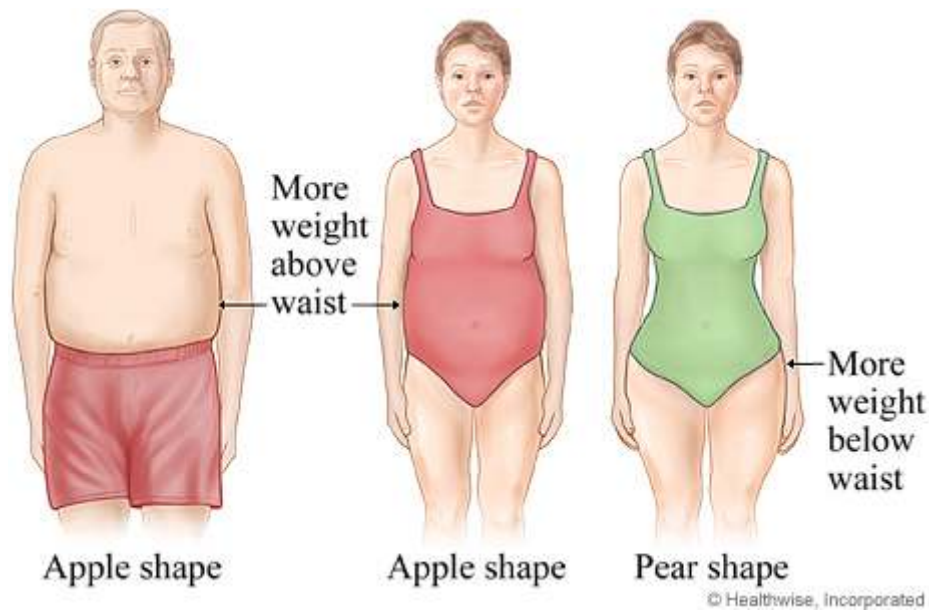


Figure 2.3: Body fat distribution of human. Due to the genetical background and environmental factors, body fat distribution varies. However, this is mostly affected by hormonal conditions. Body shape of a woman can change after menopause with the new hormonal regulations (www.emedicinehealth.com).

The distribution of adipose tissue in the body is mostly genetically determined [30]. Life style including exercise profile and food consumption habits contribute to the quantity of the adipose tissues, but not their distribution. In a person's body who consumes a lot of food, more fat storage tends to increase in adipocytes. As a result, there occurs an increase in the size of the cells and after a point, these cells begin to multiply. The adipocytes in the body which show susceptibility to grow and multiply are genetically determined. However, it is still unclear that what factors control where does a person gain the weight. Abnormal adipose tissue expansion occurs in the body in the case of obesity. Panniculus is a significant example of abnormal SFs where the adipose tissue enlarges in the tummy [31]. However, it has to be noted that the excessive weight gain does not necessarily happen in subcutaneous tissues. Even though the knowledge we have about the development of obesity increases every passing day, the exact picture is far from being clear. Open questions are which cells give rise to adipose tissues? What are the regulatory mechanisms? The answers for these questions are important issues in adipose biology, which still wait for us to be discovered.

2.2.3 Functions of adipose tissue

The most basic functions of adipose tissues were emphasized as being a thermal insulator, energy storage and a mechanical support for the body. In the organism, an increasing requirement for energy triggers lipase enzymes to hydrolyze the triglycerides stored in adipose tissues. The hydrolysis of triglycerides releases free fatty acids. Adipocytes excrete these fatty acids into the blood stream and by the circulation these metabolites are transported to muscles and the heart [32]. The fat decomposition and fatty acid release into the blood is known as lipolysis, whereas the reverse process, oil storage is called lipogenesis. Insulin triggers lipogenesis in the organism [32]. Today, in addition to the given characteristics, adipose tissue has been accepted as an important endocrine organ which plays a role for insulin sensitivity, in inflammatory reactions, energy homeostasis, and food consumption behaviors [33].

Several molecules have been identified that are synthesized in adipose tissue acting as hormones in the organism (Fig. 2.4). These molecules are generally called adipose-derived hormones, while signalling molecules from adipose tissue that have immunomodulating properties are collectively termed adipokines (see cytokine) [34].

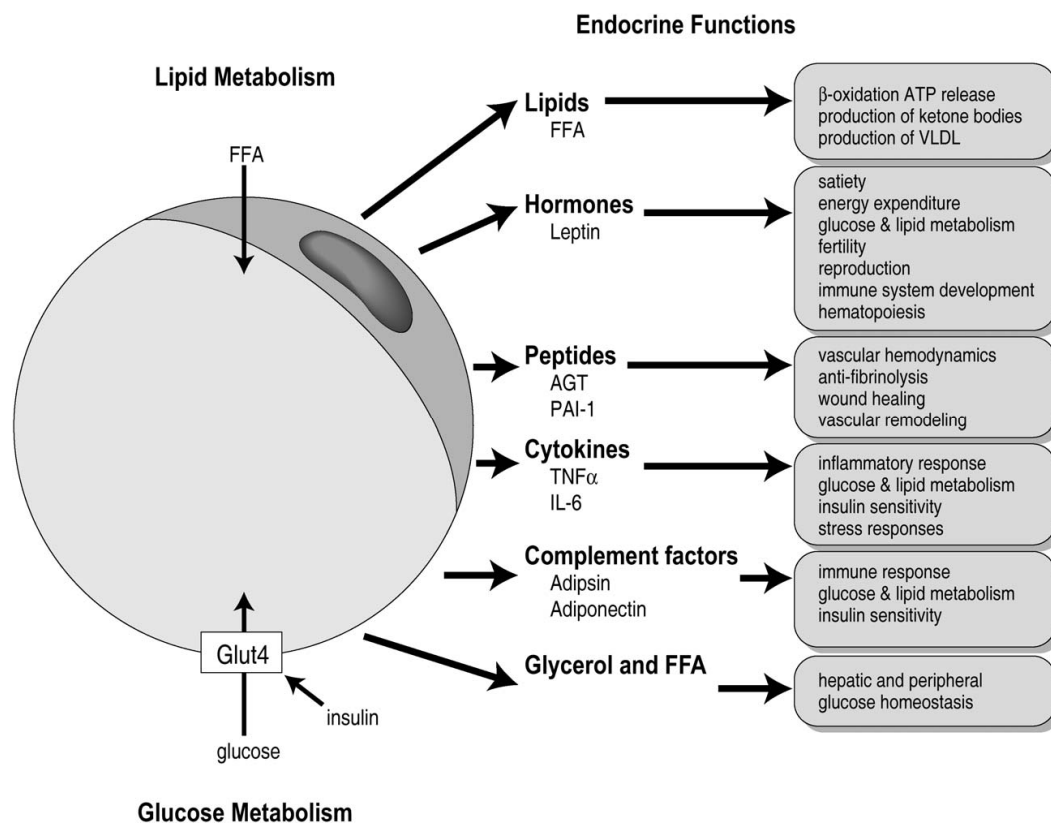


Figure 2.4: Important factors and functions of adipocytes (modified [166]).

The most well known adipose-derived hormones are adiponectin, leptin, resistin and estradiol. These hormones work generally in energy metabolism, and are directly associated with obesity and type-II diabetes. Adipose tissue has been first recognized as an endocrine organ after the discovery of leptin functions [35]. The appetite suppressant attribution is leptin's best-known feature. Estradiol is a sex hormone that is very important for gender differentiation in humans [36]. The name resistin, was given to the protein for the discovery that its injection in mice gave rise to insulin resistance [37]. In obesity, increased serum levels of resistin are found. Adiponectin is a very identified adipose-derived hormone which is secreted abundantly into the blood by adipocytes and which is crucial for glucose metabolism and fatty-acid catabolism [38].

Among adipokines secreted from adipose tissue, chemerin in particular inhibits cell growth but also cellular differentiation. David Segal and colleagues reported lower expression of chemerin in pre-adipocytes whereas after the differentiation chemerin synthesis increased [39]. Among the other important adipokines, tumor necrosis factor – alpha (TNF- α), Interleukin 6 (IL-6), and monocyte chemoattractant protein 1 can be given.

Last not least, adipose tissue-derived macrophages play an active role in the organism. In obesity, excessive accumulation of macrophages in adipose tissue has been demonstrated [40]. Though by now, adipose tissue is commonly appreciated as an endocrine organ and too many mysteries are waiting us to be discovered yet.

2.2.4 Adipose tissue structure

The discrimination between WAT and BAT is based on the features of the adipocytes as well as the tissue structures [27]. Composing 20-25% of total body weight of a healthy human, WAT is the main energy storage in the adult organism. While adipocytes represent the main cell population of WATs, there are also macrophages, fibroblasts and endothelial cells in this tissue. In addition, WAT contains a compact extracellular matrix and many small blood vessels. WATs can have both subcutaneous or visceral location.

On the other hand, BAT is known as newborns' fat [41]. Thus, it is found in abundance in neonates but also in hibernating mammals. The reason for this is that it acts as heatgenerator in the organism. This is accomplished by a high number mitochondria in the resident adipocytes (Fig. 2.5) [42].

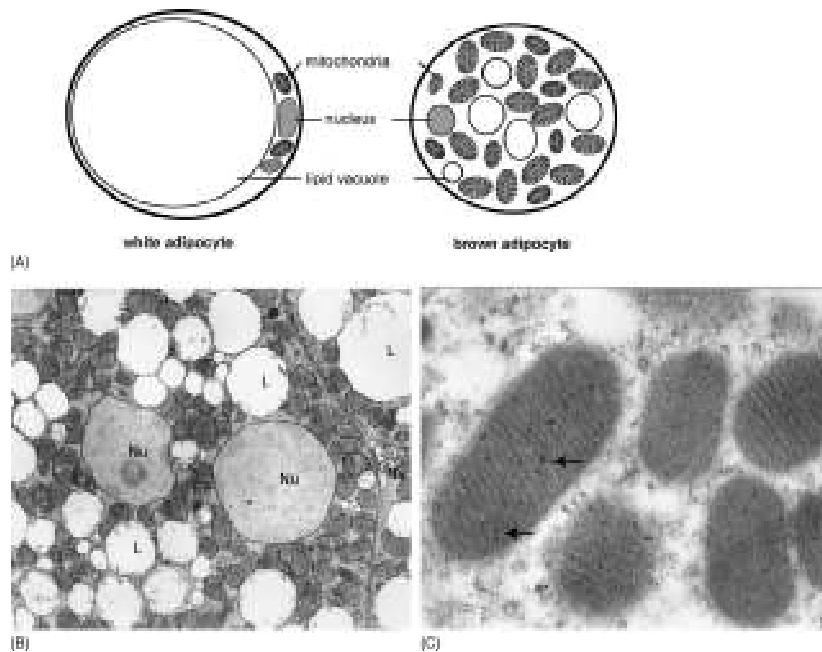


Figure 2.5: Brown adipocyte histology and UCP1 (mitochondrial proton carrier protein) function. (A) Morphological comparison of white and brown adipocytes. (B) Photomicrograph of mouse BAT. Nu—nucleus, L—lipid droplets, Nv—nerve fiber. (C) Electron micrograph of a brown adipocyte with abundant mitochondria that are labeled for UCP1. Arrows point to UCP1 labeling (immunogold, 14 nm) [42].

While WAT adipocytes harbor one single large oil drop, BAT adipocytes contain many small oil droplets scattered in the cytoplasm. Another significant difference between BATs with WATs is that BATs contain a much higher number of capillary blood vessels. Eventually, regarding the given properties, this adipose tissues get its brown color. Suprisingly, BATs have kept disappearing during the human evolutionary process. Today, the fat content of BATs is even lower than 0,1% of total body fat in adult humans.

The main element of adipose tissues is fat storing adipocytes. In addition to those cells, stromal-vascular cells are also found in adipose tissues such as macrophages, leukocytes, pre-adipocytes, and fibroblasts. The same type of adipose tissue might exhibit different activities in different regions of the body. This may rely on the properties of the adipocytes or the other cell types in the particular adipose tissues [43].

Adipose tissue's size is a consequence of the total number of adipocytes and their cell mass, therefore it can increase by proliferation of adipocytes or by lipid accumulation. Either one of these activities can dominate at different stages of an individual's lifespan. Thus, neonates are relatively fat possessing a dense and widely dispersed adipose tissues [44]. This is a result of the adipose development at late pregnancy, however, actual mechanism is not clear yet.

2.3 Adipocytes

The name, adipocyte is given to the cells storing high amounts of fat [45] which have a largely spherical shape. As mentioned above two types, WAT and BAT adipocytes can be distinguished. The majority of the fat cells in the body are WAT adipocytes, typically containing a single large oil drop in the cytoplasm and being widely distributed throughout the body. Contrarily, BAT adipocytes have a more limited distribution and their role is to generate heat for the organism by oxidizing fatty acids [42]. They have many small oil droplets in their cytoplasm. These oil droplets are found coated by perilipin proteins inside the cell [32] which happens to protect the lipids from lipase activity.

Concerning still another classification, the distinction between subcutaneous adipose tissues (SAT) and visceral adipose tissues (VAT) is not just based on their location. For example, lipolysis and fatty acid turnover are seen more frequent in VAT adipocytes [46]. Furthermore, it has been observed that VAT adipocytes produce more IL-6 than SAT adipocytes. IL-6 is an inflammatory adipokine which also suppresses the insulin response in the organism [47]. On the other hand, SAT adipocytes produce more leptin and adiponectin and these two factors intensify the insulin sensitivity. A particularly striking is that adiponectins are very specific molecules only synthesized by adipocytes but their expression differs in VAT and SAT adipocytes [38].

The question is the structural and functional differences between VAT and SAT adipocytes are due to distinct precursor cells, i.e. pre-adipocyte originated from different tissues or body regions or reflect specific differentiation traits influenced by region-specific growth and differentiation factors.

2.3.1 The origin of adipocytes

Today, emerging technologies and increasing general wealth allow us to reach easily to higher amounts of nutrients than in the past. However, this is creating serious health problems since society does not change as quick as the technologies, in adapting to these new conditions and life styles. As a result of this ambiguous progression, less moving and consuming more and unnatural foods have become inevitable reasons of obesity [49].

Obesity is an insidious disease; it causes many other diseases in the organism [8]. Increasing cases of obesity and related health problems have propelled studies on the cause of obesity. This has revealed that adipose tissue has important regulative functions beyond being just energy storage or mechanical support, consequently raising questions for the origins and development of adipocytes.

1. What triggers the formation of adipose tissue?
2. How does adipose tissue come into existence?
3. How adipose tissue formation can be reduced without impairing general health?

To answer these questions we need to understand the morphological and physiological features of adipose tissues in the context of the origin of adipocytes which, as stated before, are the main components. Question is that do specialized stem or precursor cells exist like all the other cell and tissue types in the organism, being responsible for self-renewal and repair of those structures.

During the development, the mechanism of commitment of embryonic stem cell precursors to adipocyte lineages is still unclear. WAT formation displays differences among different species but it begins before birth in all mammals and thus humans are born with relatively well developed adipose tissue. After birth, WATs experience a rapid expansion depending on the number of adipocytes [27] but this process and the factors involved are not well understood yet. Nutrient stimulation of adipose tissue expansion is also a matter still under investigation [44]. However, today concepts emerged about pre-adipocytes or precursor cells which have the potential for adipogenic differentiation. Currently the nature of those cells is investigated together with regulatory mechanisms effecting cellular differentiation.

A large part of such studies implies that certain embryonic stem cells which have capacity to differentiate into mesodermal cell types (osteoblasts, adipocytes, myocytes and chondroblasts), give rise to adipocyte lineages during development [50]. This means that some multipotential stem cells are fixed in mesodermal pathways [51]. On the other hand, there is rising evidence that a common pool of bone marrow stromal cells can differentiate into adipocytes or osteoblasts among other cell types under respective stimulation [52].

Do all adipocytes come from the same mesodermal origin? An important proposition is that adipocyte progenitors derive from cells located in vascular network, which constitute a pool of resident pre-adipocytes capable for self-renewal [53]. These cells give rise to new adipocytes in stead of the dead ones, and respresent a source for the expansion of adipose tissue. However, this is still controversial and the cells admitted as adipose-lineage committed pre-adipocytes have not been clearly defined. There are mainly two concerns, firstly a specific marker has not been discovered yet to distinguish the progenitors from other stromal cells and secondly, there are extremely heterogeneous cell populations in these regions.

One of the most important discoveries about adipocyte progenitors were made by Friedman and colleagues [54]. By fractionating adipose stroma cells using sequential flow cytometry fractionation and established stem cell markers , they were able to isolate adipocyte progenitors. One cell lineage, being CD29+, CD34+, Sca-1+, CD24+, retained the ability of adiponegenic differentiation. This cell population could also differentiate into bone, cartilage, and muscle cells. Thus, a great premise of this study was the discovery of adipose-resident pre-adipocyte progenitors. Another important contribution on this subject was made by Jensen and colleagues [55]. According to their work on, obesity, increasing adiposity in humans leads to a decrease in the number of committed subcutaneous pre-adipocytes. From these studies the conclusion can be drawn that: adipose tissues contain resident-adipocyte progenitors indeed. However, these cells have a limited proliferative capacity which requires refreshment from non-adipose tissue resident resources.

2.3.1.1 The contribution of non-adipose tissue resident progenitors to adipogenesis

After the discovery of the limited proliferation capacity of progenitor cells resident in adipose tissue, cells contributing to WAT formation became a major subject of research. Most probable candidates are bone marrow tissue progenitors [56]. Bone marrow is a tissue very rich in mesenchymal and hematopoietic stem cells. These distinct stem cell populations are multipotent having both a wide differentiation potential. Usually mesenchymal stem cells seem not to leave this site while adipocytes are also present at there. On the other hand, some of those hematopoietic stem cells which have mesenchymal characteristics, apparently leave the marrow and settling in other tissues where they might convert into adipocytes [57]. Accordingly, marrow stem cells are the primary progenitor candidates.

This has been demonstrated by two separate studies on mice and rats. In both cases, bone marrow labelled with green fluorescence protein (GFP) was transplanted into wild-type animals [58,59] which allowed to detect and trace GFP expressing adipocytes derived from the marrow graft in host adipose tissue by fluorescence microscopy. In another related investigation, Makio Ogawa and colleagues demonstrated that also hematopoietic stem cells can develop into adipocytes [57]. However, similar marrow transplantation experiments by Gou Young Koh and colleagues started a hard debate about the validity of this hypothesis [60]. Their aim was to follow the fate of the bone-marrow-derived circulating progenitor cells (BMDCPCs) and determine their adipogenic potential in vivo. But herein no unilocular or multilocular development could be observed among the cells which had migrated from bone marrow to adipose tissue. Instead, the majority of the resident BMDCPCs had become phagocytic cells, showing a weak multilocular phenotype. Thus, they did not express uncoupling protein 1 (UCP1) which is a brown adipocyte marker, whereas some macrophage surface markers were detected.

As mentioned earlier, Makio Ogawa and colleagues demonstrated the adipogenic potential of hematopoietic stem cells [57]. Psilas and colleagues have carried this investigation one step further by comparing mesenchymal and hematopoietic (myeloid) progenitor cells [61]. They have done GFP labelled bone marrow reconstitution in mice which are restricted to express LacZ in only hematopoietic cells. Since the vast majority of adipocytes detected in the animals were found

LacZ⁺, this advocates for that bone marrow-derived adipocytes descend from hematopoietic stem cells. In another study, adipocyte progenitors which expressed neither hematopoietic nor myeloid markers (CD45 – CD11b) were found, which may point to circulating fibroblasts [62]. Finally, it has to be mentioned that bone marrow-derived progenitors were frequently in VATs but very rarely in SATs in bone marrow reconstitution studies on mice.

In summary, mesenchymal stem cells seem to be the primary pre-adipocyte progenitor candidates according to the majority of published work. On the other hand, some studies suggest that hematopoietic stem cells from bone marrow might substantially contribute to adipogenesis. Still not fully established is the adipogenic capacity of fibroblastic or stromal cells, though their strong migratory phenotype and their occurrence in adipose tissues make them better progenitor candidates than their hematopoietic fellows.

2.3.2 A mysterious subject – Adipocyte Progenitors

In human development, there is a delicate balance between cell proliferation and differentiation, as a strict rule differentiating cell not being able to engage proliferation at the same time [63]. In essence, cell differentiation means to acquire a more specialized state than the progenitor cell. With initiation of embryonic development, cells begin to differentiate to form distinct tissues. However, during the life of the organism, major pools of tissue or organ specific progenitor cells are needed which are called committed stem cells.

The Latin word “Genesis” has the meaning of both initiation and creation. In biology, different types of differentiation are called with the name of the ultimate cell combined with the suffix “genesis”. Adipogenesis for example describes the process where cells differentiate eventually into adipocytes.

Adipocytes of BAT begin to evolve from mesoderm in the second half of pregnancy [44], while after birth, an increase in WATs occurs in individuals. It has been thought that pre-adipocyte progenitors begin to take positions in the organism in this timeline. As outlined in chapter “The Origin of Adipocytes”, mesenchymal stem cells are commonly regarded as the primary progenitor cells for the mature adipocytes. Fibroblasts, with some features and a strong kinship to mesenchymal stem cells, are also considered as additional candidates.

2.3.2.1 Mesenchymal Stem Cells (MSCs)

Mesoderm is one of the three main germ cell layers of mammalian embryos seen at very early stages. During development, mesoderm forms different tissues which give rise to new organs and compartments in the body. The mesenchyme being, one of these embryonic tissues, also known as loose connective tissue [64], constitutes a large part of circulatory system including heart and major blood vessels as well as bone, cartilage, the dermis in skin, and adipose tissues. The mesenchymal stem cells (MSCs) are rooted from the mesenchymal layer. They are non-hematopoietic progenitor cells which are found in virtually all adult tissues. It is believed that they have an uncommitted state and enhanced proliferative potential [65]. Musculoskeletal tissues such as cartilage, bone, muscle, ligaments, and tendons but also adipose tissue are composed by descendants of MSCs. This type of progenitors is collectively called multipotent stem cells [66].

Similar to fibroblasts, MSCs are thin elongated cells with a small cell body (Fig. 2.6). They contain a wide circular nucleus and a prominent nucleolus which serves as cell information center. In cell reproduction and differentiation the nucleolus plays a significant effects by regulation of gene expression. This may explain the marked appearance of the nucleolus in MSCs. Commonly, MSCs change their cell shape as a result of differentiation.

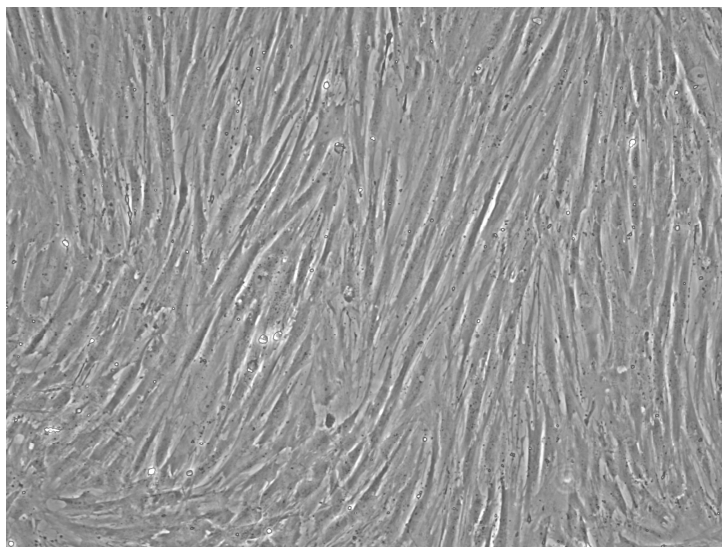


Figure 2.6: Human bone marrow-derived mesenchymal stem cell culture. Image was taken after postconfluency under 100x magnification.

The duties of MSCs vary. Their first and most important task is serving as progenitors in the organism. Thus, they give life to a variety of cells with distinct differentiation properties. For this and their regenerative features, MSCs are also essential in tissue repair (wound healing) [67], which is further fostered by their role in angiogenesis [68]. Based on that, MSCs are promising candidates for the treatment of chronic wounds. As a member of connective tissues, MSCs might enhance or improve functions of other cells in their micro-environment. As one example, Majumdar and colleagues had shown that MSCs are the stromal cells which support hematopoiesis [69]. They substantiated this hypothesis by providing extracellular matrix and specific signal molecules to cells which are committed to hematopoiesis. Today, one of the most focused tasks is the immunomodulatory activity of MSCs. First, their T-cell proliferation inhibiting properties had been discovered, but they showed similar effects also on B cells, natural killer cells, and dendritic cells [70]. These features imply that MSCs could be useful as immunosuppressors in immune-mediated diseases and after organ transplantation.

In 1966, Friedenstein and colleagues defined mesenchymal stem cells from bone marrow as colony-forming unit (CFU) fibroblast-like cells for the first time [71]. They also demonstrated that MSCs undergo osteogenic differentiation. By the passage of time, the potential of MSCs to differentiate into other tissues has been discovered, including cartilage, bone, fat, muscle, tendon, and hematopoiesis supporting marrow stromal cells. Today, several MSC specific surface markers are known which are not present in hematopoietic stem cells [52], though they can be also expressed by certain other cell types. As stated previously, the lack of markers to distinguish the adipocyte progenitors from stromal cells have not been discovered and likewise also no markers to distinguish MSCs from other cells in their environment. Typically, MSCs exist with densely packed groups of other cell types. Collectively, this illustrates how difficult processes are to isolate MSCs from humans.

Mesenchymal Stem Cell Sources in the Organism

In humans, embryonic stem cells are the most advanced cells with their great differentiation capacity. In different stages of development, stem cells are reprogrammed and as a result of this, changes occur in their differentiation capacities. For instance, MSCs which are isolated from a 30 years old individual's bone marrow are different from the MSCs which are isolated from a newborn's umbilical cord with their differentiation capacity. This difference is related to the ages of the cells, the factors they were exposed and their commitment levels. This distinction is supposed to be taken into consideration when thinking about MSC sources of the organism. The best studied source in an adult individual is bone marrow. Umbilical cord tissue and umbilical cord blood are also well known sources of MSCs [72]. Nowadays, isolation of MSCs from compartments such as adipose, muscle, dermis, trabecular bone, deciduous teeth, articular cartilage and periosteum has been known. However, MSCs isolated from different compartments can exhibit different behaviors [73].

One of the most richest MSC sources is bone marrow in humans. However, only 0,001 to 0,01% of the total number of the cells which carry nucleus are just MSCs in this environment. This is a reasonable diagnosis for two reasons:

1. MSCs are cells with enhanced self-renewal and proliferation capacity. Therefore, stimulation of MSCs for proliferation in cases of need is more acceptable rather than storing them in high amounts. MSCs are mostly found in G0 stage in vivo [74].
2. The tissues structured by mesenchymal stem cells show relatively slow turnover rates; for example, blood tissue is much more active than bone tissue. Thus, the existence of hematopoietic stem cells which give rise to blood cells in much higher numbers is a normal status.

The discovery of immunomodulatory properties of MSCs, provides important clues about their origins. Recent findings have shown the occurrence of MSCs as pericytes around wound areas [75]. Pericytes are relatively undifferentiated cells which secrete large quantities of immunomodulatory and immunotrophic factors in wound regions. These factors are very important for wound healing and angiogenesis. In addition,

their capacity to form fibroblasts, macrophages and smooth muscle cells has been discovered. This finding opens another subject to debate.

Different cells are found in different stages of differentiation. For example, pericytes are found between MSCs and fibroblasts up their differentiation stages. This is actually an imprecise but a true proposition. As discussed before, adipose tissue contains MSCs and fat-derived MSCs is an accepted fact. On the other hand, adipose tissue also contains fibroblasts. Regarding the differentiation stage hypothesis, fibroblasts are thought as terminally differentiated cells in the organism. However, as mentioned before, there are few examples which defend the fact that fibroblasts can differentiate. Based on these findings, can we tell “pre-adipocyte progenitor cells” to fibroblasts?

2.3.2.2 Fibroblast – a major member of skin

Human skin consists of two main layers. These are “epidermis” which is the outer surface of human skin and “dermis”, the layer beneath [76]. Epidermis is originated from ectodermal layers. 95% of the total cell populations are keratinocytes in epidermis. Apart from keratinocytes, epidermis contains melanocytes, Langerhan cells, Merkel cells and some other sense cells. Inflammatory functions are performed in this layer but the main task of epidermis is protecting the organism from effects of outer world. Epidermis performs its protection duty with the barrier formed by keratinocytes. These cells are capable to produce high amounts of extracellular matrix. They are originated from ectoderm layer. There are few recent studies reported the existence of keratinocyte stem cells in body [77]. Today, these stem cells are investigated to verify their existence and one step further, their usage as therapeutic agents.

The discovery of transdifferentiation skills of fibroblasts is also a very new matter in hand. Dermis is the layer located beneath epidermis. It possesses an abundant collagen content. It consists of two main compartments; papillary and reticular. These two compartments vary regarding their fibrillar content; papillary is a loose tissue whereas reticular is tighter.

Dermis tissue is very rich of collagen proteins. There are also other fibrillar proteins apart from collagens which give the flexibility to the tissue. Moreover, there exists a very dense extrafibrillar matrix in dermis. This matrix includes water, proteoglycans

and glycoproteins. Dermis contains 3 types of cells: Macrophages, adipocytes and fibroblasts; the main cell population is fibroblasts.

Fibroblasts are the most abundant cells in connective tissues. They have an advanced capacity to produce extracellular matrix. They get activated during skin growth or the cases of skin wounds. According to their status or locations, they might exhibit different phenotypes. This becomes very evident in their cell shapes [78]. They are generally spindle-shaped cells. However, fibroblasts can take a more rounded shape when they are activated in a wound case. Furthermore, amount of rough endoplasmic reticulum is much higher at their activated states [79]. This is very meaningful finding because since fibroblasts are primarily responsible for wound healing and extracellular matrix production, it is a necessity to have more rough endoplasmic reticulum regarding secretory pathways. Fibroblasts are very durable cell types with faster growth rates and longer life-spans.

Can Fibroblasts Differentiate?

Fibroblasts are originated from mesenchyme layer during embryonic development. Thus, they are not relatives of epidermal cells. However, there is “epithelial-mesenchymal transition hypothesis” (EMT) in literature [80]. This situation is characterized with loss of cellular adhesion, inhibition of E-cadherin expression and cell mobility increase in the cells. This process has very similar parts with invasion of cancer cells [81]. EMT is an important process for mesoderm formation during embryonic development. In adults, EMT was observed in stomach epithelium [82]. On the other hand, there are some “mesenchymal-epithelial transition” (MET) cases in literature [83]. MET indicates us that in appropriate cases, mesodermal originated fibroblasts might contribute to the formation of epithelia. This is a very important and interesting situation, because fibroblasts had been always considered as terminally differentiated cells [84].

Fibroblasts are located in dermis tissue with macrophages and adipocytes. These cells have a common ancestor. Moreover, fibroblasts are very similar in shape with MSCs. All these given data lead us to investigate differentiation skills of fibroblasts to adipocytes, osteoblasts and chondroblasts. The obtained results are very diverse. Yilin Cao and colleagues examined dermal fibroblasts about their differentiation potentials [84]. They cultivate the cells in vitro and treat them with different mediums for different differentiations. 6,4% of the cells showed all types of

differentiations, whereas 19,1 of the cells showed 2 kinds of the differentiations and 10,6% of the cells showed just one kind of differentiation. However, 63,9 of the total cells showed no differentiation ability. In addition, it has been reported that 1/3 of the tripotent cells (2,1% of total cells) also performed neurogenic and hepatogenic differentiation; this high transdifferentiation potential for fibroblasts is very thought provoking. But still, just 36,1% of the cells realized differentiation in the essay and this is a very small rate to talk about differentiation for a cell lineage. However, that is a fact that gives us clues about differentiation potentials of fibroblasts.

These findings have generated questions about the relationship between fibroblasts and adipocytes. The presence of fibroblasts in adipose tissue, and per contra, adipocytes in dermis layer is known. Besides, fibroblasts are in direct interactions with MSCs which are the cells forming adipocytes. In the case of wounds on body, these two cell types work together. A recent study of fibroblasts revealed that if they are cultivated with MSCs, their proliferation and migration skills tend to increase [85]. Are they also in relation with fat-derived MSCs in vivo? Do they contribute to adipogenesis in the body? If they are, how do they contribute?

Cells of connective tissues are in close and dense relations with their environments. Extracellular matrix compositions directly effect on the behaviors of the cells. The cells determined for adipogenic differentiation are usually found in a dense layer of extracellular matrix. Very different fibrillar proteins can be found in this layer, and this may affect the behaviors of progenitor cells. Therefore, an indirect influence of fibroblasts over adipogenesis can be mentioned since they are the main cell types forming extracellular matrix in connective tissues. In fact, a direct involvement to adipogenesis can be told for fibroblasts. The research which Giulio Alessandri and colleagues conducted has revealed that dermal fibroblasts have similar phenotypic features and differentiation potentials with fat-derived MSCs [86]. Accordingly, they shared some similar phenotypic profiles and displayed similar morphological appearance and growth rates. Moreover, they reported that both cell types which express mesenchymal markers, had adipogenic differentiation. This study is one of the rarest studies defending fibroblasts' adipogenic differentiation capacity. However, adipogenesis for fibroblasts is an important question still waiting to be answered today.

2.4 Adipogenesis

2.4.1 Cellular differentiation

Differentiation is a mechanism that allows the cells to take more specific tasks in the organism. Cellular differentiation continues during the entire life of the organism but particularly in development. Several phenotypic changes occur in the cell as a result. Metabolic activities of the cell, surface membrane proteins, responses to stimuli, cell mobility, the shape and size of the cell are some examples of these possible changes. An important point here is that the changes occur in phenotype of the cells, not genotypes [87]. In general, while all the cells of the organism carry the same genome, they can differentiate regarding the regulations of gene expressions. With this implication, differentiation can be mentioned as a result of the modifications of gene expression.

The cells with differentiation capacity are called stem cells in the organism. Stem cells are classified according to their differentiation capacity or the final cell type they differentiate. Zygote which is the first cell of human is a totipotent stem cell. It can differentiate into all cell types. Blastocyst cells are pluripotent cells which occur in early embryogenesis. These cells also can differentiate into all kind of human cells but they can not generate a new organism by themselves. There are 4 main stem cell types of an adult human [88]. These are muscle satellite cells, epithelial stem cells, hematopoietic stem cells, and mesenchymal stem cells. These cells are multipotent stem cells, they can differentiate into particular groups of cell types. This means that they have had a limited differentiation, but not complete. This classification system ends with unipotency and oligopotency.

Genes which are associated with the same function in the organism, are called panels or subsets. In a specialized cell, a variety of gene subsets are expressed instead of the entire genome [63]. Differentiation is a gene switch on and switch off process. Modifications, relations and regulations of genes are primarily involved in differentiation biology.

Cellular differentiation can be activated by different types of stimuli [90,91]. Signalling is the most common stimulation type in humans. Ligands, which trigger differentiation, are secreted to the extracellular environments and activate differentiation at the cells that carry appropriate receptors. Another important stimulation mechanism is direct interactions. These interactions might exist as cell-to-cell or cell-to-matrix. Today, extracellular matrix proteins are known to influence differentiation. There are also epigenetic factors effecting on differentiation [92].

2.4.2 Adipogenic Differentiation

Two main adipogenesis events occur in humans. The first one is the dense BAT formation which begins in late pregnancy and the accelerated adipose tissue formation in neonatals. This is called early adipogenesis [44]. Today, since there are ethical restrictions over the researches made with embryonic stem cells, most of the knowledge we have about adipogenesis are belong to adult adipogenesis.

In adults, multipotent stem cells are first isolated from bone marrow. After that, skin, muscle and brain were also used as multipotent stem cell sources [93]. Today, adipose tissue also has been accepted as an important source of multipotent stem cells [94]. Several studies reported that the multipotent stem cells isolated from adipose tissue could even differentiate into cardiomyocyte-like cells [95], insuling-secreting cells [96] and endothelial-like cells [97]. Late adipogenesis have been realized by multipotent stem cells like adipose tissue-derived MSCs.

Adipogenesis is a complex process that many transcription factors involve. There occur many adipose-related gene activations. Adipogenesis is associated with early-middle-late mRNAs and protein markers [98,99]. However, this distinction is not sharp. Genes of different stages are expressed together. Adipogenesis is one of the most sophisticated genetic reprogrammings in the organism. Many inhibitory or stimulatory transcription factors play important roles (Fig. 2.7).

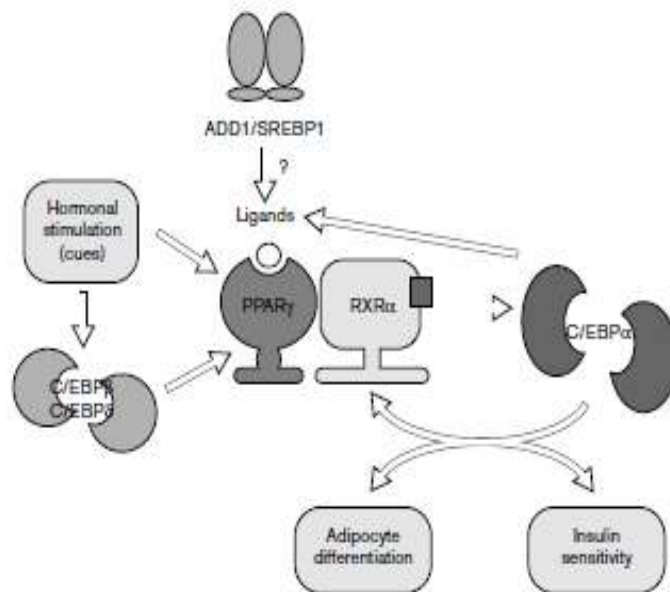


Figure 2.7: Regulatory cascade controlling adipogenesis. Adipocyte differentiation involves the interaction between and regulation of transcription factors C/EBPs and PPAR γ . C/EBP β and C/EBP δ are induced early during adipogenesis, in response to hormonal stimulation. This is followed by the induction of PPAR γ and C/EBP α . There is a positive feedback loop between C/EBP α and PPAR γ that appears to be important for the maintenance of the differentiated state. C/EBP α also appears to play a role in controlling insulin sensitivity in adipocytes. ADD1/SREBP1 may enhance PPAR γ activity through the generation of ligands for this receptor, which forms a heterodimer with retinoid X receptor (RXR) [163].

In adipogenesis, first of all, the cell is removed from cell cycle and enters into differentiation mood in stead of proliferation mood. This is called growth arrest. This change is achieved by the activation of CCAAT-enhancer binding protein (C/EBP β and δ). Activated C/EBP β and C/EBP δ directly binds to DNA, and stimulate the expression of CDK inhibitor p21 [100]. The accumulation of p21 causes hypophosphorylation of retinoblastoma proteins in the cell. Retinoblastoma protein is a tumor suppressor protein which is activated by hypophosphorylation. As a consequence of these events, the cell stops cell cycle.

On the other hand, C/EBP β and C/EBP δ activation leads to an increase in the expression of peroxisome proliferator-activated receptor- γ (PPAR γ). There are different variants of PPAR γ in the organism. These variants might have effects on adipogenesis [101]. PPAR γ increases the expression of C/EBP α in the cell [102]. Adipocyte determination and differentiation factor-1/sterol responsive element-binding protein-1c (ADD1-SREBP-1c) however increases the expression of PPAR γ

in the cell [103]. Today PPAR γ has been considered as a key factor for adipogenesis; and C/EBP α has been thought as a factor that contributes to the expression of PPAR γ to keep it at desired levels [102].

Zinc-finger DNA binding proteins GATA-2 and GATA-3 also involve in adipogenesis [104]. They serve with their suppressant characteristics. GATA-2 and GATA-3 synthesis in progenitor cells holds the cell at pre-adipocyte stage. In a research, genetically obese mice have grown from GATA-3 deficient embryonic stem cells. Individuals were prone to develop adipose tissues. In another study, overexpression of cAMP responsive element binding protein (CREB) in active form was sufficient to induce adipogenesis [105]. Accordingly, cAMP can be mentioned as a modular that regulates adipogenesis. Many other growth hormones, cytokines and factors are reported which have modulatory effects on adipogenesis.

Adipogenesis is a gene expression regulation event. Today, PPAR γ , perilipin and adiponectin are the proteins/markers which are identified with directly adipogenesis. These proteins are used to recognize adipocytes. A better understanding of the mechanisms and interactions of these proteins will be very descriptive about details of adipogenesis.

2.4.2.1 PPAR γ

Peroxisome proliferator-activated receptors (PPARs) are members of type-2 nuclear hormone receptor family. They form heterodimer structure with retinoid X receptors (RXR) [106]. 3 subtypes of PPARs are known: PPAR α , PPAR γ and PPAR δ . PPAR γ among these forms is directly related with adipogenesis [107]. PPAR γ gene is found on chromosome 3, the location 3p25 [164]. Humans have 3 forms of PPAR γ . While PPAR γ 1 is found almost in all tissues except muscle, PPAR γ 2 is only found in adipose tissues. Between these two forms, there is a 30 amino acid difference at their N-terminal end, PPAR γ 2 is longer than PPAR γ 1. It is believed that these forms of PPARs arise as a result of RNA splicing.

PPAR γ is activated before many adipocyte specific proteins. Studies have shown that only PPAR γ expression is sufficient for growth arrest [108]. As noted before, PPAR γ takes role with C/EBP α . Transgenic animal studies have shown that PPAR γ knock-out mice have revealed a sharp decrease in the expression of C/EBP α , whereas PPAR γ expression is very slightly effected in C/EBP α knock-out mice [102].

Furthermore, overexpression of PPAR γ tolerates the defect of C/EBP α . These findings indicate the supporting role of C/EBP α and the leading role of PPAR γ in adipogenesis.

Today, synthetic and natural PPAR γ ligands are identified. The drug “thiazolidinediones” is one of these ligands. After binding, this drug activates PPAR γ , and enhances insulin sensitivity of the organism. This action stimulates adipose conversion [109]. The most surprising information about thiazolidinediones is its strong effect on fibroblasts [110].

The effects of saturated and unsaturated fat on cardiovascular health is an issue that scientists work on for many years. According to general belief, saturated fatty acid is more dangerous for our health. However, a study suggests that unsaturated fatty acid might be dangerous as much as saturated one [111]. Unsaturated fatty acid stimulates adipogenic differentiation of pre-adipocytes more than saturated fatty acid. It is thought that unsaturated fatty acid performs this influence by acting as a PPAR γ ligand precursor in the organism.

Though other PPAR family members are not considered as functionary in adipogenesis, their abundant expressions in adipose tissues lead scientists to investigate their potentials in adipogenesis [112]. Until now, many proteins have been found in interaction with PPAR γ [164]. Given duties of these proteins show us how important PPAR γ is for adipogenesis.

2.4.2.2 Perilipin

One of the most characteristic aspects of adipocytes are the significant lipid droplets they contain. These subcellular structures are covered with a phospholipid monolayer. Lipid droplets do not just serve as triacylglycerol storages, they also play role in cellular lipid metabolism [113]. Today, they are considered as organelles which exist in many eukaryotic cell types [114]. Lipid droplet biogenesis is not a subject well clarified. As well as the formation of golgi apparatus, there might exist proteins which interact with surface membrane and give rise to lipid droplets. Proteomics studies identified proteins which are involved in lipid droplet biogenesis and metabolism. In mammals there are diversified proteins on lipid droplets which function in lipid metabolism and membrane traffic. However, great majority of these

proteins are PAT (perilipin/ADRP/TIP47 – perilipin super family) protein family members [115].

Phosphoprotein perilipin is the main member of PAT family, selectively expressed only in adipocytes and steroidogenic cells. They are essential for cAMP-dependent lipolysis via protein kinase A (PKA) [116]. They prevent the access of lipase enzymes to reach to lipid droplets when the cell stands in basal conditions. In humans, epinephrine hormone is secreted to blood stream in cases such as increased heart rate and decreased insulin secretion. These events cause a higher energy demand in organism. β -adrenergic receptors can be activated by epinephrine and their activation leads to phosphorylation of perilipin and hormone sensitive lipases (HSL) in the cells and prepare the conditions for lipolysis. Because phosphorylated perilipins cease holding the lipid droplets and with the new appropriate circumstances, HSLs which are activated by phosphorylation anchor to lipid droplets [117].

Perilipin genes are dispersed in human genome [161]. Today, 5 different perilipin subtype is defined in humans. These subtypes can be mentioned either with numbers or letters. Perilipin 3 (perilipin C) gene is located on chromosome 19. Perilipin 3 proteins interact with mannose 6-phosphate receptors (MPRs) and assist to the transfer of lysosomal hydrolase enzymes from golgi apparatus to endosomes. Perilipin 4 and perilipin 5 are generally considered as lipid droplet coating proteins. They are expressed from perilipin 3 gene and taken their latest status with splicing. These proteins are not adipocyte specific markers.

Perilipin 2 (perilipin B) gene is located on chromosome 9. Although the protein serves in lipid droplet metabolism, it is not considered as an adipogenic marker since it is expressed in different types of cells. Cell cultivation studies revealed that perilipin 2 proteins are encountered in Sertoli cells, epithelial and endothelial cells .

Perilipin 1 (perilipin A) is an adipogenic marker for humans, it is just synthesized in adipocytes. The gene is found on chromosome 15, the location 15q26. It is a basic substrate of cAMP-dependent protein kinase. Therefore, after a signalling series which activate this kinase, it gets phosphorylated by the enzyme [116]. This event allows the access of lipases to lipid droplets and starts lipolysis.

Gouri Ranganathan and colleagues reported expression of perilipin might trigger and increase obesity [118]. On the other hand, this might also be mentioned as increased gene expression of perilipin is found in cases of obesity. However, Ranganathan could not find a direct relation between perilipin expression and insulin resistance. The question is, does which one trigger the formation of the other one? As a result, perilipin, as a causing factor of obesity, is an issue waiting to be understood.

There is an interesting subject needs to be told at this stage. Mesenchymal stem cells (regardless they are derived from bone marrow or adipose tissue) and fibroblasts are spindle shaped cells. Adipogenesis makes the cells spheroidal shaped. In addition, with increasing adipogeny, lipid droplets occur in the cells that can be seen under microscope. These are phenotypic factors that allow the selection of adipocytes. However, as is understood from given statements, perilipin 2 expression and lipid droplet formation can occur in the cells which are not adipocytes. This prevents the use of lipid droplets as adipogenic markers. Microscope images of oil particles need to be verified with gene expression analyses. Nevertheless, perilipin 1 expression is an important adipogenic marker.

2.4.2.3 Adiponectin

Adiponectin (Acrp30, apM1, GBP28, AdipoQ) is the most commonly synthesized protein in adipose tissues. In a given time, adiponectin composes 0,01% of the total proteins in human's circulatory system. This concentration is even 1000 times bigger than the most frequently produced hormone [119]. Adiponectin is only synthesized in adipose tissue (Fig. 2.8). Because of the explained features, adiponectin was first called adipose most abundant gene transcript (apM1).

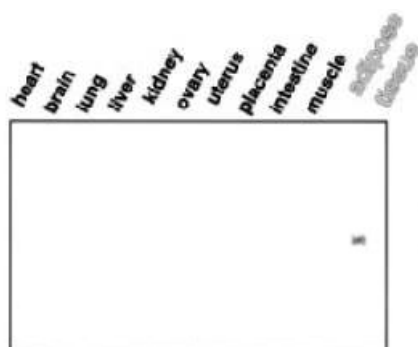


Figure 2.8: Adiponectin expression profile in human. By using northern blotting, different human tissues were investigated to find adiponectin mRNA. It is just observed at adipose tissue (modified [165]).

Adiponectin is secreted into blood stream by WATs. In obesity and type-2 diabetes, plasma adiponectin levels are reduced and insulin resistance increases [119]. It takes active roles in glucose metabolism and fatty acid catabolism [38]. It stimulates NO synthesis from endothelial cells [120]. In type-1 diabetes, high plasma adiponectin levels are encountered and this indicates that adiponectin supports glucose intake [121]. Adiponectin blocks protein kinase-A (PKA) activity by effecting on platelet-derived growth factor subunit B (PDGFB) in smooth muscle cells. As a result, cell proliferation and migration stop [122]. What is more, adiponectin has important immunomodulatory functions in the organism [120]. In various cancer types, serum adiponectin levels remain lower than normal [123]. Lipid metabolism is just one the functions of adiponectin and these data offer that adiponectin takes on many other important duties in humans.

Adiponectin is a product of apM1 gene. This gene is found on chromosome 3, the location 3q27 [162]. It has 3 exons and 2 introns. Expression of adiponectin is under control of various intracellular and extracellular factors such as C/EBP β , PPAR γ , Nuclear factor-Y (NF-Y), insulin activity and β -adrenergic stimulation. Its synthesis is higher in SATs [124]. It includes 4 different subunits. These are the signal sequence at N-terminal part, the variable domain beside, a collagen-like domain and lastly, a globular domain. Within this structure, adiponectin resembles to tumor necrosis factor – alpha (TNF- α) and complement factor C1q [125]. Adiponectin molecules are attached together and structure a polymer form in circulation. However, adiponectin polymer forms differ between women and men.

Although there are myriad of receptors affected by adiponectin in the organism, just 2 adiponectin receptors were cloned [126,127]. These are ADIPOR1 and ADIPOR2 proteins. Activation of these molecules induces PPAR α and AMP-activated protein kinase (AMPK) activation in the cell. AMPK is an important cellular enzyme for energy homeostasis which is actively produced in several tissues. It inhibits cholesterol synthesis whereas stimulates fatty acid oxidation and lipogenesis. Furthermore, AMPK serves as a regulatory factor in insulin secretion. Surprisingly, It can either inhibit lipolysis and lipogenesis in adipocytes [128]. This means adiponectin can prevent adipose tissue expansion in organism. Therefore, it is believed that only terminally differentiated adipocytes synthesize adiponectin.

2.4.3 PPAR γ , perilipin and AdipoQ – early or late markers?

Though these proteins have very different functions and features, the common character they share is their belonging to adipose tissue. PPAR γ is very active at initiation and maintenance of adipogenesis. Perilipin is very important for structural transformation of the cell. By supporting lipid droplet constitution in the cell, they provide important contributions to adipogenic phenotypes. Adiponectin is even explanatory in itself about how important adipose tissues are for humans. It is one of the most important tools for adipocytes to perform their tasks in organism.

Accordingly, PPAR γ is a protein which is involved in both early and late stages of adipogenesis. They are associated with differentiation of adipocytes rather than their adipogenic duties in the organism. Thus, PPAR γ expression in the cell is expected to be in stable levels. Perilipin expression starts right after settling the appropriate conditions in the cell which allow the cell to produce lipid droplets. Depending on the lipid metabolism in the cell, perilipin expression would increase or decrease. Adiponectin is an instrumental protein for adipocytes to carry out their duties. Therefore, only completely differentiated adipocytes are expected to synthesize adiponectin molecules and this leads adiponectins to be known as late markers.

2.5 Extracellular Matrix

2.5.1 What is extracellular matrix?

Extracellular matrix (ECM) layer is one of the most important features of connective tissues [129]. It is classified under two types. The first one is interstitial matrix. This matrix can be considered as a lute material between cells. It is formed by proteoglycans, non-proteoglycan polysaccharides and fibrous proteins which are the molecules that cells secrete to extracellular environments or directly locate onto their surfaces. These molecules fill the interstitial space. Second group of ECM is known as basement membrane [130]. It is the large fibrillar structure lying beneath epidermis layer.

ECM structure differs according to its location in the organism. The organs, tissues and cells that ECM is in contact are determining about this issue. Basement membrane occurs between epidermis and dermis layers. In addition, outer surfaces of many organs and blood vessels are surrounded by basement membrane. It is an

advanced pool of mostly fibrillar proteins. The merger of basal lamina that is formed by epidermis cells and reticular lamina which is formed by reticular cells, composes basement membrane structure. Reticular cells are special fibroblasts. Adipose tissues are held together by reticular fibers and so basement membrane [131]. The most abundant proteins in basement membrane are collagen fibrils and laminin proteins [129].

Interstitial matrix serves as the filling material in connective tissues. Skin hypodermis layer is very abounding in interstitial matrix. It is especially produced by fibroblasts. It separated the tissues. Moreover, it controls intercellular communication in these tissues. It is vital for processes such as growth, fibrosis, wound healing [132]. As conclusion, interstitial matrix can be thought as a workspace for cells.

ECM layer is crucial for growth; it enables signalling traffics of cells. Today, the unique importance of ECM for development is started to be understood. This is related with the included molecules about how they affect other cells. For example, adipocytes and progenitor cells are surrounded by a thick ECM layer in adipose tissues. The discovery of paracrine and endocrine features of adipocytes proves us how important this layer for the continuity of the organism. Because adipocytes need ECM to realize their duties. But on the other hand, are there effects of ECM on progenitor cells? This is the question of whether ECM regulates or disturbs differentiation in the organism. Above all, differentiation is a very complicated process which can be effected by many factors, and it would be normal if behaviors of progenitor cells are effected, since ECM is a layer which they are in continuous contact. Today it is a matter of interest if the protein content of ECM influences adipogenic differentiation in humans.

2.5.2 Proteins of Extracellular Matrix

ECM proteins are secreted by exocytosis from the cells [133]. In addition, extracellular surface proteins might also involve in ECM. Fibrillar collagens are the main proteins of this layer [132]. Also, fibrillar elastins are found in big amounts in ECM, they give the elastic nature to ECM. By the attribute of elastins, ECM can return to its original state after being bended [134]. The most important non-fibrillar

proteins are fibronectins in ECM. Besides, laminins are also another important non-fibrillar proteins in this layer.

2.5.2.1 Collagens

Collagen is an animal protein which is especially found in connective tissues of mammals. It is the most abundant protein and very vital for the survival of organisms [135]. From contractions of muscles to seeing process, collagens are involved in many cases.

Collagens is a protein family which share a similar trimeric structure and functional properties [136]. There are 5 subgroups of collagens. These are fibrillar collagens (type I, II, III, V, VI collagen), short chain collagens (type VIII, X collagen), basement membrane collagens (type IV collagen), fibril-associated collagens with interrupted triple helices (FACITs) (type IX, XII, XIV, XVI, XIX collagen) and membrane-associated collagens with interrupted triple helices (MACITs) (type XIII, XVII collagen). All collagen subtypes contain triple helical domains, which are in left-handed helix forms. These domain formations are generally structured by Gly-Pro-X or Gly-X-Hyp repeat amino acid sequences. Glycine is known as the smallest amino acid which does not have a side group. Therefore, it is seen in every third position in collagen helices. This triple structures create the appropriate conditions for hydrogen bonds and provide the stability of collagen fibrils.

The most abundant form of collagens is type-I collagens. It is the main component of organic parts of bones [137]. Moreover, collagen type-I constitutes endomysium structures in muscles and therefore, it is very important for healthy functions of them. This protein is also in abundant in tendons [138]. They are mainly synthesized by fibroblasts. In wound healing cases, there occurs an excessive increase in collagen production. The abundant occurrence of collagen type-I proteins in organic parts of bones brings the idea if they can effect on cellular differentiation of mesenchymal stem cells.

Collagen type-IV is located in basement membrane [139]. Glycine amino acids which are required in every third position for a tight structure do not exist in collagen type-IV subunits. This causes a sheet-like structure for these proteins in stead of a helical structure. Thus, formation of basal lamina occurs.

The effects of collagens (collagen type-I) have been identified over osteogenesis [21]. Considering the important role of basement membrane in maintaining the adipose tissue formation, the possible effects of collagen type-IV on adipogenic differentiation would not be a surprise.

2.5.2.2 Fibronectins

Fibronectins are found in glycoprotein form in ECM; they are big molecules with an average weight of 450kDa. Fibronectins can bind collagens and at the same time, they can bind cellular surface proteins, integrins and allow the cell movement. Fibronectin proteins have relatively complicated structures. They are found in dimeric formations [140]. The dimeric subunits are formed in 3 modules and these modules are formed in 2 β -sheet peptides. These modules determine the functions of fibronectins. Different forms of fibronectins can be produced by alternative splicing. Two main types of fibronectins are synthesized in humans which are soluble and insoluble. Insoluble form is one of the basic components of ECM and they are mainly synthesized by fibroblasts.

Fibronectins are involved in the formation of thrombus (blood clot) in wound areas [141]. Construction of thrombus is the last step of blood coagulation. The separation of soluble and insoluble fibronectins are very indicative in this process. Soluble fibronectins give rise to thrombus structure. However, in wound healing stage, fibroblasts start to excrete protease enzymes to the area for recovery. This application causes the general digestion of the proteins in that area. After that, exchange of plasma fibronectins with cellular fibronectins which are insoluble are done by fibroblasts. This mechanism is essential for wound healing and furthermore, ECM assembly.

The effects of fibronectins in cell movement lead the scientists to investigate their possible roles over invasions of cancer cells. However, before invasion, their roles in carcinogenesis were discovered [142]. Jesse Roman and colleagues found the stimulation of gonadal steroids by fibronectins in their research. Gonadal steroids interact with vertebrate androgen receptors and control the expression of cyclin D in the cells. Therefore, fibronectins can indirectly change the cell cycle status of the cell. In another study with fibronectin knock-out transgenic mice, lack of embryogenesis has been showed [143]. Deprivation of fibronectin leads to the

defects in mesodermal and vascular development. It is a matter of wonder if fibronectins with such important roles on growth might have effects on cellular differentiation.

2.5.2.3 Laminins

Laminin proteins are located in basement membrane in trimeric structures. Occurrence of α , β and γ chains allows the diversity of laminins [144]. They create spider web-like networks instead of fibrillar forms. They can interact with ECM molecules or cell surface proteins. Collagen type-IV is one of these molecules.

Laminins play roles in cell attachment [145]. They are very important in terms of cell shape and cell motility. Moreover, laminins are the only proteins whose relation with cellular differentiation has been demonstrated among the mentioned ECM proteins in this work. They are commonly used in cell cultures [146]. It is possible to make pluripotent cell cultures with the usage of laminins. Laminins are important tools for understandings of cellular interactions with extracellular matrix. The uses of laminins in cell culture essays led to the studies which investigate the effects of laminins on differentiation. However, the findings over this issue are observational discoveries, intracellular effects are not well known yet today. However, in some neural development experiments, few clues were found about their intracellular activities. Accordingly, it is thought that laminins cause changes in the level of intracellular cAMP amounts [147].

3. MATERIALS AND METHODS

In this research, the main subject was understanding the adipogenic differentiation potentials of mesenchymal stem cells (MSCs) and dermal fibroblasts. According to this intention, the cell lines were cultured in adipogenic differentiation and control mediums. In addition, cultivation of MSCs in coated vessels with extracellular matrix (ECM) proteins were done. Lastly, two main detection techniques were performed:

- Quantitative real time polymerase chain reaction (qRT-PCR)
- Immunofluorescence Staining

3.1 Materials and equipment

3.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A

3.1.2 Compounds, kits and buffers

The compounds and commercial kits used during the study are listed in Appendix B; the recipes of buffers and media used in the experiments are given in Appendix C.

3.2 Methods

3.2.1 Cell culture and differentiation

3.2.1.1 Isolation of MSCs

Two mesenchymal stem cell strains derived from bone marrow (BM72 & BM78), one from adipose tissue (LA47) and one human forehead dermal fibroblast culture (DF24) were provided by Karen Bieback and colleagues from Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, University of Heidelberg. In short explanation, concerning the ethical issues [73], bone marrow aspirates and lipoaspirates were treated with a series of digestion, dilution and centrifugation steps in order to assort the cells. Afterthat, for the suspension of MSCs, cell isolates were seeded in MSC-Growth Medium (Lonza, Cologne, Germany) at a density of 1×10^6 cells/cm² into T75 or T175 culture flasks (Nunc, Rochester, NY, USA; Greiner, Frickenhausen, Germany). After the first medium change, non-adherent cells were removed and fibroblastoid cells kept proliferating in culture.

3.2.1.2 Preparation of ECM coated culture vessels

In order to perform adipogenic differentiation on ECM-proteins, culture flasks were coated by collagen type-I (rat tail tendon; Roche), collagen type-IV (human placenta; Sigma-Aldrich), fibronectin (human plasma; Roche) and laminin (mouse EHS-tumor; Roche) proteins.

Laminin was supplied 0,5 mg/ml in 0,15 M NaCl, 2 mM EDTA and 0,05 M Tris HCl solution (pH 7,4). Flask surface was incubated with 5 µg/cm² laminin for 1h at 37°C. Solution was aspirated from the vessels and cell were added.

Fibronectin was diluted with 5 ml sterile water for 1 mg/ml last concentration. Afterwards, flask was coated with 5 µg/cm² solution for 1h in incubator at 37°C. Then solution is removed very gently and vessels is washed for one time with medium and cells were added. Collagen type-I was dissolved in 0,2% acetic acid for the last concentration 2 mg/ml. Coating was performed with 5 µg/cm² solution in incubator at 37°C for 1h.

Then collagen solution was aspirated and surface was washed with PBS for 2 times. Afterwards, cells were added. Collagen type-IV coating was made with the same conditions with collagen type-I coating. However, dissolving collagen type-IV is a harder process, thus, dissolution in the solution took several hours.

3.2.1.3 Cell culture and proliferation

MSCs were proliferated in MSC-GM whereas fibroblasts were cultured with 1x DMEM medium containing 10% FCS and 1% penicillin/streptomycin. Incubations were performed in humidified atmosphere at 37⁰C, 5% CO₂, 95% air in T175 culture flasks (Greiner Bio One, North Carolina, USA).

Cells were split until we had enough number of cells for the experiments. For this purpose, 1 x trypsin-EDTA (PAA) solution was used for passaging the cells. The cultures which reached 70-80% subconfluency were treated with 1 x trypsin-EDTA solution for one minute and moved to fresh media. The media include inactivating agents for the enzyme, thus trypsin in the solution gets inactivated. This is very important because, trypsin is a protease which cleaves peptid residues [148]. In biotechnology, we use this enzyme to re-suspend the cells from vessel surface. However, treatment the cells more than one minute causes the cleavage of the cell surface proteins by trypsin and this is harmful for the cells.

3.2.1.4 Cell Counting

Regarding the standardization concerns, the number of the cells which are used for the cultivation of differentiation is an important issue. Cultures need to be initiated with the same number of cells, therefore population density will effect the behavior of the cells in the same manner. According to this, the cells were seeded after counting.

The number of the cells which were used for seeding was counted using an automatic cell counting system (Fig. 3.1, Vi-Cell XR Cell Viability Analyzer, Beckman Coulter, Miami, USA). This system uses conventional trypan blue dye exclusion method. However, the well automated system takes 50 photographs of each cell suspension and gives the average number of the cells. It indicates the viability ratios of the cells indeed. Accordingly, 1000 µl cell suspensions were counted with dilution factor 2.0.



Figure 3.1: Vi-Cell XR Cell Viability Analyzer, Beckman Coulter, Miami, USA (www.beckmancoulter.com).

3.2.1.5 In vitro differentiation of MSCs and fibroblasts

MSCs and fibroblasts were seeded to the flasks for differentiation experiments. The initiation number of MSCs were 2×10^4 cells/cm² whereas fibroblasts were $1,5 \times 10^3$ cells/cm² in T25 culture flasks (Greiner Bio One, North Carolina, USA). On the other hand, fibroblasts were seeded onto glass coverslips (SPI supplies, West Chester, USA) with $0,9 \times 10^5$ cells/ml (3ml in total) for immunofluorescence staining purpose.

All the cells were kept in growth media until they reached to confluency. The necessity of growth arrest for differentiation has been mentioned before [63]. The cells can not differentiate if they keep proliferating. Therefore, it is a must to wait untill confluency in cell culture. When the cells reach to confluency, they are ready to start differentiation.

After observation of 80% population concentration in the flasks, adipogenic differentiation was initiated by adipogenic induction medium (AIM, Lonza). This medium contains adipogenesis stimulation factors such as dexamethasone, indomethacin, recombinant human insulin and 3-isobutyl-1-methylxanthine (IBMX). 3 days later, AIM was removed and cells were treated with adipogenic maintenance medium (AMM, Lonza) for two days. This medium lacks the differentiation factors IBMX, dexamethasone and indomethacin. This set of treatments were done for three times during 14 days. The daily schedule of differentiation essays is listed in Table 3.1 via fibroblasts.

During the experiment, unstimulated control groups were kept in growth media.

Table 3.1: Adipogenic differentiation plan of fibroblasts.

	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13	d14
A.D.	AIM	AIM	AIM	AMM	AMM	AIM	AIM	AIM	AMM	AMM	AIM	AIM	AIM	AMM	AMM
FibAD1	>	>	X*												
FibAD2	>	>	>	>	>	X									
FibAD3	>	>	>	>	>	>	>	X							
FibAD4	>	>	>	>	>	>	>	>	>	>	>	>	>	>	X
FibAD5	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>
Control	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13	d14
	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM	GM
FibC1	>	>	X												
FibC2	>	>	>	>	>	X									
FibC3	>	>	>	>	>	>	>	X							
FibC4	>	>	>	>	>	>	>	>	>	>	>	>	>	>	X
FibC5	>	>	>	>	>	>	>	>	>	>	>	>	>	>	>

*X refers to sampling days. A set of 3 days AIM and 2 days AMM treatments were performed for three times

3.2.2 RNA isolation and reverse transcription reaction

3.2.2.1 RNA isolation

RNA isolation is the first and one of the most important steps of a successful gene expression analysis. Since RNA is a very diversified and sensitive molecule, it needs to be handled very carefully.

In this study, after trying a few RNA isolation kits, we decided to use Qiagen RNeasy Kit (Qiagen, California, USA) regarding to its performance. At sampling times, cells were washed with 5 ml PBS (PAA) for two times and lysed in 600 µl RLT buffer (Qiagen) including 0,6 µl β-mercaptoethanol (Applichem). This is a special solution of the kit to loosen the cells from surface. During the exposure with RLT buffer, surface is disturbed with a sterile spatula for an efficient gathering of the cells. This cell suspension is filtered in Qiashredder spin-columns (Qiagen) to homogenize the cell lysates. Afterwards, the filtered solution is treated with a set of buffers in RNeasy mini spin columns (Qiagen) to separate and reserve the total RNA content of the cells. Eventually, RNAs are solved in 40 µl RNase-free water (Qiagen).

3.2.2.2 Quantification of the isolated RNAs

RNA is a very unstable molecule. It is open to the negative effects of RNase enzymes which is located almost everywhere and it is very easy to lose RNA molecules during the processes. Thus, we need to be sure about the healthy existence of RNAs in our tubes. On the other hand, for a standart reverse transcription reaction, same amount of sample RNAs need to be used. Therefore, a healthy measurement of these RNAs is a vital process for the fate of the study.

According to the concerns, sterile working issues had been followed especially by using RNase disinfectant solution (RNase-ExitusPlus, Applichem, Denmark) on the working areas. RNAs are measured with Nanodrop ND 1000 V 3.3 (Thermo Scientific, Wilmington, USA) spectrophotometer.

3.2.2.3 Reverse transcription (rt) reaction

After some trials of different reverse transcription reaction kits, Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) was chosen. 1 µg RNA was determined as the standart amount of RNA for every reaction. 1 µg RNA was reverse transcribed using 1 µl anchored-oligo(dT)₁₈ primer (2,5 µM).

The cDNA was snythesized from total RNA using the high fidelity kit in two steps. In first step, reaction is performed to open RNA molecules and create efficient conditions for the proper annealing of the primers. In second step, reverse transcription of the RNA into cDNA is performed by the enzyme.

In a 20 µl standart reaction tube, there are 4 µl of buffer (5 x concentration), 0,5 µl of RNase inhibitor (40 U/µl), 2 µl of dNTP (10 mM each), 1 µl of DTT (5 mM), 1,1 µl of transcriptase enzyme (10U/l), 1000 ng RNA and enough PCR degree water. Overview of the reaction is presented in Figure 3.2. Reaction conditions are mentioned in Table 3.2.

Table 3.2: Conditions of rt reaction for quantitative PCR.

Amount Concentration Element			
STEP - I	Variable	1000 ng	RNA
	1 μ	2,5 μ M	Primer
	Variable	-	Water
Total	11,4 μ l		

Amount Concentration Element			
STEP - II	4 μ l	5 x conc.	Buffer
	0,5 μ l	40 U/ μ l	RNase Inh.
	2 μ l	10 mM each	dNTP Mix
	1 μ l	5 mM	DTT
	1,1 μ l	10 U	Transcriptase
	11,4 μ l	-	Reaction tube
Total	20 μ l		

10 Min' at 65⁰C Incubation

30 Min' at 55⁰C & 5 Min' at 85⁰C

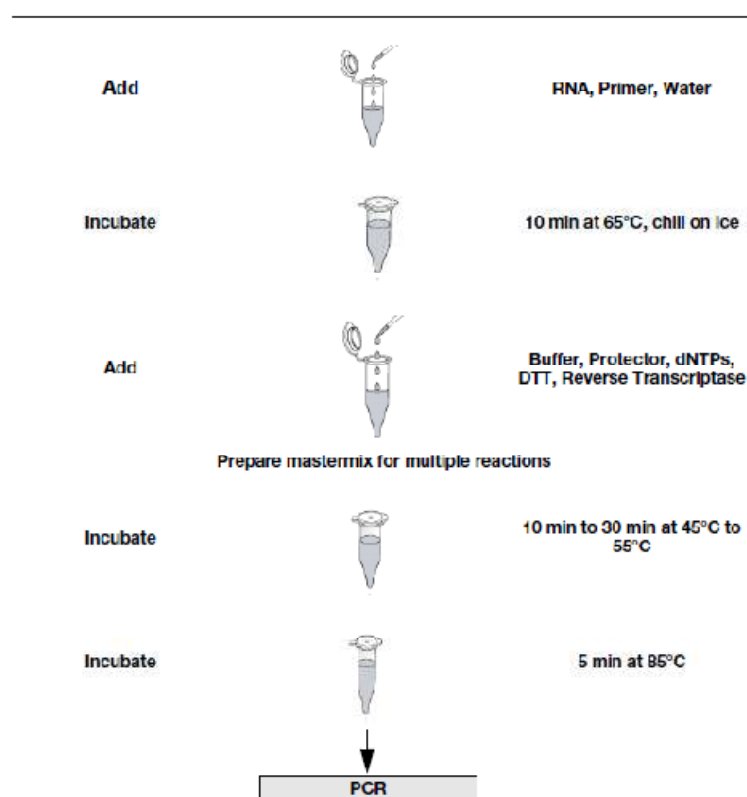
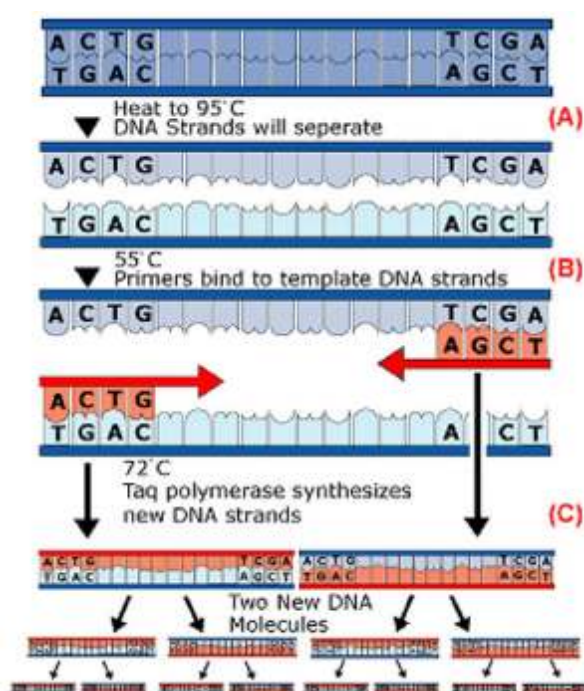


Figure 3.2: Main steps of the rt reaction (from Transcriptor High Fidelity cDNA Synthesis Kit manuel).

3.2.3 Quantitative real time polymerase chain reaction (qRT-PCR)

3.2.3.1 A brief overview of qRT-PCR

The basic polymerase chain reaction (PCR) is a molecular biology technique which allows scientists to amplify the demanded DNA sequence in vitro [149]. Accordingly, a special thermostable polymerase enzyme catalyzes the amplification reaction with the guidance of specific primers (Fig. 3.3). Template DNA and four main type of deoxynucleoside triphosphates (dNTP) are needed for the newly synthesized millions of strands. There are also additional compounds to support the reaction such as buffers, ions and performance-enhancing molecules.



With time, very different and advanced types of PCR techniques have been developed [150]. Some progressions even let the scientists to analyze their target sequences via RNA molecules. For this purpose, with a special enzyme, RNA template is converted into DNA molecules and it is called complementary DNA (cDNA). This started to be achievable after the discovery of in vitro usage of reverse transcriptase enzyme [151].

Figure 3.3: PCR starts with the denaturation of double strand DNA. After denaturation, primers anneal to their complementary sequences on single strands. Synthesis of the new strands is catalyzed by special polymerases. In every reaction turn, two daughter double-stranded DNA is produced from the parent strand.

cDNA synthesis from RNA molecules is called reverse transcription reaction. This is a milestone finding for molecular biology because following the gene expressions inside the cell on real-time is done today by rt reaction.

Real-time polymerase chain reaction is a kind of PCR with an additional feature that includes continuous collection of fluorescence signals from every reaction turn. So, RT-PCR contains special probes and fluorophore systems in addition. The analysis of the fluorescence signals in every each turn and their conversions into numerical values by a developed software make the reaction quantitative [152]. Today, there are several types of different probe & signalling systems in use. For instance some hybridization probes, which bind to the target sequences, start to give fluorescence signals after hydrolysis during the PCR. On the other hand, there are special fluorescence dyes which only bind DNA double-strand non-specifically. This means, the more synthesis of double-stranded DNA in reaction tube, the more stronger fluorescence signals the system detects [152]. LightCycler 480 Real-Time PCR and Universal ProbeLibrary systems are used for quantitative gene expression analyses in this study (Roche Diagnostics, Mannheim, Germany).

3.2.3.2 Locked Nucleic Acid (LNA) based probes

The term, probe, refers to an oligonucleotide sequence in molecular biology. The most common types of qRT-PCR is done with a probe system which contains a signal molecule on the 3' end and a dark quencher dye on the 5' end of the molecule. The quencher molecule absorbs excitation energy (the signal in this case) from the fluorophore molecule as long as the probe stands as a whole. However, the probe binds to its complementary sequence which is located in somewhere but on the way of DNA polymerase enzyme. As a matter of fact, probe is removed and fragmented by the enzyme regarding its 3'-5' exonuclease activity. Disintegration of the probe causes the separation of fluorophore and quencher molecule, and therefore it gets possible to collect the signals from reaction tube (Fig. 3.4). The signal strength shows a logarithmic increase after every qRT-PCR turn if the reaction goes well.

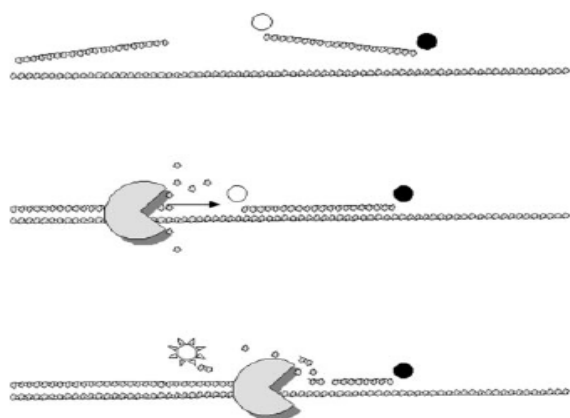


Figure 3.4: White circle refers to the fluorophore molecule whereas black circle refers to the dark quencher dye. After the disintegration and fragmentation of the probe by the enzyme DNA polymerase, it gets possible to collect the signals from reaction tubes [150].

Universal ProbeLibrary system (UPL) consists of 165 short hydrolysis probes which are designed with the mentioned probe structure [153]. There is fluorescein molecule (FAM) on the 3' end of UPL probes. FAM is the most common type of fluorophore molecules in use in qRT-PCR. UPL probes perform an extensive transcript coverage regarding their prevalent and 8-9 nucleotides short sequences. Moreover, they are designed complementary to the exon-exon junction spanning sequences to eliminate the difficulties caused by sequential obstacles. Determination of the UPL probes are set by ProbeFinder Assay Design Software (Roche Diagnostics, Mannheim, Germany). The matter which makes these probes special is the incorporation of locked nucleic acids (LNA) into the sequences.

LNA is a bicyclic RNA analog where the ribose sugar is structurally constrained by a methylene bridge between the 2'-oxygen and the 4' carbon atoms (Fig. 3.5). Molecular form of LNA makes it more thermostable, therefore the LNA content raises the denaturation temperature of the probe [154]. This feature increases accuracy rates [155]. Because DNA polymerase catalyzes the reaction at 59-61⁰C temperature which is a high degree for the sequential mismatches in reaction.

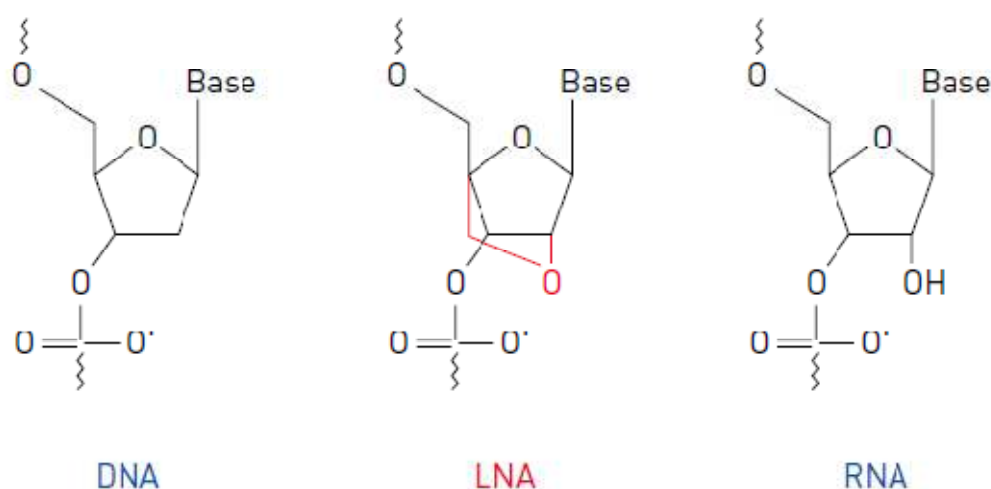


Figure 3.5: Molecular structures of DNA, LNA, and RNA [156].

3.2.3.3 qRT-PCR conditions and primers

qRT-PCR is performed with LightCycler 480 Real-Time PCR instrument (Roche Diagnostics, Mannheim, Germany). Reaction conditions are listed in Table 3.3.

Table 3.3: Conditions of qRT-PCR.

	[°C]	Time	Ramp rate [°C/s]	Cycles
Denaturation	95	10 min	4,8	1
Amplification	95	10 sec	4,8	45
	60	30 sec	2,2	
	72	1 sec	4,4	
Cooling	40	10 sec	1,5	1

Besides the three marker genes, five housekeeping genes were tested and three of them were determined as the most stable expressed genes [157]. This step was necessary to calculate the normalization factor which gives us the general gene expression profile of the cells. The final gene expression results of marker genes were found by relative comparisons with the three housekeeping genes' expressions. These genes are β_2 microglobulin (B2M), glyceraldehyd-3-phosphate dehydrogenase (GAPDH) and TATA box binding protein (TBP). Primer sequences and UPL probe numbers of the complete set of genes are given in Table 3.4.

Table 3.4: Genes, primer sequences and UPL probes.

Genes	Primer Sequences [5'-3']	UPL probes*	Amplicon length	Tm ⁰	GC %
B2M	s_ttctggcctggaggctatc	42	86 nt	64,3	57,8
	as_tcaggaaatttgactttccattc			62,9	34,7
GAPDH	s_tccactggcgtcttcacc	45	78 nt	65,3	61,1
	as_ggcagagatgatgaccctttt			64,2	47,6
TBP	s_cccatgactcccatgacc	51	108 nt	64,1	61,1
	as_tttacaaccaagattcactgtgg			62,6	39,1
PPAR γ	s_caggaaagacaacagacaaatca	7	94 nt	63,0	39,1
	as_ggggtgatgtgtttgaacttg			64,1	47,6
perilipin	s_ggacacagtgggtgcattacg	64	70 nt	64,1	55
	as_gtcccggaattcgtcttc			64,8	61,1
ADPQ	s_ggtgagaagggtgagaaagga	85	61 nt	64,4	52,3
	as_tttaccgatgtctcccttag			62,9	47,6

* ProbeFinder Assay Design Software (Roche Diagnostics, Mannheim, Germany)

Primer efficiencies and normalization factors were also other important tools which are considered during the determination of the primers [157]. Gene expression analyses were duplicated for every gene in each sample in order to gain more reliable data and the average Ct values of duplicates were used in calculation. A cDNA mix of three adipocyte samples were used as positive control. The general layout of the qRT-PCR plates is shown in Table 3.5. The application of master mix is listed in Table 3.6.

Table3.5: Plate layout of qRT-PCR.

Plate		TBP	TBP	B2M	B2M	GAPDH	GAPDH	PPAR γ	PPAR γ	Perl.	Perl.	ADPQ	ADPQ
S*		1	2	3	4	5	6	7	8	9	10	11	12
S	A	x	x	x	x	x	x	x	x	x	x	x	x
S	B	x	x	x	x	x	x	x	x	x	x	x	x
S	C	x	x	x	x	x	x	x	x	x	x	x	x
S	D	x	x	x	x	x	x	x	x	x	x	x	x
S	E	x	x	x	x	x	x	x	x	x	x	x	x
S	F	x	x	x	x	x	x	x	x	x	x	x	x
(-)*	G	x	x	x	x	x	x	x	x	x	x	x	x
(+)*	H	x	x	x	x	x	x	x	x	x	x	x	x

*S: Samples, (+): Positive control, (-): Negative control

Table 3.6: The content of reaction in every well.

cDNA	2 μ l	50 ng
H ₂ O	6 μ l	-
Primer/Probe mix	2 μ l	0,2 μ M primer / 0,1 μ M probe
Probes master	10 μ l	-

As emphasized before, detection of the fluorescence signal accumulation is important for a successful qRT-PCR. However, this is not an easy process even for well developed detection systems of the instrument. Therefore, signals can be detectable after exceeding background level of fluorescence and this happens after a number of reaction cycles. Cycle threshold (Ct) value refers to the total number of cycles which signals start to be detectable. This means Ct values are considered inversely with the amount of target gene expression.

In other words, if there is a high frequently expressed gene, the number of cycles will be lower to catch the threshold cycle. So, the lower Ct value, the higher amount of nucleic acid.

29 and lower Ct values mean an abundant occurrence of gene expression product. 30 – 37 mean positive reactions with a moderate expression of the gene. 39 – 40 Ct values exhibit no expression. An example of Ct value graphic is presented as Figure 3.6. This example belongs to a MSC sample's perilipin expression which was at day 14 of adipogenic differentiation.

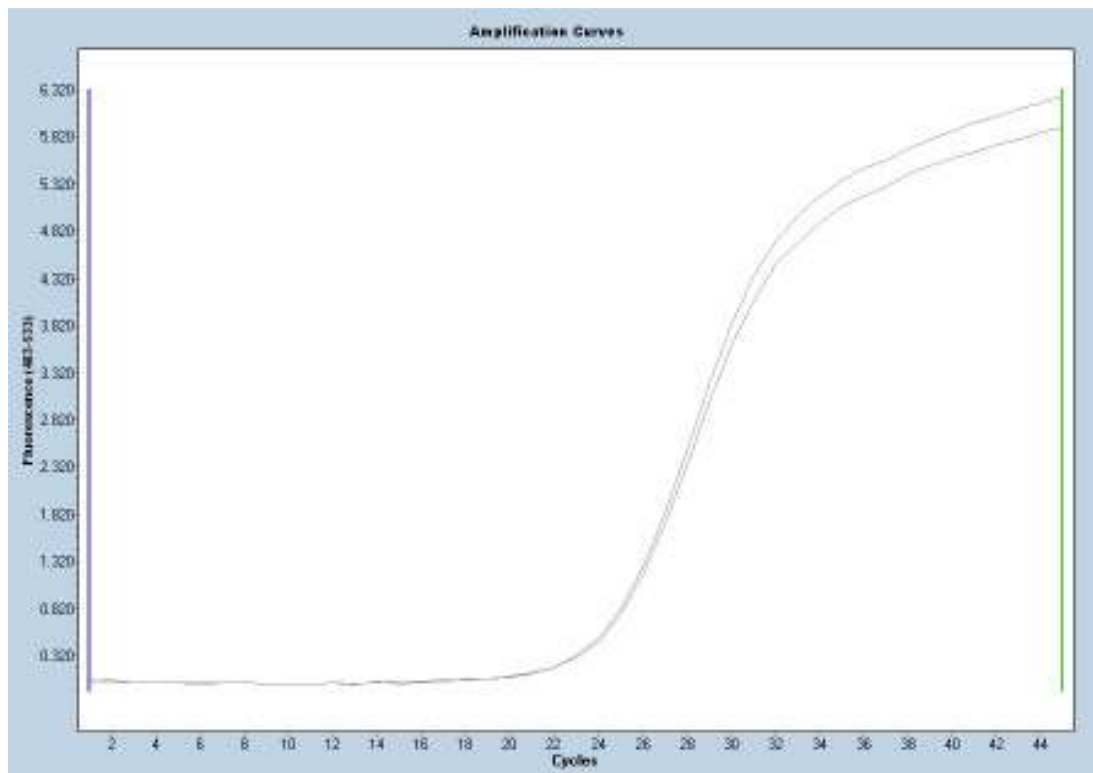


Figure 3.6: Perilipin expression of a BM-MSC sample at day 14 of adipogenic differentiation. The reaction is done for two times with the same sample. Ct value of the first curve is 24,60; Ct value of the second curve is 24,58. Signal amplification can be seen.

3.2.3.4 Calculation from Ct values

Normalization process

To date, gene-expression analysis has changed the progress of many researches in molecular biology. It allows scientists to look inside of complicated regulatory networks, signalling pathways, implication of diseases and relate the genes with biological processes.

Though there are several ways, two recently developed techniques get ahead of gene expression analysis. These are microarrays and qRT-PCR.

These techniques reveal different advantages. qRT-PCR provides the measurement of gene expression simultaneously [152]. This feature can be made for many different samples but for a limited number of genes. Its high throughput, speed and automation levels make it much more advantageous comparing with conventional techniques. However, no matter how advanced technology is used, this approach needs the normalization of mRNA quantification [157].

Efficiencies of enzymes and primers, amounts of starting material, differences of cells and their metabolic activities are the several variable factors which need to be controlled for an informative gene expression analysis. Standardization of these variations is called normalization. This process actually starts from the beginning of the experiments. For instance, using same amount of RNA of each sample for rt reaction is a tool of normalization. Even though there is a hard debate if RNA mass quantity can be mentioned in that way, it is not hard to predict that using 3 ng/ μ l and 50 ng/ μ l might effect the general results.

Today, internal control genes are most frequently used factors to normalize the mRNA fraction. Although housekeeping gene expression can exhibit differences considerably, they are the best candidates which are in use to calculate the normalization factors [159]. Jo Vandecompele and colleagues chose 10 commonly used house-keeping genes to evaluate their skills for normalization [157]. Accordingly they used 13 different types of human tissues. At the end of their study, they ranked the control genes in order of their expression stability. The genes and the primers, β_2 microglobulin (B2M), glycerinaldehyd-3-phosphate dehydrogenase (GAPDH) and TATA box binding protein (TBP) were chosen regarding these findings.

Calculation of normalization factors

Since there is no such unit that can refer the gene expression stages, the general metabolic activity needs to be calculated to talk about standart gene expressions. This calculation gives us normalization factor. Relative quantification is realized through normalization factor.

The geNorm algorithm (VBA applet for MS Office Excel) validates the gene expression normalization factor based on the geometric mean of reference genes. The mathematical equations and formulas are mentioned in Jo Vandecompele's study [157]. The geNorm software is the application of the set of these formulas for regular usage.

Table 3.7: Primer efficiency values.

B2M	1,829
GAPDH	1,796
TBP	1,722
PPAR γ	2,037
perilipin	1,688
ADPQ	1,765

Real time primer efficiencies are as important as Ct values for a healthy calculation of normalization factor. Primer efficiency values are calculated with a serie of qRT-PCR trials which are made with the primer of interest and housekeeping genes. Mean and standart error of the mean of reactions give us primer efficiencies. The values for primers are listed in Table 3.7. Average of positive controls of every plate is also another important tool of normalization factor calculation. To conclude, normalization factor is found with calculating Ct values of control housekeeping genes' expressions with geNorm algorithm. Consideration of general metabolic activity of the cell, abundance of the genes and other flexibilities by using normalization factor provide a relative quantity of the expressions of the genes.

3.2.4 Microscopic analysis

3.2.4.1 Phase contrast microscopy

Cells were followed during the cultivation by using phase contrast microscope and camera system (Axiovert 25 Zeiss & AxioCam Icc). The cells were visible in cell culture flasks, therefore no further fixation and staining process were applied. Imaging was done under 100x magnification.

3.2.4.2 Fluorescence microscopy

For fluorescence staining DF24 cells were grown on glass coverslips which were seeded with $0,9 \times 10^5$ cells/ml (3ml in total) concentration and incubated with growth medium. After confluency, differentiation started with adipogenic differentiation media (Lonza). Coverslips were taken at day 0, day 5, day 7, and day 14 and fixated with PFA solution for fluorescence staining. For fixation, they were washed with PBS twice for 5 minutes and treated with 3,7% PFA solution for 10 minutes. The coverslips were kept at -80°C until microscopy day.

To understand adipogenic differentiation, we analyzed two different factors; the cell shapes and the occurrence of oil droplets inside the cells. Accordingly, vimentin filaments were stained by using indirect fluorescence technique. It is called “indirect staining” because the labelling reagents does not directly bind the target proteins; instead, they bind the primary and secondary antibody molecule complex which recognizes vimentins in this case (Table 3.8). Vimentins give green color and display the cell shapes in the images.

Table 3.8: General features of antibodies.

Primary Antibody				
Antigen	Type	Produced in	Supplier	Dilution
vimentin	polyclonal	Guinea pig	Progen	1:100
Secondary Antibody				
Fluorophore	Specifity	Produced in	Supplier	Dilution
Cy2 (Cyanine)	Guinea pig	Donkey	Dianova	1:100

Another factor that we stained was DNA molecules in the cell. For this purpose, we used Hoechst 33258 (Serva) which intercalates into DNA and emit a blue fluorescence light. Since this reagent binds DNA, it visualizes the nucleus in the cell. Thus, we can see nucleus with in a blue color. This is an important tool to distinguish the cell bodies. This type of staining is an example of direct fluorescence technique. Since adipocytes have a great capability to store oil particles, staining these cell components is one of the most important markers to understand adipogenesis.

For this purpose oil red o fluorescence dye (Fisher Scientific) was used. This molecules interacts with triglycerides in cell culture, regardless if they are in the cell or outside and give red points at the image. General outline of staining is listed in Table 3.9.

Table 3.9: Staining protocol.

1.	Fixated cells on coverslips were rehydrated in PBS
2.	They were treated with 0,4% BSA for 15 min. And rinsed in dH ₂ O
3.	Following treatment was made with primary antibody (1:100) for 2 hours at RT in dark
4.	Coverslips were rinsed in dH ₂ O and washed with PBS for 3 times
5.	They were treated with 5% BSA for 15 min and rinsed in dH ₂ O
6.	Following treatment was made with secondary antibody (1:100) for 1 hour at RT in dark. At the same time, coverslips are also treated with Cy2 and Hoechst (1:50)
7.	They were rinsed in dH ₂ O and washed with PBS for 3 times for 10 min.
8.	Coverslips were incubated in ORO Working solution for 30 min. in dark
9.	They were rinsed in dH ₂ O for 3 times for 30 sec.
10.	Eventually coverslips were rinsed under running tap water for 10 min.
11.	Coverslips were mounted on microscope slides and let dry in dark

Fluorescence staining is analyzed with Leica DM-R epifluorescence device. The basic parameters about imaging are mentioned at Table 3.10.

Table 3.10: Imaging parameters.

Component	Color	Fluorophore	Exposure time
DNA (nucleus)	blue	Hoechst	~120 ms
vimentin	green	Cy2	~650 ms
Oil particles	red	oil red O	~200 ms

Scale bar : 100 μ m

4. RESULTS & DISCUSSIONS

4.1 Gene expression analyses of adipogenic differentiation

4.1.1 Establishment of cell cultures

Two mesenchymal stem cell strains derived from bone marrow, one from adipose tissue and one dermal fibroblast culture were provided by Karen Bieback and colleagues from Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, University of Heidelberg. Cell cultures for adipogenic differentiation were initiated with the conditions mentioned at Table 4.1.

Table 4.1: General features of cell cultures

Source	Cell Type	Name	Passage No	Seeding Conc.
Bone Marrow	MSC	BM72	4	2×10^4 cells/cm ²
Bone Marrow	MSC	BM78	4	2×10^4 cells/cm ²
Adipose Tissue	MSC	LA47	3	2×10^4 cells/cm ²
Forehead Dermis	Fibroblast	DF24	24	$1,5 \times 10^3$ cells/cm ²

It took approximately 3 weeks for MSCs and 2 weeks for fibroblasts to reach to the postconfluency. Before the assay started, day 0 samples were taken for each sample. Afterwards, cells were treated with adipogenic differentiation media (AIM and AMM, Lonza) for 14 days. Cultures of BM72, BM78 and DF24 were established for samplings at day 2, 5, 7 and 14, in the case of LA47 for samplings at day 2, 5 and 14. In addition, cultures of cells fed just with growth media were established as controls.

4.1.2 Total RNA isolation results

The yield of isolated RNAs are listed in Table 4.2, 4.3, 4.4 and 4.5 for each sample.

Table 4.2: Isolated RNA amounts of BM72

Samples	RNA [ng/μl]
BM72 day 0	547,6
BM72 day 2 AD+	485,3
BM72 day 5 AD+	163,4
BM72 day 7 AD+	409,4
BM72 day 14 AD+	717,9
BM72 day 2 C	333,9
BM72 day 5 C	515,3
BM72 day 7 C	298,8
BM72 day 14 C	157,9

Table 4.3: Isolated RNA amounts of BM78

Samples	RNA [ng/μl]
BM78 day 0	472,6
BM78 day 2 AD+	371,6
BM78 day 5 AD+	287,2
BM78 day 7 AD+	565,8
BM78 day 14 AD+	445,6
BM78 day 2 C	350,8
BM78 day 5 C	405,5
BM78 day 7 C	387,7
BM78 day 14 C	369,7

Table 4.4: Isolated RNA amounts of LA47

Samples	RNA [ng/μl]
LA47 day 0	237,5
LA47 day 2 AD+	414,6
LA47 day 5 AD+	536,1
LA47 day 14 AD+	808,4
LA47 day2 C	284
LA47 day 5 C	340,1
LA47 day 14 C	441,6

Table 4.5: Isolated RNA amounts of DF24

Samples	RNA [ng/μl]
DF24 day 0	53,6
DF24 day 2 AD+	76,7
DF24 day 5 AD+	68,9
DF24 day 7 AD+	113,2
DF24 day 14 AD+	183,1
DF24 day 2 C	86,4
DF24 day 5 C	77,4
DF24 day 7 C	95,5
DF24 day 14 C	124,3

The absorbance ratio at 260 nm and 280 nm is used to estimate the purity of the RNA samples. All the RNA isolates display 260/280 ratios between 1,95 and 2,10 which is close to the theoretical value of 2,00, indicating high purity of the RNA.

RNA amounts

Although the RNA amounts per se are not too informative they are reflecting to some extent the metabolic activity of the cells. In fact, they can give us a hint if the response of the cells to differentiation is reasonable or coincidental. We expect a close correlation among the RNAs isolated at the same sampling days from the same type of cells. Accordingly, RNA amounts of BM72 and BM78 were largely correlating, whereas was not the case for RNA samples of DF24 which represents a totally different cell type (Fig. 4.1).

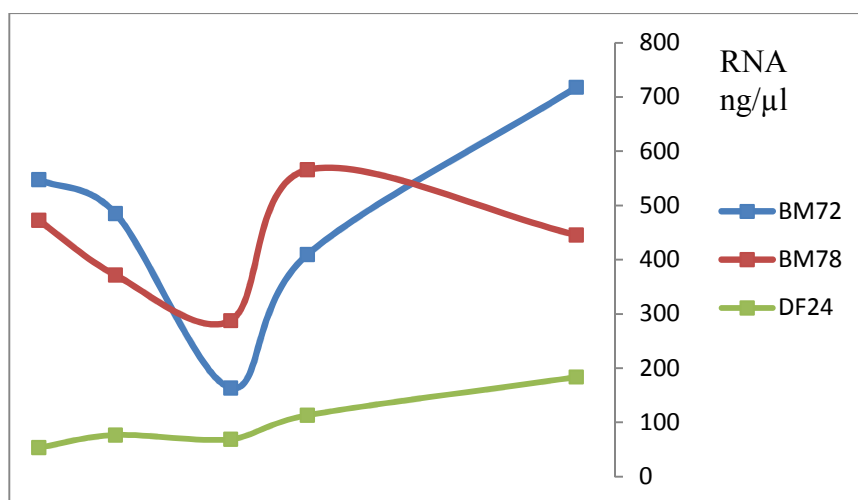


Figure 4.1: The comparison of RNA amounts isolated from different cells. The marks refer to the time of harvesting at day 0, 2, 5, 7 and 14.

The amounts of RNA which emphasizes the metabolic activity via transcriptome is compatible between two bone marrow-derived MSCs. The curves reveal a similar shape in a rough calculation. This indicates that these cells respond to adipogenic differentiation in a comparable manner. Most probably, until day 5 most of those genes are suppressed which are involved in early stages of adipogenic differentiation. After day 5 metabolic activity raises in the cells as well as the expression of adipogenic marker genes. These gene expression rearrangements may influence the RNA profiles resembling between the samples of the two related cell strains. This is further substantiated by the qRT-PCR data, but this issue will be discussed in later sections. On the other hand, a totally different response of DF24 can be seen. However, it has been anticipated and it is highly plausible that the response of MSCs to induction medium is much higher than in fibroblasts. Nevertheless, it is hard to make a reliable prediction about the differentiation capacity of the fibroblasts based on that graph.

4.1.3 Quantitative RT-PCR results

Each sample was analyzed by qRT-PCR for expression of 3 housekeeping genes and 3 adipogenic marker genes with determined primers. All the qRT-PCR analyses were performed two times for each sample; hence, there were 2 Ct values of induced and uninduced samples. The mean Ct values and standard deviations for all samples are listed at Appendix D. Normalization factors were calculated by using the raw data (Ct values) of housekeeping genes, averages of positive control values, and primer efficiencies. Conclusive gene expression results were possessed by the estimation of adipogenic Ct values with geNorm algorithm.

4.1.3.1 BM72 cells

The mesenchymal BM72 cell strain, derived from bone marrow is fully competent for adipogenic differentiation. Gene expression of adipogenic markers PPAR γ , perilipin, and ADPQ in cultures of BM72 cells is shown in Figure 4.2. PPAR γ which takes a part in almost every step of adipogenesis is expressed in high amounts compared to the control group (Fig. 4.2A). As previously stated in detail, this protein is important for growth arrest and differentiation in different aspects. Treating the cells with AIM triggers differentiation and synthesis of PPAR γ which is strongly increasing until day 3. Two days of AMM treatment follow the induction step and this is the reason of the decrease seen at day 5. Apparently at that time point adipogenic differentiation was not fully established yet and dependent on the presence of adipogenic factors. It is known that gene expression often is regulated even in the manner of hours, thus an adipogenic rearrangement also might be the reason of this decrease in PPAR γ expression at day 5. After resuming induction the expression increases again reaching the top value at day 14. Of note, the time point day 14 follows directly AMM treatment like day 5. Consequently, the cells had already reached an advanced stage of adipogenic differentiation at 13 days, hence expression of PPAR γ was no longer affected by this medium change. However, PPAR γ is also crucial for the basal metabolism of cells and thus, we see a minimal to modest production of PPAR γ in control groups. The peak value at day 5, declining again thereafter, may indicate that metabolic or general cell activity was highest then in cultures with control medium.

Perilipin synthesis is correlated and presumably required for the production of oil droplets. So, under relevant conditions when the cells become capable to store oil droplets, perilipin expression starts. In fact perilipin is a middle-to-late marker for adipogenic differentiation and its expression displays a corresponding pattern (Fig. 4.2B), showing a slight delay in comparison to PPAR γ . On the other hand, perilipin is definitely not crucial for cell survival which is underlined by its very limited production in the control group.

The gene for ADPQ is only expressed in adipocytes, representing a late marker for adipogenesis. In accordance with this, significant ADPQ expression was not seen before day 7 but it was strongly raised at day 14 whereas there was virtually no expression in the control group (Fig. 4.2C)

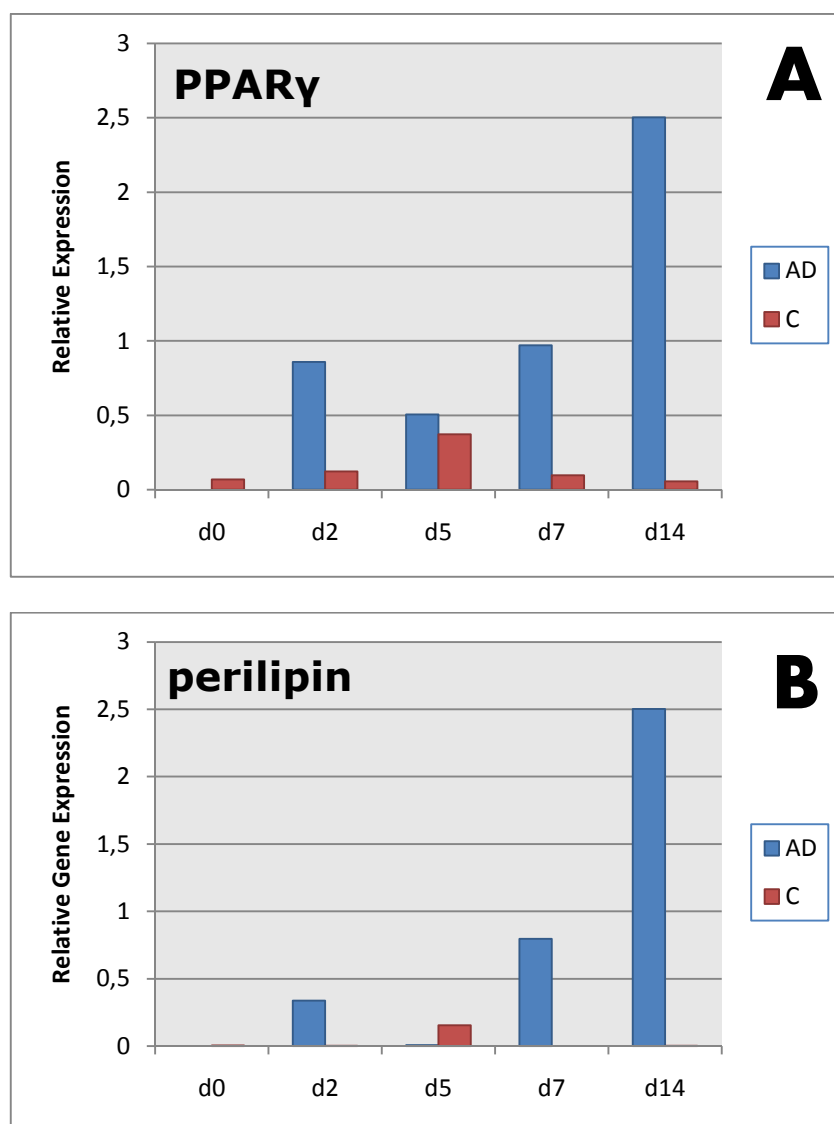


Figure 4.2: Relative adipogenic gene expression profile in BM72 cultures.

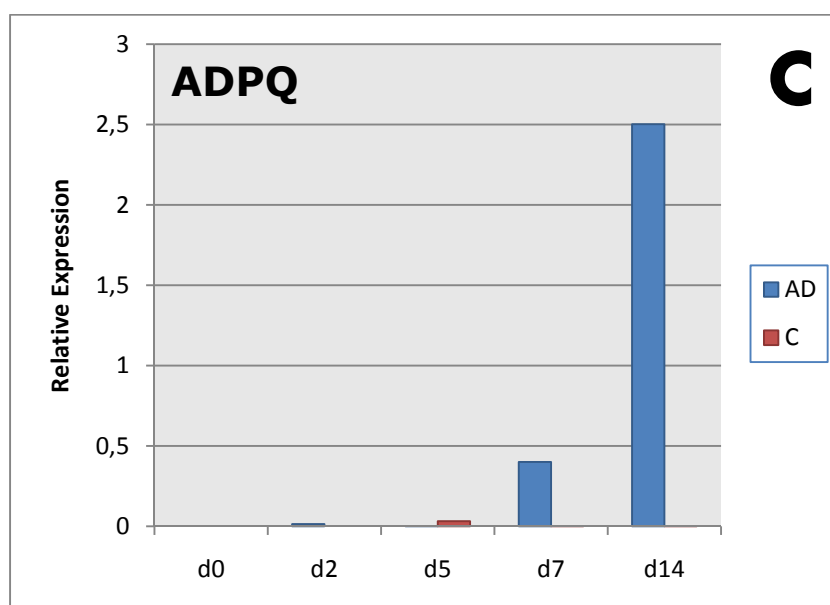


Figure 4.2: (contd.) Relative adipogenic gene expression profile in BM72 cultures.

4.1.3.2 BM78 cells

The BM78 cells are like BM72 derived from bone marrow and consequently should reveal comparable changes in gene expression upon induction. Thus in general, the profiles of adipogenic marker expression are similar between these samples, though some differences in expression intensity are apparent. Since the cell lines used in this study were provided complying with the ethical standards, we had limited knowledge about the donors. Thus, the observed differences may be caused by individual factors such as general health conditions or donor age.

Figure 4.3 shows the expression of the adipogenic markers PPAR γ (Fig. 4.3A), perilipin (Fig. 4.3B) and ADPQ (Fig. 4.3C) for BM78. Despite the rather minute differences to the BM72 samples, similar conclusions can be drawn from both sets of data and therefore the comments on BM72 above are also valid for BM78.

PPAR γ expression of both samples BM72 and BM78 exhibits a pattern which is appropriate to adipogenic differentiation (Fig. 4.2 and Fig. 4.3A). As stated before, the decrease at day 5 reflects the withdrawal from induction medium, intermittent treatment with AMM, and indicates a still not fully committed state which reached later on (day 14). Perilipin expression figures the lipid droplet storage potential of the cells (Fig. 4.2 and Fig. 4.3B). Lastly, since the cells presumably had finished adipogenesis at day 14, the drastically increased expression of ADPQ meets

expectation for being a late marker (Fig. 4.2 and Fig. 4.3C) expression of ADPQ in high amounts was an expectation to come.

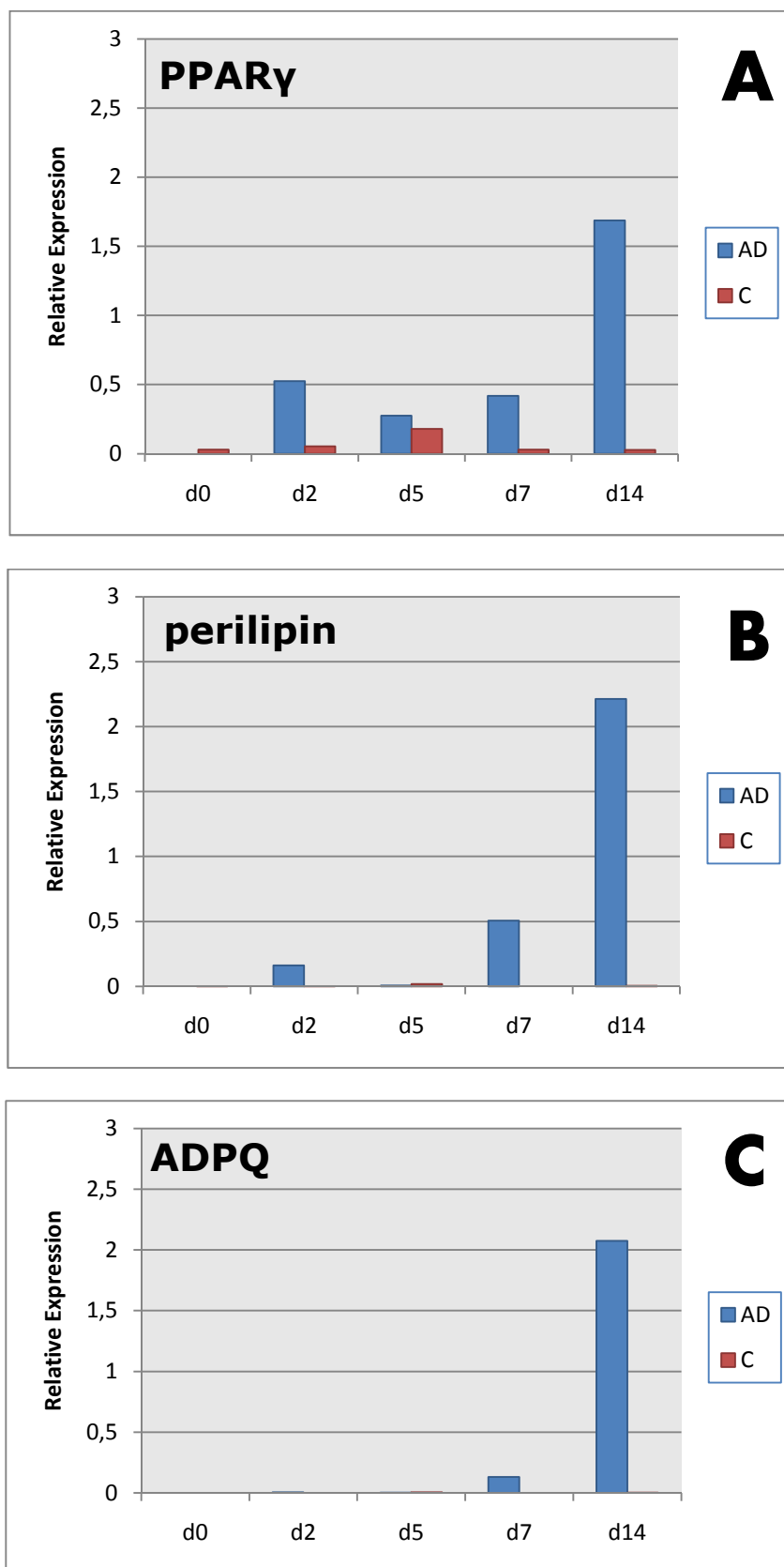


Figure 4.3: Relative adipogenic gene expression profile in BM78 culture.

4.1.3.3 LA47 cells

These adipose tissue-derived mesenchymal cells exhibit some differences in adipogenic gene expression in comparison to the bone marrow-derived samples (Figure 4.4). Nevertheless, the general outline reveals also for LA47 a high potential of adipogenesis.

PPAR γ expression suits expectations for adipogenic differentiation (Fig. 4.4A). Accordingly, after the adipogenic induction with AIM, the quantity of gene's product raises very fast and significantly higher than in the control group. This shows that adipogenesis is triggered immensely for this cell strain. As observed before, there was a decrease at day 5 due to the cycling AIM/AMM treatment and a marked increase again, while in the control group PPAR γ was constantly expressed at low levels to meet the metabolic functions of the cells.

The expression level of perilipin increases very fast after starting adipogenic induction while it remains low in the control at day 2 (Fig. 4.4B). The stable expression levels between 0,5 and 1 indicate that these cells gain quickly the lipid droplet storage capability being stable for 14 days. Minimal expression in the control groups imply absence of adipogenic differentiation.

ADPQ is a late marker of adipogenesis but as stated before, 14 days are enough time for transformation into adipocytes. Thus, during the early stage of differentiation there is no significant change in the minute expression levels of ADPQ, while expression becomes very prominent at day 14 (Fig. 4.4C)

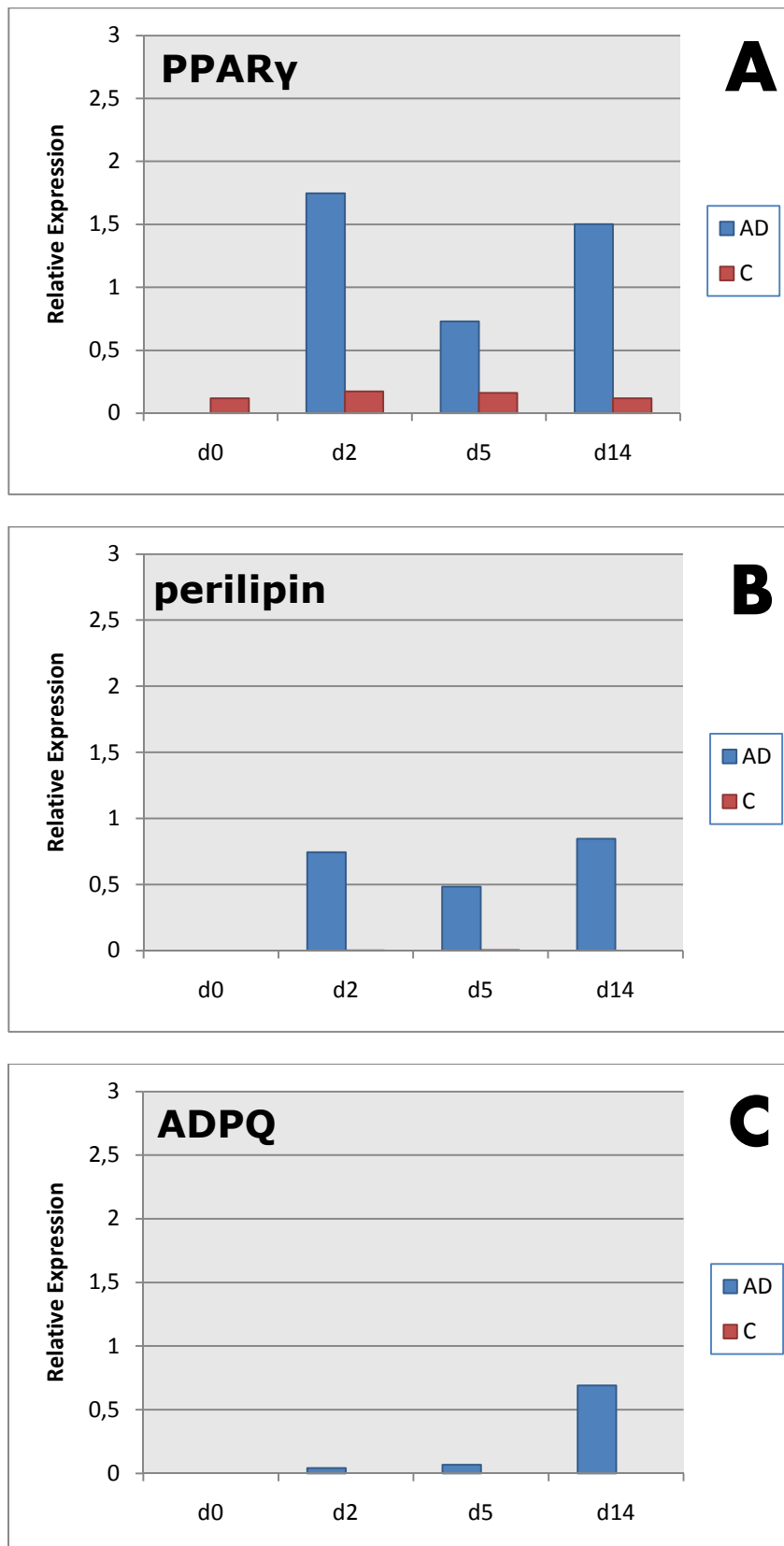


Figure 4.4: Relative adipogenic gene expression profile in LA47 culture.

4.1.3.4 Comparison of marker expression in BM72, BM78 and LA47 cells

PPAR γ

By the adipogenic induction, PPAR γ expression is raised enormously in the cell. This receptor is involved in cell cycle arrest and adjustment of the metabolic activities of cells undergoing differentiation. Therefore, it is actively synthesized during the whole process of this transformation but also initially at low levels for the basal metabolism of adipocyte precursors.

The relative expression of PPAR γ in BM72, BM78 and LA47 complies principally with this statement (Fig. 4.5). However, analysis by qRT-PCR reveals some significant differences in amount and kinetics of PPAR γ expression.

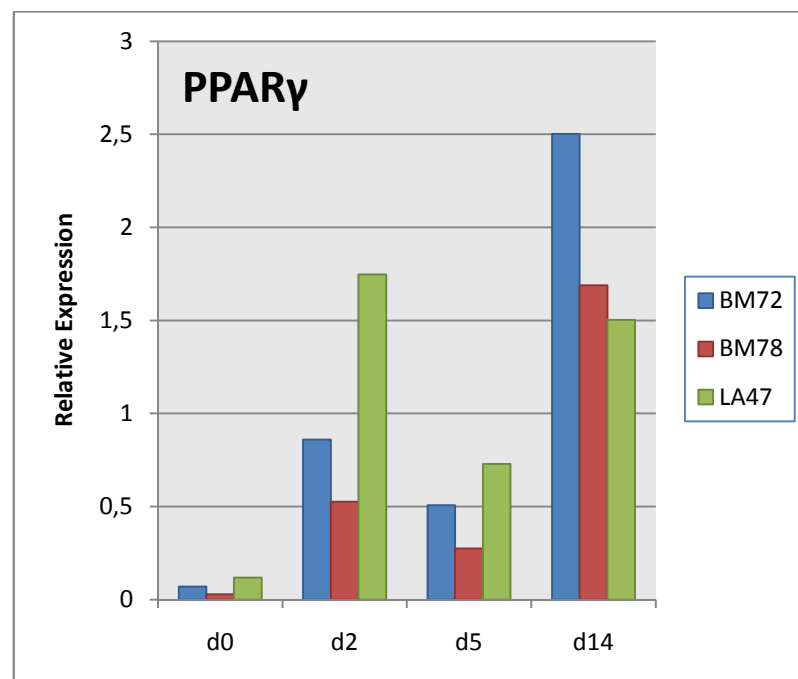


Figure 4.5: PPAR γ expression levels of BM72, BM78 and LA47 during adipogenesis.

Bone marrow and adipose tissue-derived MSCs are both classified as multipotent stem cells. They have a great potential to differentiate into various cell types. But regarding the data presented herein, they are not really equal in their functional properties. In part this may mirror initial adaptation to the different environments (bone marrow versus fat tissue) or due to distinct inherent traits. Thus, the inequality in PPAR γ expression may reflect adaptational changes or different degrees of commitment. At day 2, PPAR γ expression of LA47 is almost two times higher than in the other samples. At day 5, after two days of factor withdrawal, LA47 cells still

synthesize more PPAR γ mRNA than the other cell strains. The need to survive in adipose tissue might have triggered this modification in response. Moreover, the PPAR γ expression level around 1,5 at day 14 is apparently sufficient for LA47 cells, whereas the bone marrow-derived cells may need more expression. This is important because the modifications which cause the fluctuations of PPAR γ gene expression might be the factors for the conversion of early progenitors into committed pre-adipocytes.

Perilipin

There is a similar difference between bone marrow and adipose tissue-derived MSCs in perilipin expression as seen for PPAR γ (Fig. 4.6). Perhaps, the susceptibility skills for adipogenesis provide this difference. Accordingly, after 3 days of adipogenic induction, LA47 cells express perilipin almost two times higher than the other samples. Furthermore, at day 5, expression is still around 0,5 for LA47 whereas there is virtually no expression in the bone marrow-derived samples. On the other hand, there is an extreme increase in BM72 and BM78 at day 14 whereas in LA47 the level is only moderately raised, thus showing a quite steady perilipin expression level throughout.

Perilipin is necessary to store oil droplets inside the cell. With the synthesis at day 2, LA47 cells start to constitute oil vesicles earlier than the other cells. The expression at day 5 and day 14 is needful and enough to maintain the existence of oil particles. On the other hand, perilipin expression in BM72 and BM78 starts after a couple of metabolic adaptations to adipogenesis. The ability of the cells to store oil droplets closely corresponds with the abrupt increase in perilipin synthesis.

This is a very important finding because it may be explicative for the differences between progenitors and their progeny, the pre-adipocytes. The adipose tissue-derived MSCs, generally accepted as pre-adipocytes have exhibited a much faster reaction to adipogenic induction than the bone marrow-derived MSCs which are considered as pre-adipocyte progenitors. This firmly underlines the view that there is an additional rearrangement step to reach a stage corresponding to adipose tissue-derived adipocyte precursors.

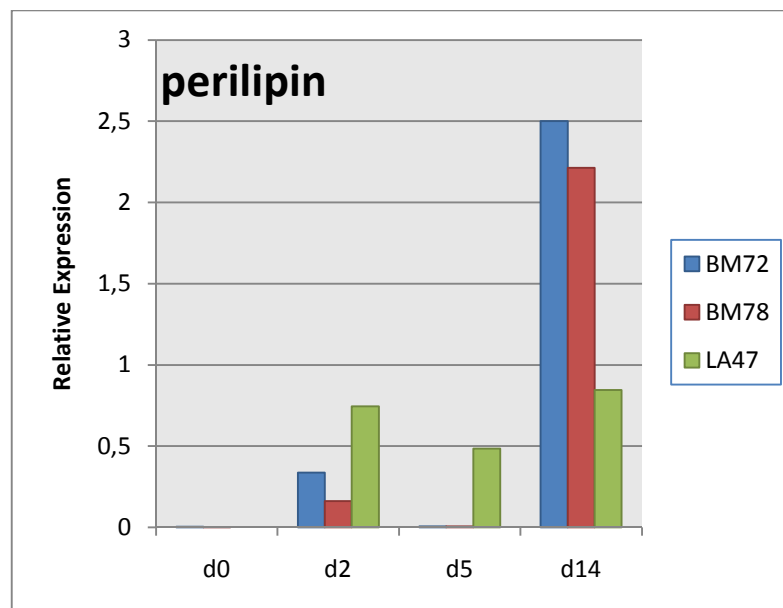


Figure 4.6: Perilipin expression levels of BM72, BM78 and LA47 during adipogenesis.

Adiponectin

Adiponectin (ADPQ) is a marker for advance adipogenesis, therefore the relatively late expression, being very strong at day 14 fits into that scheme (Fig. 4.7). This is an expressive finding for the potential of these cell strains to undergo adipogenesis.

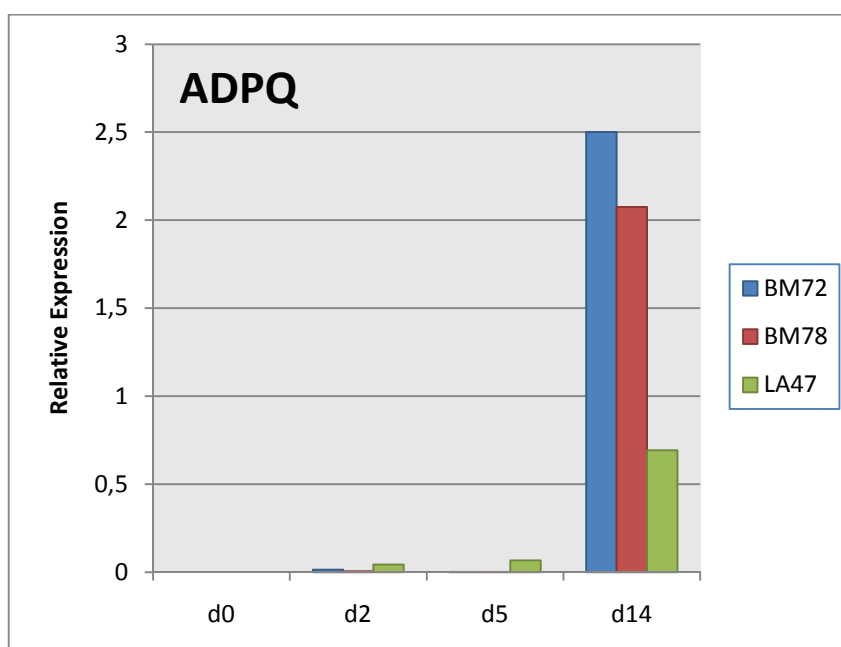


Figure 4.7: Perilipin expression levels of BM72, BM78 and LA47 during adipogenesis.

Nevertheless the reason is elusive why ADPQ expression is too much higher in the bone marrow-derived samples than in LA47 which is completely against expectation. But the following theoretical approach may enlighten this paradox.

As discussed before, though we accept all the MSCs as multipotent stem cells, the classification of these cells needs a more detailed system. For example, we observe the adaptation to the adipogenic induction in LA47 faster than in BM72 and BM78 according to PPAR γ and perilipin expression. These factors are important for adipogenesis and the regulation of other intracellular processes. They do not serve as signal molecules between the cells, while adiponectin protein serves as a adipocyte-derived hormone in the organism. Therefore we can suppose that adiponectin is important for the missions of the adipocytes but not for their own maintenance in the first place.

Today we accept adipose tissue as an endocrine organ. On the other hand, adipocytes are found in different regions of the body such as bone marrow. The thing is, MSCs from different regions of the body might be modified in a different manner. The adipocytes in bone marrows might assume more regulatory duties comparing with adipose tissue-resident fellows which mostly focus on lipid storage. Thus, the high adiponectin expression in BM72 and BM78 at day 14, far exceeding that of LA47, is presumably not directly related to intimate adipogenic function.

Conclusion

The results of relative gene expression demonstrated that MSCs from both bone marrow and adipose tissue were capable for complete adipogenic differentiation. Whereas BM72 and BM78 cells revealed similar gene expression profiles, the profile of the LA47 sample was slightly different. In the samples from bone marrow MSCs we could not observe significant changes in gene expression initially, but an increase at day 7 with further enhancement at day 14. Contrarily, in the samples from adipose tissue MSCs, we observed a higher expression of adipogenic marker genes at the beginning of adipogenic induction, while at day 7 and 14 expression raised only modestly, being lower than in BM72 and BM78. Considering all these cells alike multipotent stem cells, which they were actually supposed to be, then what might be the reason for these differences in expression? While this issue remains still elusive, the findings on individual expression of the adipogenic markers, provide some clues for this question. Since PPAR γ is important in regulation of adipogenesis, the high

PPAR γ expression score of LA47 at day 2 indicates a quicker response to adipogenic stimuli. Later on these cells might need less amounts of this protein to further proceed in adipogenesis. This is apparently also the case for perilipin.

On the other hand, ADPQ is a late marker of adipogenic differentiation and thus its expression had to be looked at especially on day 14 which gave relative expression scores of 0,7 – 2,5 – 2,1 LA47, BM72, and BM78 in that sequence. Hence, the scores of the BM samples are at least three times higher than that of the LA sample which strongly suggests that they represent two distinct subtypes [160].

In human, generally there two types of bone marrow: Red bone marrow and yellow bone marrow. Red marrow is basically responsible to produce blood cells. Yellow marrow is referred as yellow because of the existence of adipocytes in this tissue. Though yellow bone marrow is not directly related with hematopoiesis, the signalling and immunomodulatory factors secreted primarily from adipocytes and other cells in this tissue contributes to this mechanism. Besides, yellow marrow contains MSCs in abundance. These multipotent stem cells can differentiate into a number of cell types including especially osteoblasts and adipocytes.

Adiponectin is an essential hormone for the organism produced by adipocytes, playing an important regulatory role for the energy metabolism. Moreover, it is controlling adipogenesis and can function as suppressor function. After full maturation, adipocytes yellow bone marrow, derived from MSCs start to synthesize ADPQ which upon a certain level prevent adipogenesis of neighbouring stem cells. Thus, excessive fat cell production in marrow is suppressed. The lower levels of ADPQ in adipose tissue could explain that adipogenesis is not limited as much as in bone marrow, which is compatible with the observed differences in our MSC samples.

4.1.3.5 DF24 cells

The fibroblast line DF24 was investigated for its adipogenic differentiation capacity by determining the expression of PPAR γ , perilipin and adiponectin (ADPQ) after adipogenic induction. The cells were treated with adipogenic induction and adipogenic maintenance medium using alternating induction cycles as for the MSC cells. The data of relative gene expression are given in Figure 4.8.

Expression of PPAR γ is increasing immediately under the treatment with induction medium over 14 days as seen for MSCs (Fig. 4.8A), while the basal expression remains rather low throughout in the range between 0.2 and 0.3. Under the influence of induction, expression levels raise to 0.7 or 0.8 at day 2 and 5, reaching a peak of 1, at day 7 (7 times higher than the basal levels) with a modest decline at day 14. This clearly demonstrates that also the DF24 cells, though representing fibroblastic lineage, are able to respond to adipogenic induction by increased expression of this early marker.

Also expression of perilipin was inducible in DF24 by adipogenic induction, as demonstrated by the sharp increase at day 7 (Fig. 4.8B). Similar to PPAR γ , there was a mild decrease observed at day 14. Accordingly, DF24 cells started to form oil vesicles inside which, however remained restricted in size.

While the control group is cultivated in growth medium with a high content of glucose, the adipogenic induction medium contains in addition insulin, IBMX, and dexamethasone, which trigger adipogenic differentiation in MSC cultures. While insulin stimulates glucose intake, IBMX stimulates cAMP production which is a crucial promoter for adipogenesis in MSCs. But the point is that not every event in this scenario does have the same consequences in another cell type. Thus, cells can adapt to these new conditions without undergoing cellular differentiation. Accordingly, DF24 cells were storing small oil droplets in response to these factors without proceeding further in this specific type of differentiation. Confirming proposition, the late differentiation marker ADPQ was not at all expressed in DF24, neither in growth nor in adipogenic induction medium in contrast to matured adipocytes.

These findings document that some rearrangements in gene expression occur in dermal fibroblasts (DF24) under the influence of adipogenic factors. However, cells seem not to respond to this stimulus with a regular adipogenic program which was underlined by further investigations using a different approach (see below).

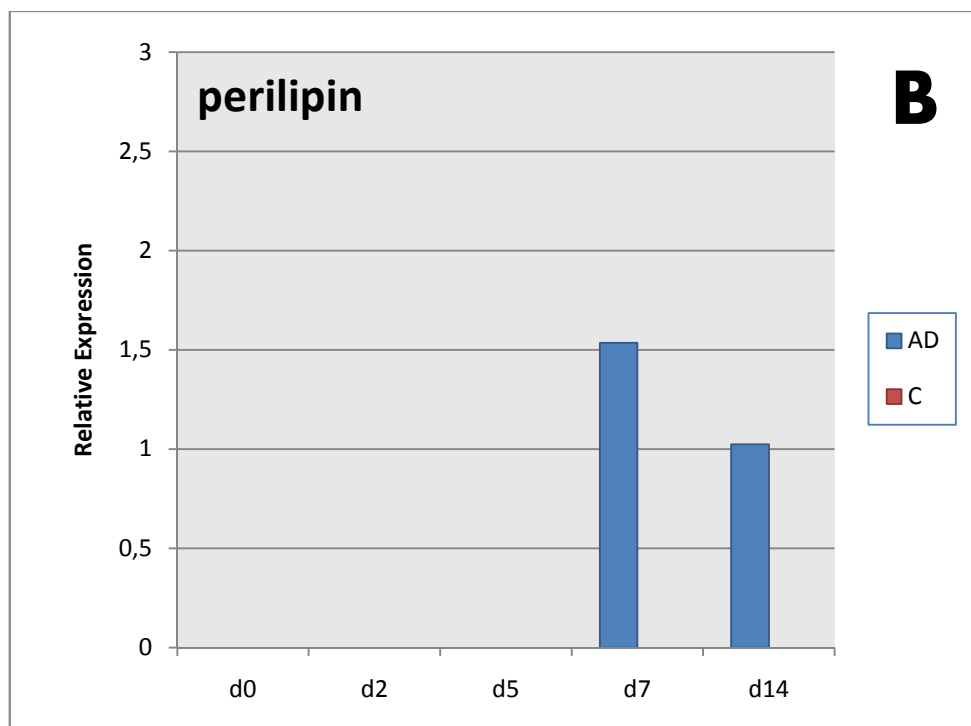
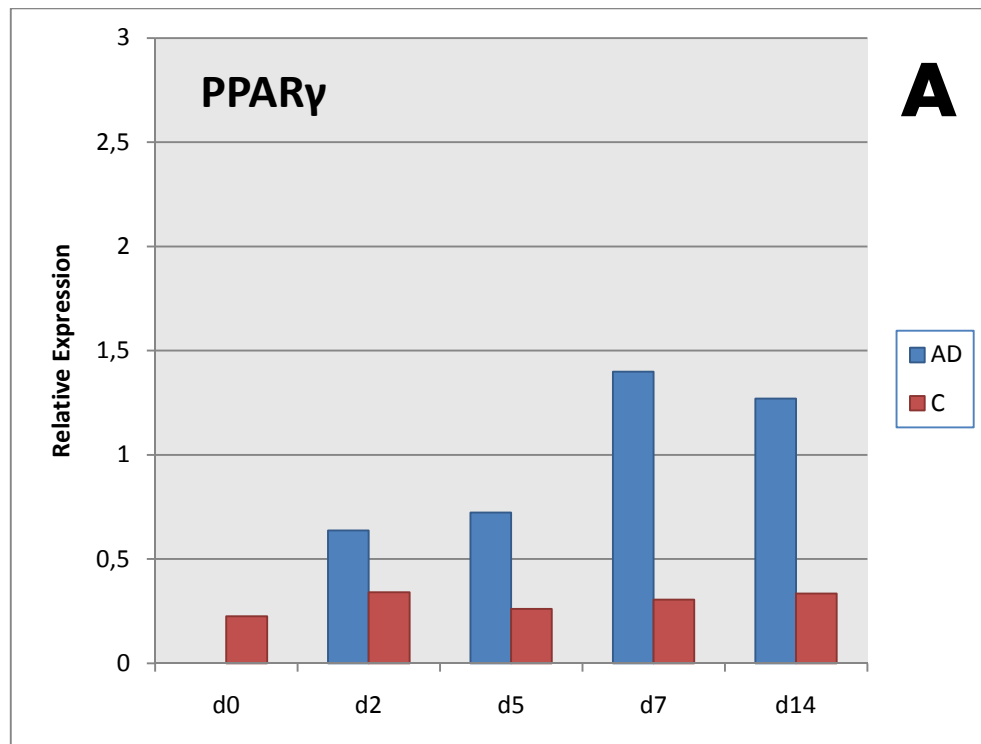


Figure 4.8: Relative adipogenic gene expression profile of DF24 culture.

4.2 Immunofluorescence staining of DF24 for adipogenic markers

4.2.1. Cultivation on coverslips and adipogenic differentiation

For better understanding of the adipogenic differentiation potential of DF24 cells, they were analyzed by immunofluorescence staining in parallel to gene expression assays. For this purpose, DF24 cells of passage 24 were suspended at a density of $0,9 \times 10^5$ cells/ml and 3 ml were seeded on 6 cm cell culture dishes with glass coverslips.

Adipogenic differentiation was stimulated after the cells reached confluency, using the same protocol as for the gene expression. Accordingly, cells were treated for 3 days with AIM, followed by 2 days in AMM, which was repeated two more times. Treated and control cells were sampled at day 0, 2, 5, 7, and 14. After fixation of the specimens, coverslips were stored at -30°C until analysis.

4.2.2 Following the fibroblasts exposed to adipogenic factors

Taken as reference, the image of an MSC-derived adipocyte culture exhibits a closed layer of largely spherical shaped cells, partially overlapping (Fig. 4.9). Accordingly, morphological changes of DF24 cells were followed during treatment with adipogenic medium by phase contrast microscopy (for methods by Axiovert 25 Zeiss Microscope and AxioCam Icc 1 camera system). The comparison of treated and control cultures of DF24 is presented in Figure 4.10.

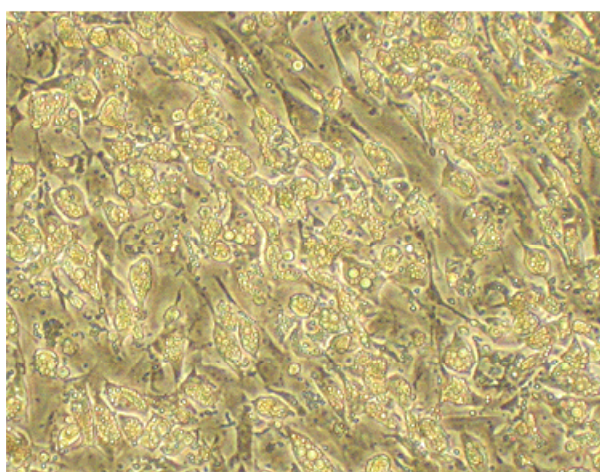


Figure 4.9: Human adipocyte cell culture (www.cellapplications.com).

Generally, ~80% confluency of cultures is sufficient to initiate differentiation. But, despite the subconfluency, infrequent mitoses were noted from time to time among the control group cultures. Accordingly, cells suppressed partially their active proliferation, though an increase in density was observed, while maintaining their spindle-shaped structure. Any abnormal events such as contamination or necrosis were not reported during the treatment.

The adipogenic factor treated group revealed some alterations through adipogenic treatment, however, these variations were limited only to individual groups of cells. Thus, from day 5 some cell accumulations appeared resembling somehow clusters of adipogenic cells at that state. While eventually most cells became less elongated, cell bodies acquired a more roundish shape. This type was maintained through day 14, but no further morphological transformations could be observed which would have been indicative for adipogenic differentiation process.

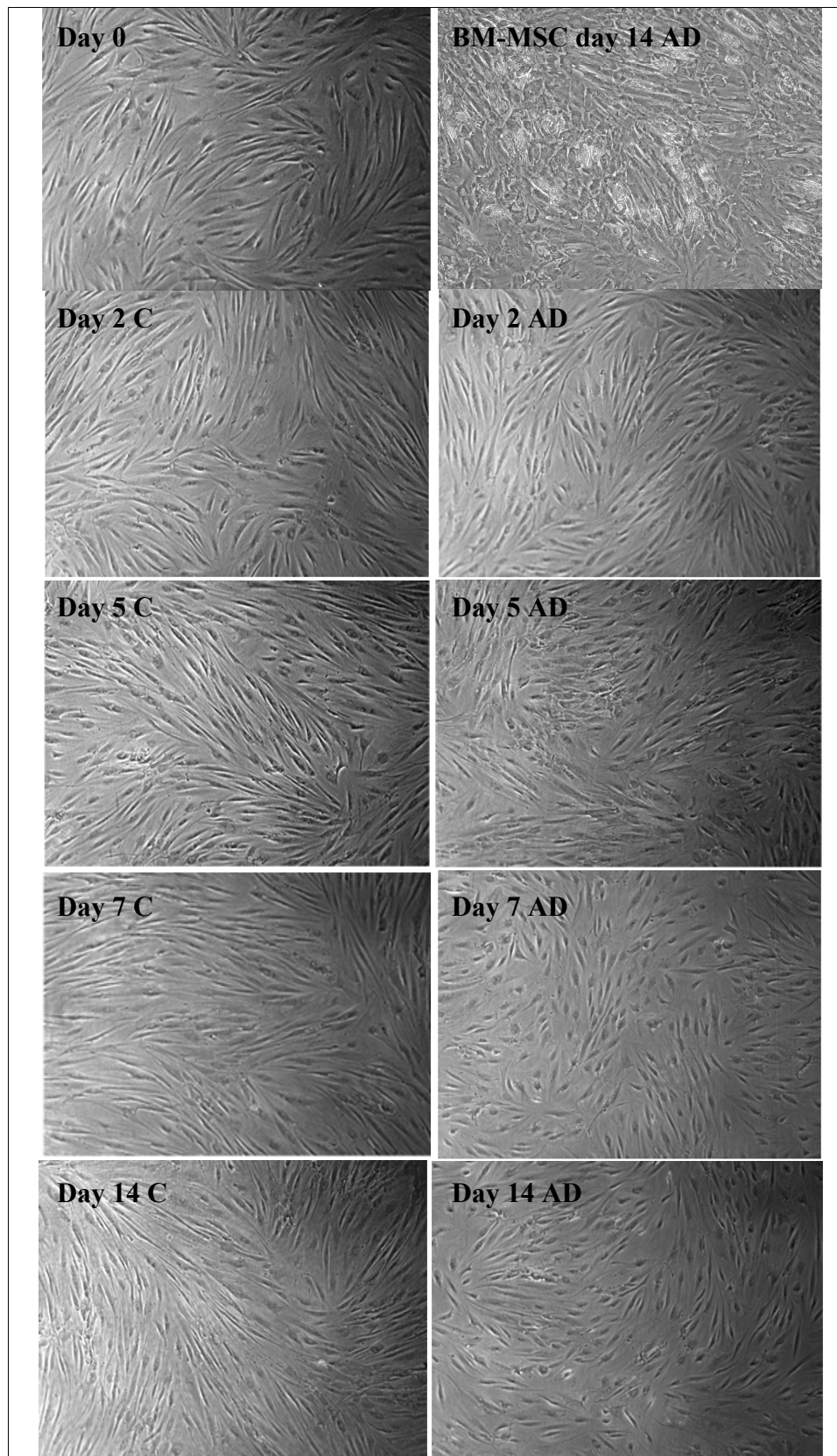


Figure 4.10: Phase contrast microscopy of adipogenic induced and control DF24 cells. Left panel shows control samples cultivated in complete growth medium whereas the right column cells cultivated under adipogenic stimuli. 100x mag.

4.2.3 Immunofluorescence staining

DF24 cells were analyzed for the formation of lipid droplets as morphological indicator of adipogenic differentiation by immunofluorescence staining, in comparison to gene expression. For evaluation of integrity and cellular shape, also vimentin, a typical cytoskeletal component in mesenchymal cells, was visualized by fluorescence staining and in addition cell nuclei by Hoechst DNA-stain. The changes during treatment are shown in Figure 4.11.

At day 7, initiation of a slight accumulation of fat vesicles can be seen, which becomes very pronounced at day 14. This type of distribution is also observed in multilocular cells like brown fat adipocytes. This finding also corresponds to the gene expression data. As a reminder, expression of perilipin which is essential for oil particle formation has started at day 7 and remained until day 14. Thus what we see in the images fits to these findings. On the other hand, we observed a progression from spindle to spherical shape also by immunofluorescence staining.

Though there are findings that would support in vitro adipogenesis, we could not get a definite proof that dermal fibroblasts initiate and proceed in this type of differentiation. Therefore, we believe that these cells can realize some structural and genetic rearrangements under adipogenic stimuli, however, terminal adipogenic differentiation seems far from being realized.

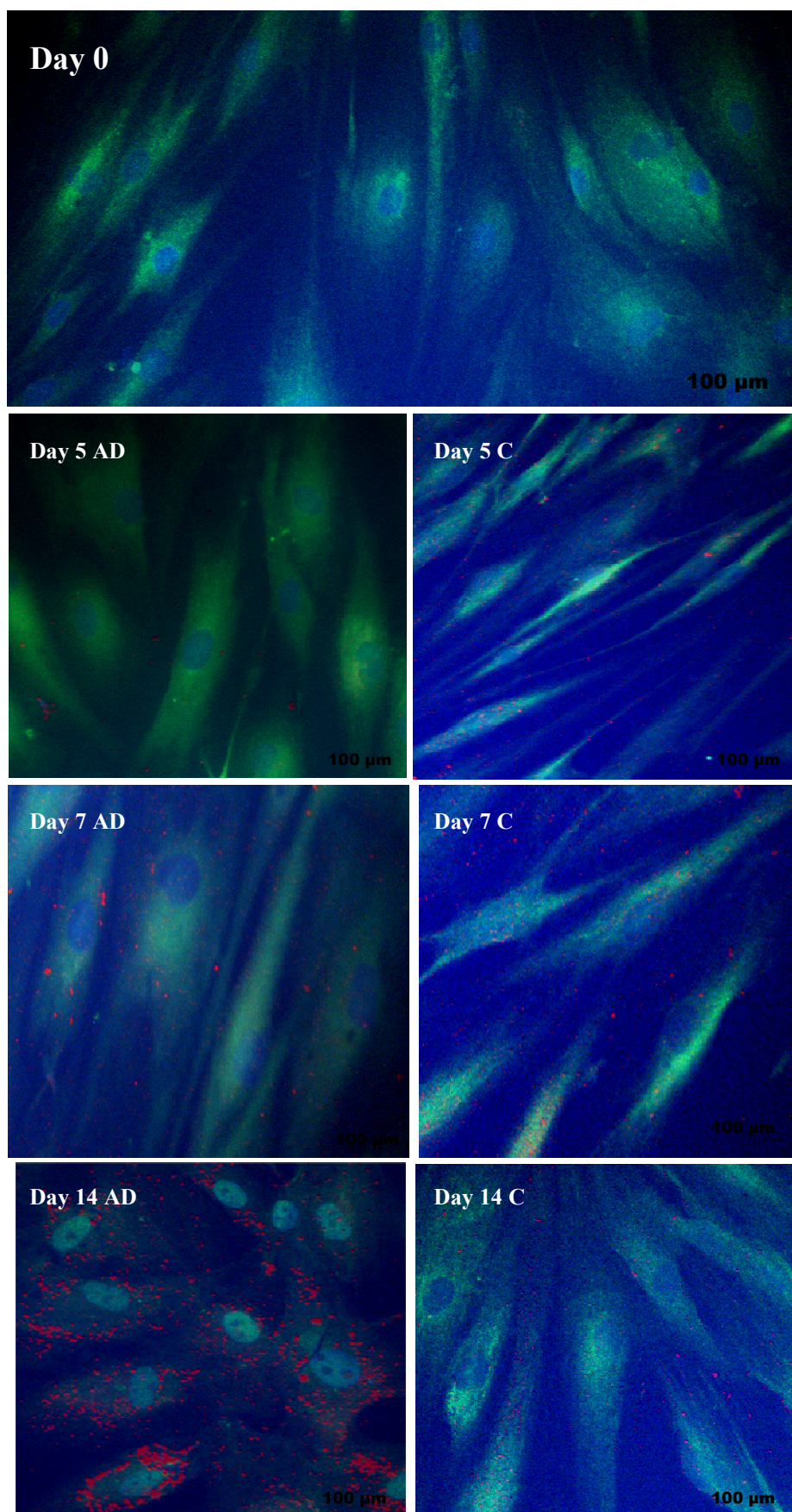


Figure 4.11: Immunofluorescence staining images of DF24.

4.3 Adipogenic differentiation on ECM proteins coated surface

Culture flasks were coated with ECM proteins as described in methods. In previous work the bone marrow-derived mesenchymal stem cells used herein had been demonstrated to possess high potential for adipogenic differentiation. Thus, we chose BM72 cells (passage 6) for this assay using a seeding density of 2×10^4 cells/cm². A general outline about cultivation conditions is provided in Table 4.6.

4.3.1. Preparation of cell cultures and RNA isolation

Culture flasks were coated with ECM proteins with the methods noted in related section. In previous work, bone marrow-derived mesenchymal stem cells were examined about their adipogenic differentiation skills and they exhibited a high capacity of adipogenesis. Thus we chose BM72 samples for this assay. According to this purpose, we use passage 6 BM72 cells with 2×10^4 cells/cm² seeding concentration. General information about cultivation of the cells are noted in Table 4.6.

Table 4.6: General features of cell cultures on coated surface.

Coating protein	Sample	Passage No	Seeding Conc.
Collagen type I	BM72	6	2×10^4 cells/cm ²
Collagen type IV	BM72	6	2×10^4 cells/cm ²
Fibronectin	BM72	6	2×10^4 cells/cm ²
Laminin	BM72	6	2×10^4 cells/cm ²
Null [PBS]	BM72	6	2×10^4 cells/cm ²

Total RNA isolation results of BM72 cultures are listed in Table 4.7.

Table 4.7: Isolated RNA amounts of samples.

Sample				ng/ μ l
BM72	d0	Col1	GM	34,8
BM72	d0	Col4	GM	29,1
BM72	d0	FN	GM	23,5
BM72	d0	LN	GM	41
BM72	d0	PBS	GM	34
BM72	d2	Col1	GM	68,8
BM72	d2	Col1	AIM	61
BM72	d2	Col4	GM	73,7
BM72	d2	Col4	AIM	132,1
BM72	d2	FN	GM	58,8
BM72	d2	FN	AIM	42,8
BM72	d2	LN	GM	86,5
BM72	d2	LN	AIM	126,2
BM72	d2	PBS	GM	61,6
BM72	d2	PBS	AIM	105,2
BM72	d5	Col1	GM	73,2
BM72	d5	Col1	AIM	15,3
BM72	d5	Col4	GM	76,1
BM72	d5	Col4	AIM	22,2
BM72	d5	FN	GM	82,6
BM72	d5	FN	AIM	19,3
BM72	d5	LN	GM	58,3
BM72	d5	LN	AIM	14,2
BM72	d5	PBS	GM	50,9
BM72	d5	PBS	AIM	57
BM72	d7	Col1	AIM	135,6
BM72	d7	Col4	AIM	71,1
BM72	d7	FN	AIM	118,9
BM72	d7	LN	AIM	89,7
BM72	d7	PBS	AIM	66,8
BM72	d14	Col1	GM	49,1
BM72	d14	Col1	AIM	180,3
BM72	d14	Col4	GM	64,8
BM72	d14	Col4	AIM	14,1
BM72	d14	FN	GM	42,9
BM72	d14	FN	AIM	218,6
BM72	d14	LN	GM	94
BM72	d14	LN	AIM	28,9
BM72	d14	PBS	GM	62,2
BM72	d14	PBS	AIM	14,5

The cells were cultivated under adipogenic stimuli for 14 days. After the cells reached to postconfluency, differentiation was initiated and sampling was done at day 0, 2, 5, 7, and 14. Isolated RNA amounts and 260/280 ratios were measured by Nanodrop ND 1000 V 3.3 spectrophotometer system (Thermo Scientific) which revealed a high RNA purity by general ratios around 2,05. However, a marked restriction in RNA production (or recovery) was observed over time, though the reason of this remains unclear.

4.3.2. Phase contrast images of adipogenic induction on ECM coated surfaces

All MSC cultures were followed up during 14 days of adipogenic treatment by phase contrast microscopy. The effects of the applied ECM proteins are shown in the following figures.

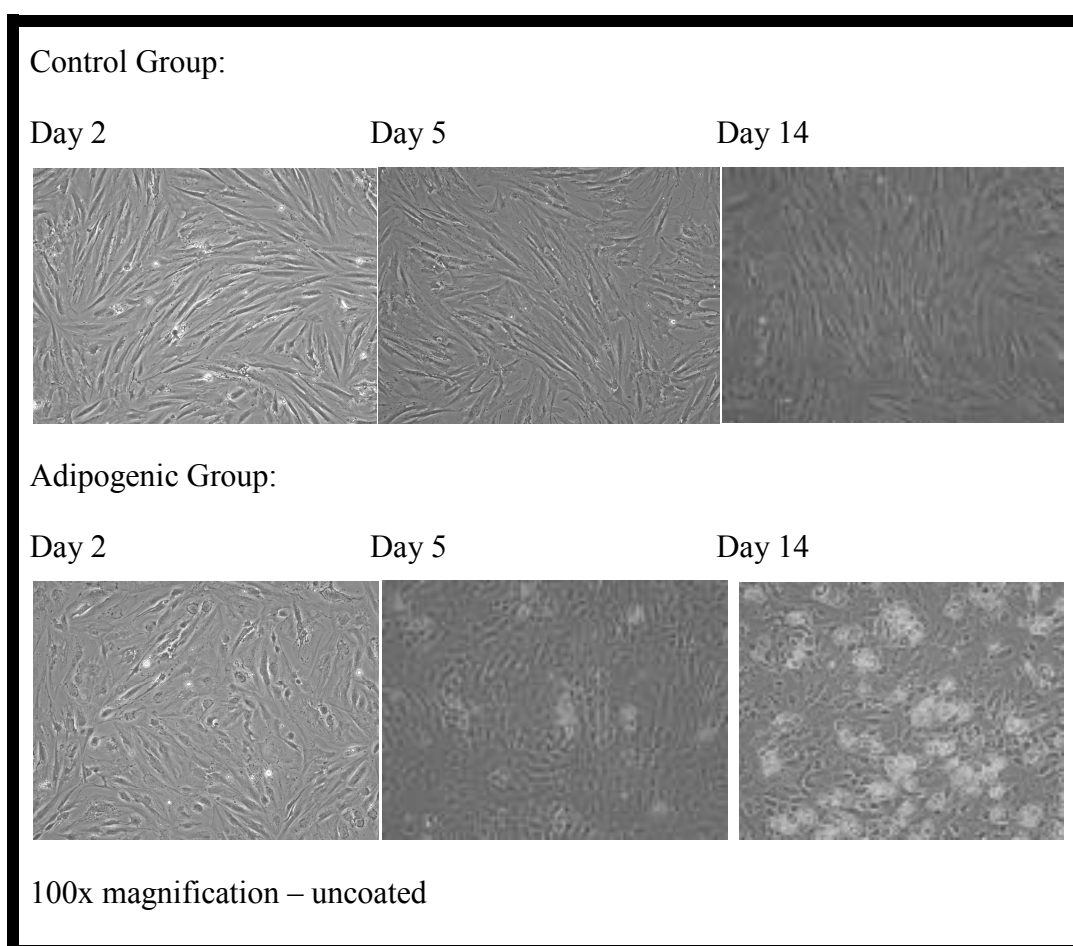


Figure 4.12: Morphological changes of BM72 cells after adipogenic induction cultivated without surface coating. Phase-contrast 100x mag.

The successful induction of adipogenic differentiation in cultures without coating is demonstrated in Figure 4.12. The profound changes in cell shape and dense accumulations of oil particles at day 14 are strong evidence for an advanced stage in adipogenesis. In contrast, MSCs cultivated with growth medium maintained their initial cellular characteristics during the assay as expected.

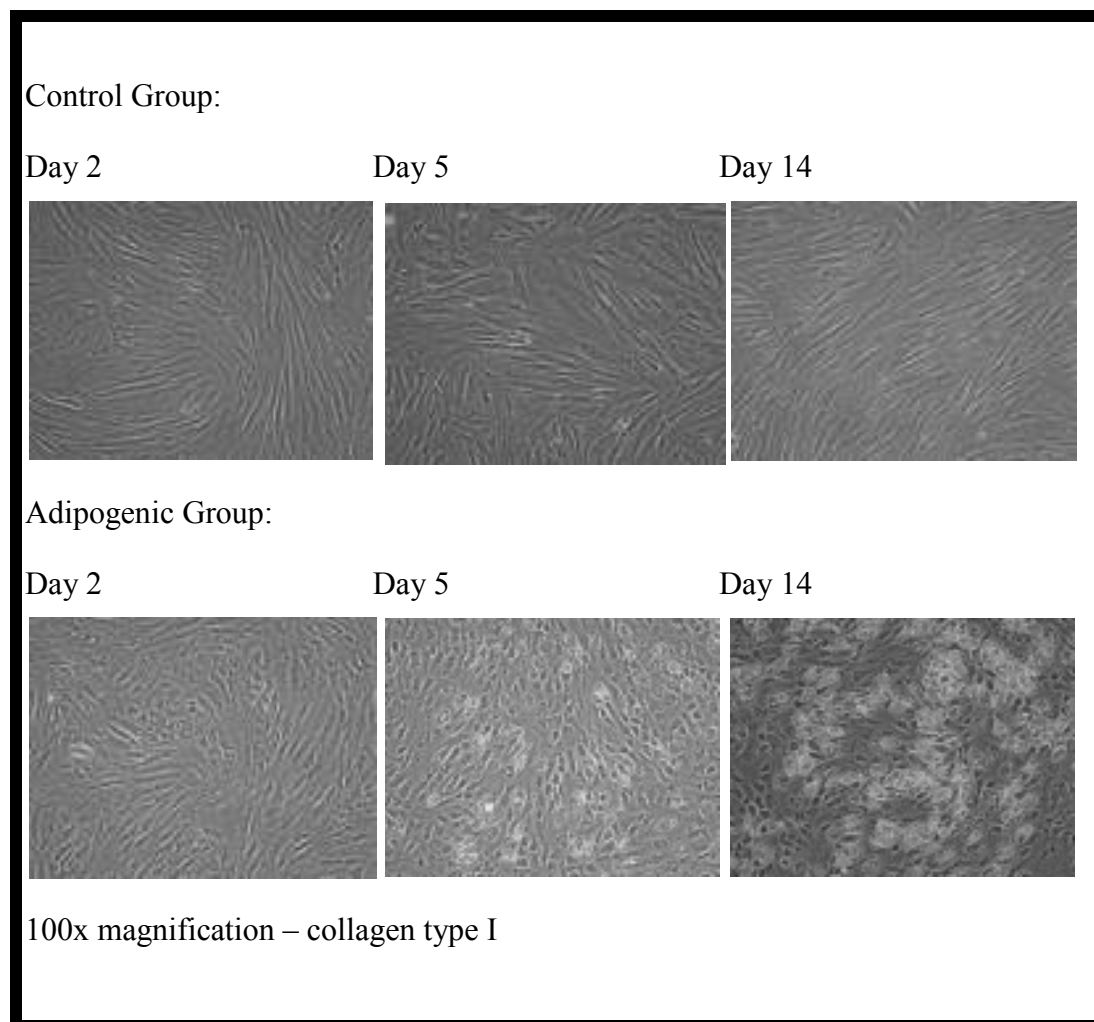


Figure 4.13: Morphological changes of BM72 cells after adipogenic induction cultivated on collagen type I coated surface. Phase-contrast 100x mag.

Considering the endpoints day 14 of the adipogenic groups at day 14, we can state that all coatings revealed similar effects on adipogenesis, being rather modest at best. Only the effect of collagen type I (Figure 4.13) seemed to be slightly stronger, while on laminin coating the shape changes became apparent somewhat earlier, clearly visible at day 2 (Figure 4.16). This shows that phase contrast images allowed only a limited evaluation.

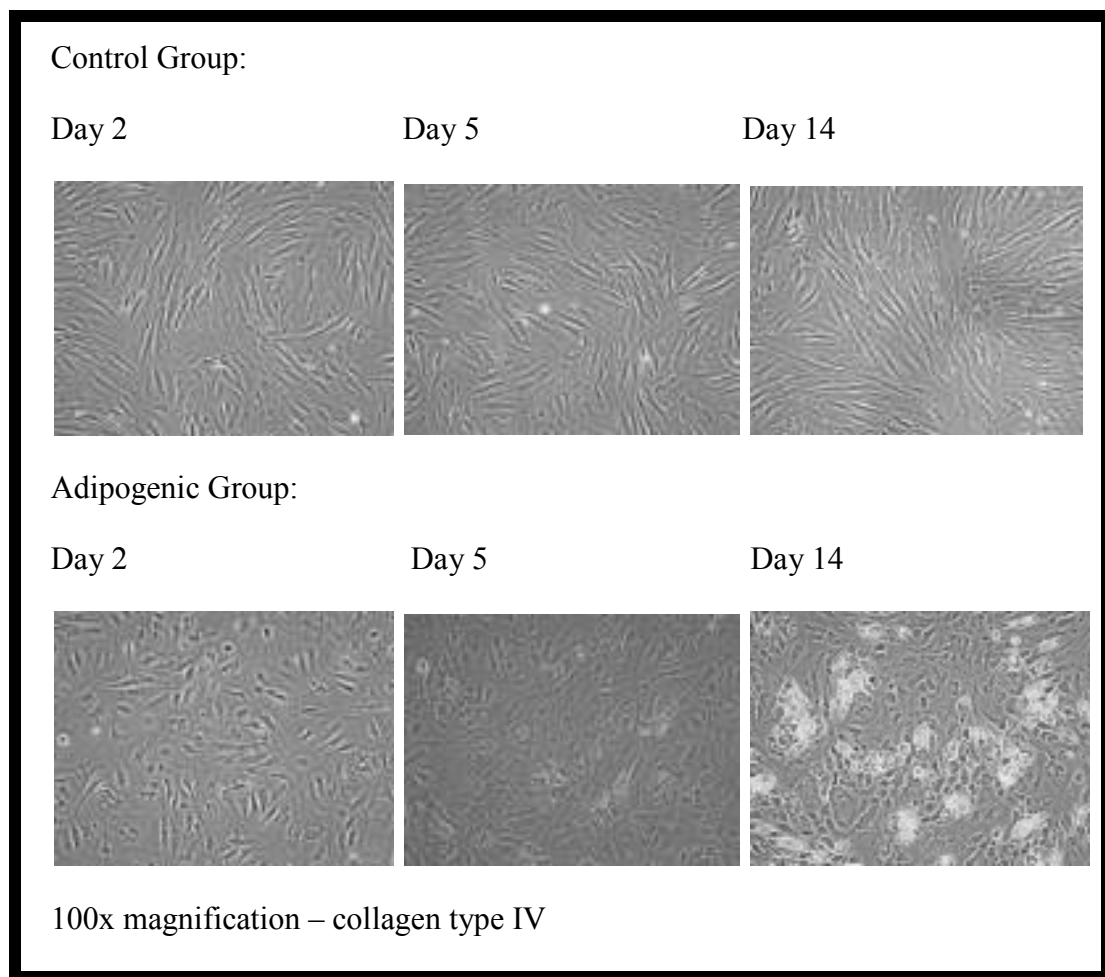
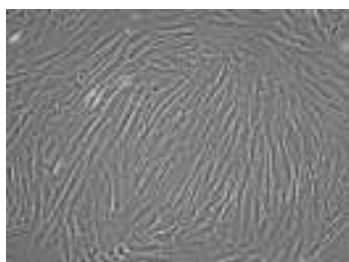


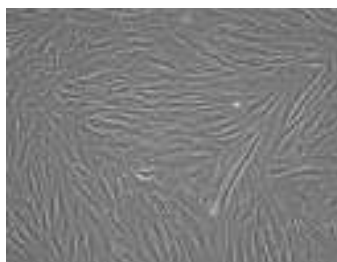
Figure 4.14: Morphological changes of BM72 cells after adipogenic induction cultivated on collagen type IV coated surface. Phase-contrast 100x mag.

Control Group:

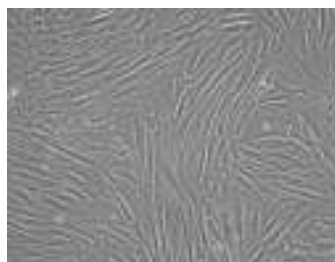
Day 2



Day 5

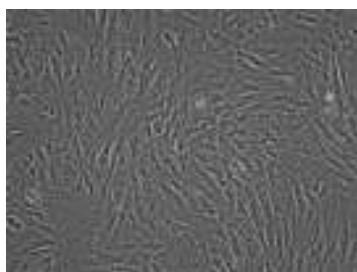


Day 14

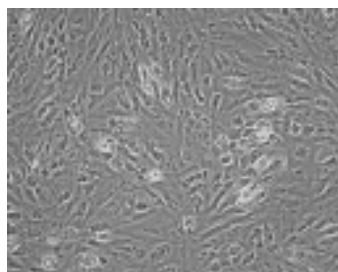


Adipogenic Group:

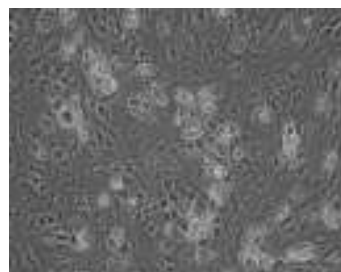
Day 2



Day 5



Day 14



100x magnification - fibronectin

Figure 4.15: Morphological changes of BM72 cells after adipogenic induction cultivated on fibronectin coated surface. Phase-contrast 100x mag.

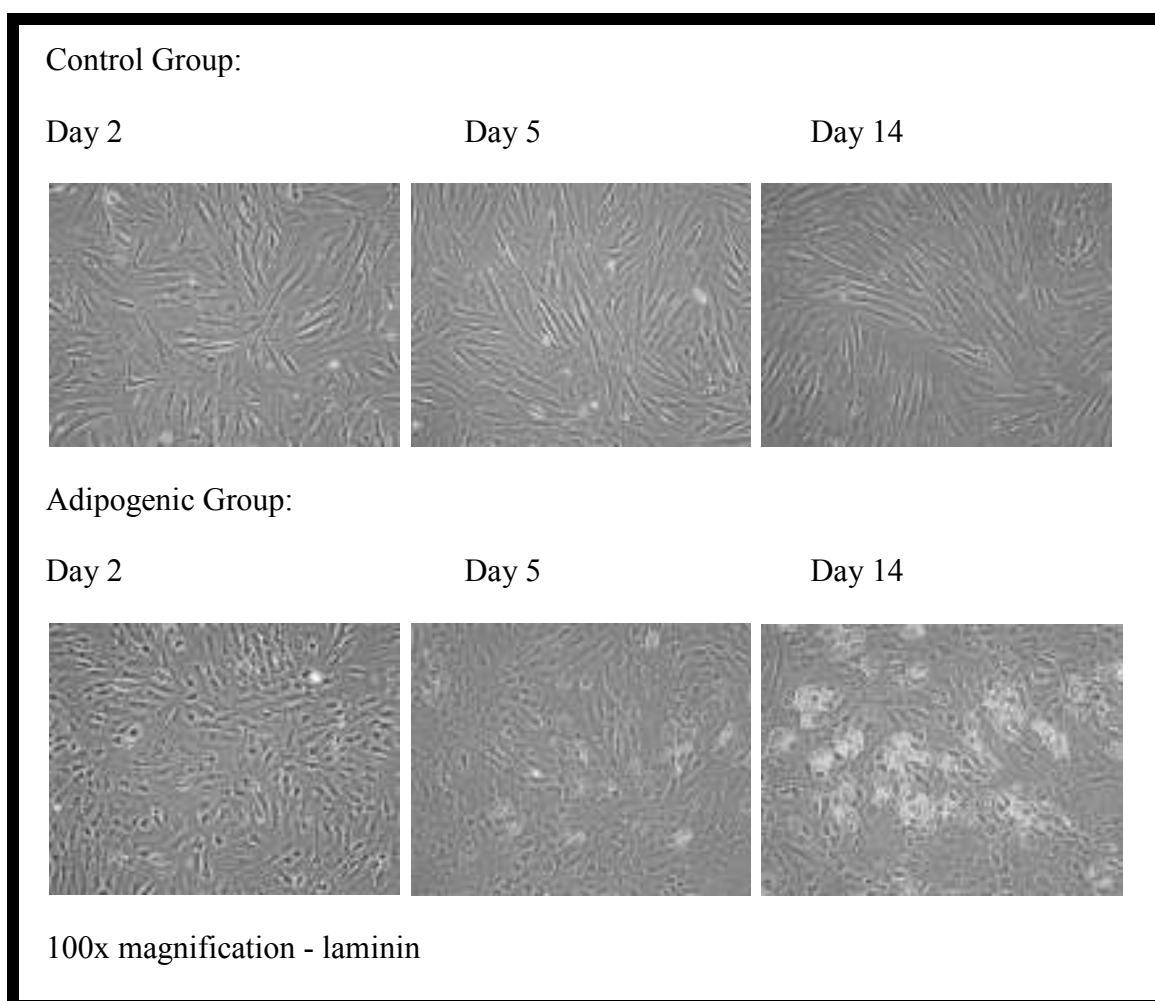


Figure 4.16: Morphological changes of BM72 cells after adipogenic induction cultivated on laminin coated surface. Phase-contrast 100x mag.

Collectively, BM72 cells were able to terminally differentiate regardless of the proteins that we have used to coat the surface. However, as stated before, this evaluation by phase contrast allows only a very crude prediction of the effects of the ECM proteins on differentiation behavior. Thus, more conclusive results should be gained by relative expression analysis of adipogenic marker genes.

4.3.3. Influence of ECM coating on relative gene expression

Expression of the adipogenic marker genes PPAR γ , perilipin, and ADPQ was analyzed as shown before, providing a quantitative estimation of the effects of these ECM proteins on adipogenesis. The qRT-PCR analysis of all samples was carried out using the same housekeeping genes and primers as in the previous assays. The mathematically calculated relative expression is presented in this section for every coated surface.

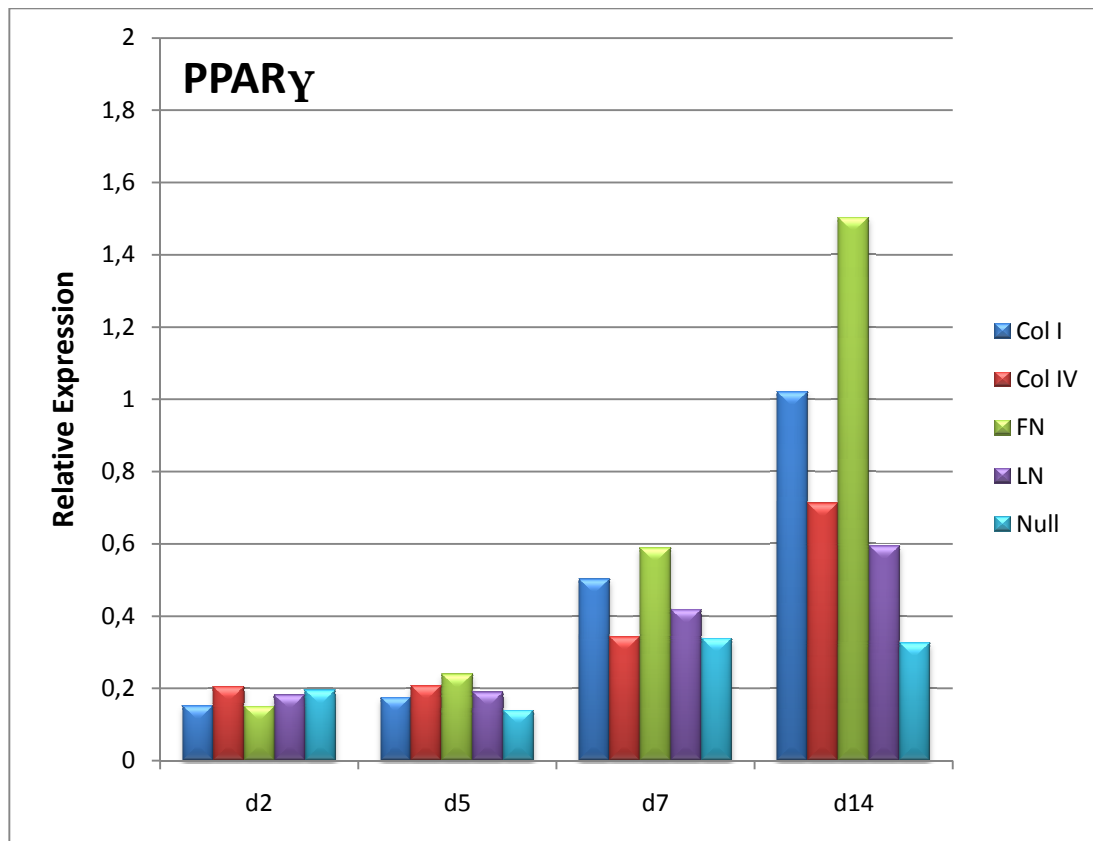


Figure 4.17: Kinetics of PPAR γ expression of BM72 cells after adipogenic induction, grown on different protein coatings.

Figure 4.17 shows that on all ECM protein coatings BM72 cells express initially similar amounts of PPAR γ which is essential factor for early steps in adipogenesis. However, with time significant differences become apparent among the samples cultivated on different ECM proteins. In detail, the similar expression levels at day 2 approve a proper application of the assay. In these cultures treatment has just been started and the levels of PPAR γ mRNA are almost the same showing only marginal changes at day 5. In marked contrast, at day 7, after 6 days of adipogenic induction, ECM proteins were beginning to exert marked effects on PPAR γ expression, in accord with the positive regulatory effects of fibronectin reported by other laboratories (-mentioned in Introduction). This ECM protein involves to a series of mechanisms such as wound healing, vascular development or cell growth in the organism. Therefore, this finding complies with our expectations. Also strong, though less pronounced was the effect of collagen type I which is the most abundant protein in the majority of ECMs and also the whole vertebrate organism. Contrarily to the more dynamic function of fibronectin, abundance of this collagen rather reflects a steady or quiescent state of tissues. Thus, most mesenchymal cells are

surrounded by this protein in their habitat and kept in place without being stimulated by this protein. This is compatible with the niche concept for MSCs or derived precursor cells, ‘feeling more comfortable’ in such type of microenvironment. Like fibronectin the existence of collagen type I may favor adipogenic differentiation, though with some delay, reflected by a higher expression of PPAR γ synthesis in the presence of ECM, while the most effective protein is fibronectin, followed by collagen type I which indicates a diverse ECM specificity in the angiogenic process.

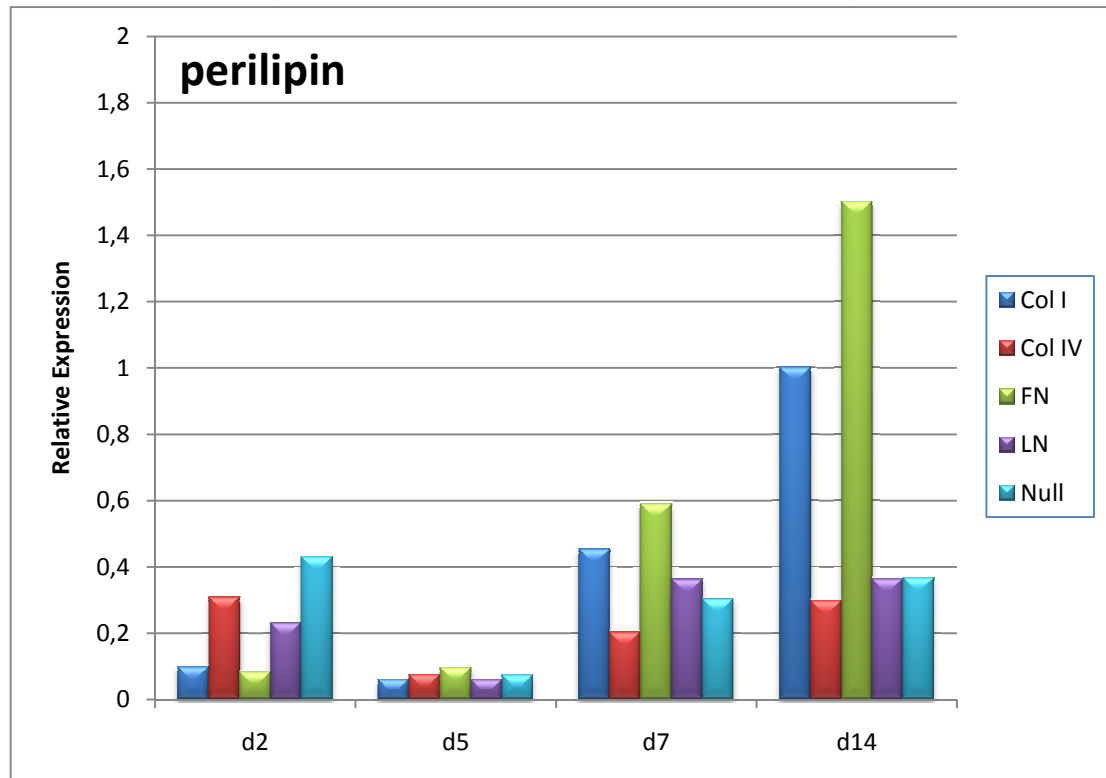


Figure 4.18: Kinetics of perilipin expression of BM72 cells after adipogenic induction, grown on different protein coatings

Perilipin serves important functions in regulation of lipid storage and lipid metabolism in the cells. It is essential for lipid droplet formation. Thus it is one of the most characteristic proteins of adipocytes.

The data on relative gene expression indicate that fibronectin and collagen type I stimulate perilipin synthesis in BM72 cells (Fig. 4.18). This occurs in a similar time frame and intensity as demonstrated for PPAR γ . Thus, fibronectin obviously supports adipogenic differentiation through perilipin production, so does collagen type I which is clearly seen at day 7 and still about two times higher at day 14. In marked contrast, however, laminin and collagen type IV do not seem to have any

positive influence on adipogenic processes, not even detectable at day 14. Collagen type IV gave a relatively lower expression score for perilipin indeed, but this difference was too low to be significant for implying a modest suppressive effect. To conclude, fibronectin and collagen type I exert positive differentiation stimuli attributed to perilipin expression, whereas laminin and collagen type IV are the best neutral in the aspect.

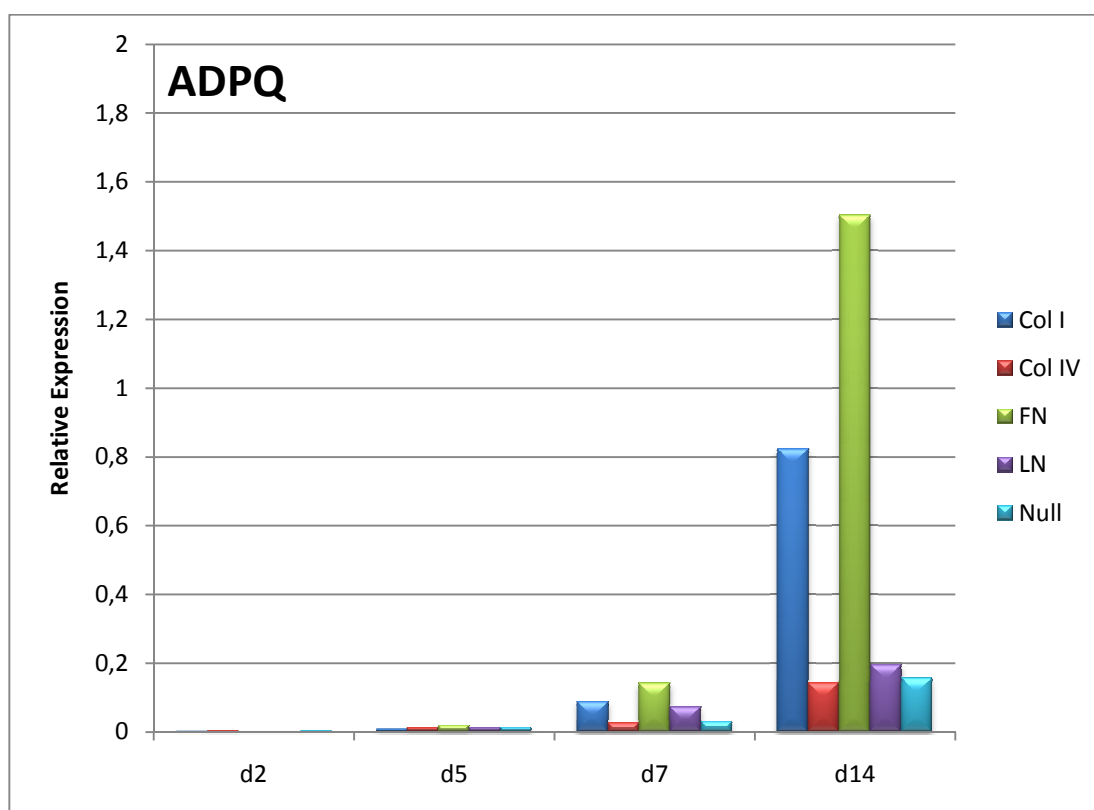


Figure 4.19: Kinetics of ADPQ expression of BM72 cells after adipogenic induction, grown on different protein coatings.

Adiponectin (ADPQ) is a late marker of adipogenesis, which explains again that significant expression of this gene is not observed before day 7, which is further raising until day 14. As seen for the other genes above, ADPQ synthesis is influenced by ECM proteins in different ways.

At day 2 and day 5 there is virtually no ADPQ expression, at best at day 5 cells produce minimal levels of ADPQ. At day 7, some differences become apparent, showing slightly higher ADPQ mRNA levels especially for fibronectin samples. This means once more that cells on fibronectin advance faster in adipogenic differentiation than cells on the other ECM proteins.

At day 14, adiponectin mRNA levels reach the top, showing that primarily fibronectin and, consistently second place, collagen type I supports synthesis of ADPQ like that of the other marker genes. With this adequate finding, the notion that both proteins supports adipogenic differentiation becomes irrefutable. Whereas the maximal stimulation by collagen type I is only about 30% lower than by fibronectin, coating with collagen type IV or laminin results in similar ADPQ expression profiles as on plain dishes as seen before for perilipin synthesis.

Collectively, fibronectin primarily but also collagen type I apparently provide suitable support for long-lasting adipogenic differentiation of MSCs, at least for populations derived from bone marrow.

As final critical synopsis of experimental work, it has to be pointed out again that the results of these qRT-PCR data are relative. In every single reaction, the expression level of regular/current house keeping genes is employed as basic internal standard. In the first series of qRT-PCR reactions, the results of fibroblast and MSCs derived from different tissues were added to the calculation of normalization factors. On the other hand, in the second round of qRT-PCRs, only BM72 cells were analyzed, growing on different coated surfaces or without ECM coating. The important thing is that the lower scores of this second set of reactions, exhibiting relatively lower grades, do not mean these cells express lower amounts of adipogenic markers.

Conclusion

According to the results of relative gene expression analysis, fibronectin was found as the most effective protein even more than collagen type I. But interestingly, at day 2, expression was even lower on collagen type I and fibronectin, implying a delayed onset of differentiation. The cells cultured on fibronectin-coated surface gave an average of 24 Ct values at day 14 for three marker genes with a maximum ratio of 0,04 standard deviation. Day 14 results of fibronectin group and control group are given at Figure 4.20.

Table 4.8: Relative gene expression results of FN, Col IV and control groups at day 14

Day 14	PPAR γ	perilipin	ADPQ
fibronectin	1,50	1,50	1,50
collagen type IV	0,71	0,30	0,14
no coating	0,33	0,37	0,16

Considering these results we can state that fibronectins strongly induce adipogenic differentiation. After fibronectin, collagen type I protein was found to be the most effective. Comparing with control group results, expression levels of marker genes increased approximately threefold at day 14. Laminin protein showed a relatively neutral characteristic, the results were very similar with control group.

A marked effect of the ECM proteins collagen type IV and laminin was clearly restricted to PPAR γ expression and only seen at the late phase (two times higher than control), So this should not be related to adipogenesis and reflect other PPAR γ functions. Day 14 results of collagen type IV group and control group are also given at Figure 4.20. Decreases are occurred in very small rates. Therefore, an inference through the inhibitory effects of collagen type IV on adipogenic differentiation would not be healthy. This protein also demonstrates relatively neutral effect on adipogenesis like laminin protein.

Both experiments were done with BM72 cell strain. In the second assay, the group cultivated on no coating has had the same conditions with the cells at the first assay. The only difference was that ECM proteins investigation was carried out with 2 passages older cells (p6). This is an important situation because since these are primary cell strains and naturally tend to be very sensitive, it is hard to foresee their behaviors in invitro environment. Therefore, their comparison also done as the last step of the thesis, by using their Ct values and internal control house-keeping genes. Results are given at following figures.

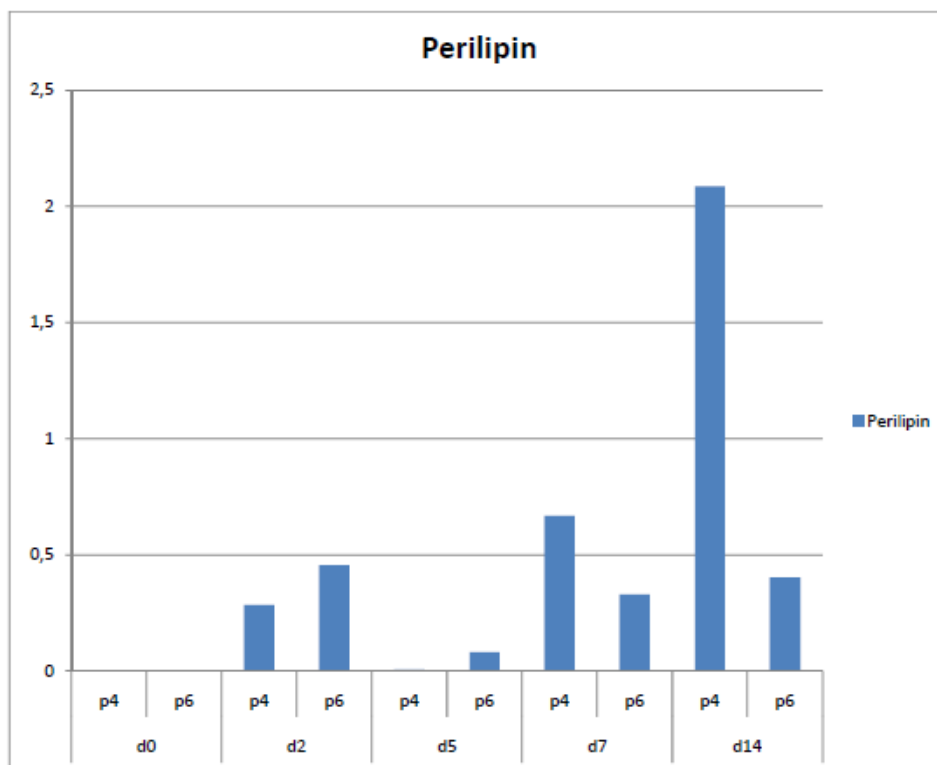
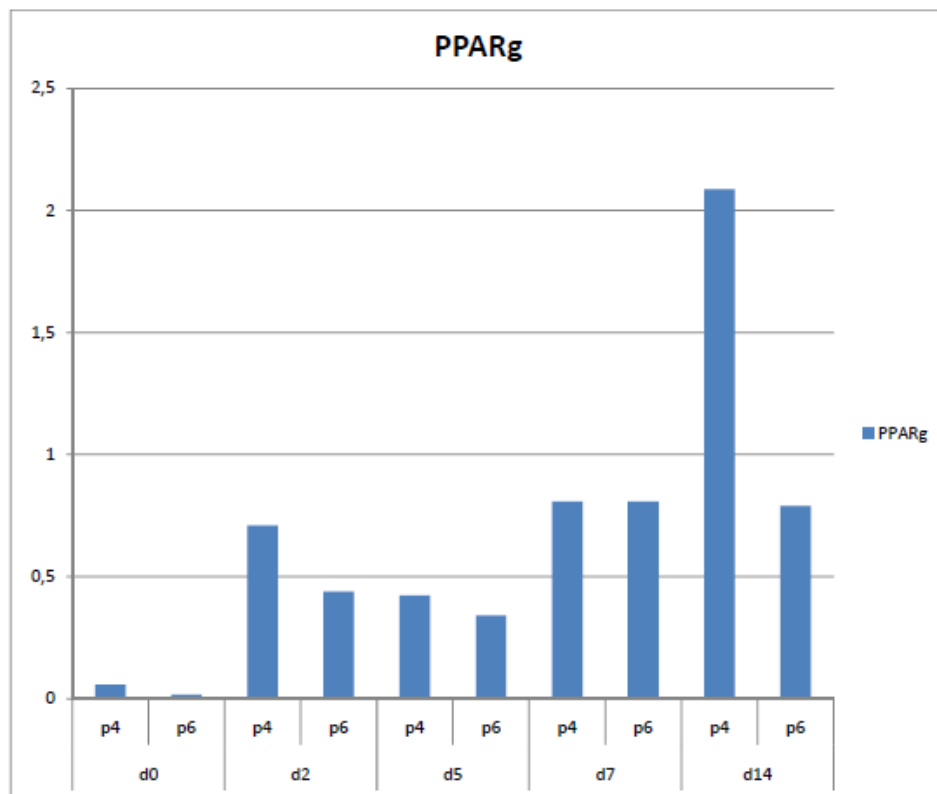


Figure 4.20: Comparison of passage 4 and passage 6 BM72 cells under adipogenic induction due to their relative gene expression results

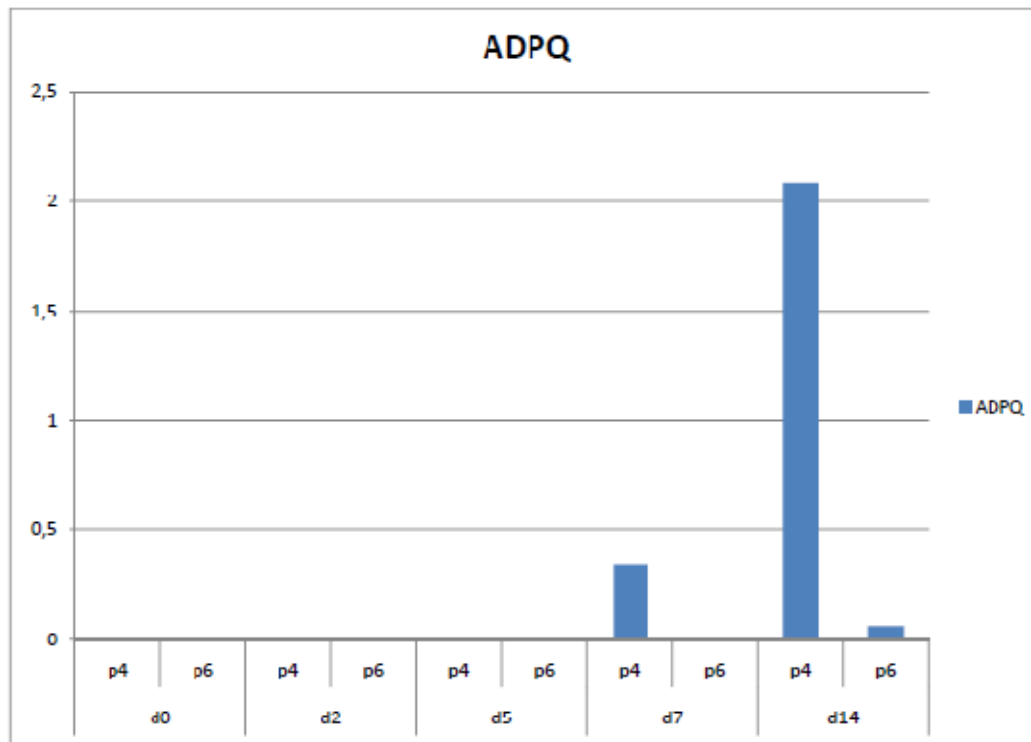


Figure 4.20: (contd.) Comparison of passage 4 and 6 BM72 cells under adipogenic induction due to their relative gene expression results

As observed, BM72 cells tend to lose their differentiation capacity after two times of passaging. In this context, fibronectin and collagen type I, as the abundant proteins of bone marrow matrix, assist to the cells to keep their high differentiation capacity. On the other hand, collagen type IV and laminin are among the basic proteins of basement membrane which is not a part of bone marrow ECM, thus the mesenchymal stem cells cultivated on these proteins showed a decrease in their differentiation potential curve as the cells cultivated without any coating. To conclude, comparison of gene expression results of passage 4 and 6 BM72 cells cultivated with the same conditions might mean that collagen type I and fibronectin proteins accomplish a partial rescue of adipogenic differentiation.

5. CONCLUSION

Though there was a minimum level of PPAR γ expression in MSC control groups, we witnessed an excessive increase in the PPAR γ expression of the cells treated with adipogenic induction. Meanwhile, we observed a reaction in induced fibroblasts but the effects were lighter in comparison with MSCs. Like PPAR γ expression profile, perilipin expression was also seen among the cell types, nevertheless factor dependent skills of this protein points to an adaptational rearrangement more than being a differentiatinal process. MSC samples expressed high amount of ADPQ, a late marker of adipogenesis, whereas we could not observe any expression of ADPQ in fibroblasts. High adipogenic differentiation potential of MSCs were obvious, however, qRT-PCR results revealed some significant differences in kinetics of the adipogenic marker gene expressions. As it is known, all MSCs are classified as multipotent stem cells, but regarding the data herein; they were not equal in their functional properties. This inequality in gene expressions may reflect different degrees of commitment and would be explicative for the conversion of early progenitors into committed pre-adipocytes.

Fibroblasts, on the other hand, needed to be confirmed by further assays including immunofluorescence staining and cell proliferation kinetics. Accordingly, we could not observe any particular change in cell cultures neither for induced group nor control group in phase-contrast microscope images. Closer immunofluorescence staining images revealed a more round cell shape which was normally elongated for fibroblasts, and occurrence of oil vesicles was in correlation with gene expression analysis. Eventually, we believe that though fibroblasts have shown a high adipogenic adaptation capability, they were far from true adipogenesis.

As demonstrated for bone marrow MSCs, fibronectin and collagen type I promote/maintain adipogenesis, possibly also by expansion of intermediate precursor cells, presumably a crucial aspect for homing and tissue repair and regeneration. In contrast, laminin and collagen type IV, being neutral in this respect, are likely candidates for providing a genuine docking site for MSC maintenance in bone marrow or perivascular location. Further studies on this subject would be highly rewarding for tissue engineering and translational medicine.

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APPENDICES

APPENDIX A: Laboratory Equipment

APPENDIX B : Kits & Chemicals

APPENDIX C : Buffers & Medi

APPENDIX D: Mean Ct and Standard Deviation Values of Samples

APPENDIX A

Laboratory Equipment

Micropipettes	Eppendorf Research 2µl 10µl 20µl 100µl 200µl 1000µl
Pipettes	Sarstedt Serological Pipettes 2 ml, 5 ml, 10 ml, 25 ml
Multipipette	Eppendorf Multipipette stream + Combitips
Centrifuge	Hettich ROTINA 420 Tabletop Centrifuge
Minicentrifuge	Eppendorf Centrifuge 5415 D Eppendorf Minispin Plus Labnet Minicentrifuge
Vortex	IKA MS3 Basic & Yellowline TTS2
Balance	Kern Scales 440
Microwave	Clatronic MW 771
Water Bath	Medingen Water Bath WBT22
Laminar Flow	Thermo Scientific HERAsafe Cabinets Class II
Incubator	Thermo Scientific Heraeus Function Line
Refrigerators	Liebherr +4 ⁰ C, FRYKA -30 ⁰ C, Kirsch -30 ⁰ C New Brunswick Scientific -80 ⁰ C
PCR Thermocyclers	Bio Rad DNA Engine Dyad Peltier Thermal Cycler Eppendorf Mastercycler Thermal Cycler
Spectrophotometer	Thermo Scientific Nanodrop ND 1000 V 3.3 + Software
Cell Counter	Beckman Coulter Vi-Cell XR Cell Viability Analyzer + Software
qRT-PCR System	Roche LightCycler 480 Real-Time PCR System Software + geNorm algorithm data calculation
Autoclave	Systec V-150
Heat Sterilizator	Jouan Oven
Microscope	Axiovert 25 Zeiss + AxioCam Icc 1 + Software
Epifluorescence	Leica DM-R

APPENDIX B

Kits & Chemicals

Disinfectants	Bacillol AF - Cellculture
	Applichem RNase-ExitusPlus
Ethanol	Carl Roth 70%, 99,9% (RNA purpose)
Trypsine	PAA
PBS	PAA Dulbecco's PBS (1x) without Ca & Mg
β-mercaptoethanol	Applichem β -mercaptoethanol (RNA purpose)
DMEM	PAA DMEM High Glucose (4,5g/L) with L-Glutamine
FCS	Invitrogen
Penicillin/Streptomycin	Biochrom
Differentiation Media	Lonza Adipogenic Induction Medium
	Lonza Adipogenic Maintenance Medium
Collagen type-I	Roche
Collagen type-IV	Sigma-Aldrich
Fibronectin	Roche
Laminin	Roche
Paraformaldehyde	Sigma-Aldrich
BSA	Roche
Primary Antibody	GF53 – Vimentin specific, Progen Biotechnik
Secondary Antibody	Dianova cat# no 706-226-148
Fluorophore	Cyanine Dye 2 (Cy2), Serva
Fluorophore	Hoechst 33258, Serva
Lysochrome	Oil red o, Fisher Scientific cat# no 50-121-0480
RNA Isolation	Qiagen Rneasy Kit
RT Reaction	Roche Transcriptor High Fidelity cDNA Synthesis Kit
qRT-PCR	Roche LightCycler 480 Probes Master (2 Conc.)
Probes	Roche Diagnostics Universal ProbeLibrary
Primers	Sigma-Aldrich

APPENDIX C

Buffers & Media

Growth Medium	500ml DMEM HighGlucose (4,5g/L) with L-Glutamine 50 ml FCS 5ml 100x Penicillin/Streptomycin
Differentiation Media	<u>Adipogenic Induction Medium</u> (AIM) - Complete Kit Lonza AIM, h-insulin, L-glutamine, MCGS, Dexamethasone, Indomethacin, IBMX (3-siobuty-1-methyl-xanthine), GA-1000 <u>Adipogenic Maintenance Medium</u> (AMM) – Complete Kit Lonza AMM, h-insulin, L-glutamine, MCGS, GA-1000
Trypsine Solution	Trypsin/EDTA (0,025% / 0,01%) Trypsin 125 mg, EDTA 50 mg 500 ml HBSS
PFA Solution	7,4 g PFA (3,7% last concentration) 200 ml PBS pH 7,4 (Dissolve @70 ⁰ C)
BSA Blocking Solution	0,1 g BSA (0,1% last concentration) 100 ml PBS pH 7,4

APPENDIX D - Mean Ct and Standard Deviation Values of Samples

Genes	ADPQ		B2M		GAPDH		perilipin		PPARγ		TBP	
<i>Samples</i> \ <i>Values</i>	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
BM72 p4 day 0	-	-	23,51	0,24	22,14	0,06	37,22	0,35	29,34	0,14	28,22	0,36
BM72 p4 day 2 AD	33,00	0,03	23,41	0,15	22,49	0,04	29,10	0,00	26,23	0,01	29,57	0,34
BM72 p4 day 5 AD	36,45	0,01	21,32	0,07	21,24	0,09	34,84	0,07	25,68	0,01	28,16	0,05
BM72 p4 day 7 AD	26,10	0,01	22,62	0,07	20,94	0,05	26,39	0,06	25,28	0,07	29,06	0,06
BM72 p4 day 14 AD	23,43	0,01	23,45	0,03	21,92	0,04	24,81	0,00	24,40	0,01	28,84	0,06
BM72 p4 day 2 C	-	-	23,53	0,02	22,36	0,03	37,51	0,22	28,95	0,03	29,57	0,01
BM72 p4 day 5 C	31,90	0,02	23,93	0,01	22,38	0,04	30,99	0,11	27,70	0,02	30,27	0,05
BM72 p4 day 7 C	-	-	22,80	0,00	20,96	0,06	-	-	29,11	0,04	31,18	0,02
BM72 p4 day 14 C	36,81	0,47	22,93	0,05	21,65	0,03	37,13	0,14	29,32	0,09	28,10	0,02
Genes	ADPQ		B2M		GAPDH		perilipin		PPARγ		TBP	
<i>Samples</i> \ <i>Values</i>	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
BM78 p4 day 0	-	-	24,04	0,01	22,32	0,04	-	-	30,99	0,06	28,96	0,01
BM78 p4 day 2 AD	24,61	0,03	23,45	0,42	22,02	0,01	30,25	0,01	26,74	0,01	29,84	0,02
BM78 p4 day 5 AD	37,93	0,02	21,94	0,05	21,53	0,04	35,51	0,10	26,96	0,01	28,80	0,05
BM78 p4 day 7 AD	29,31	0,09	23,90	0,01	22,05	0,04	28,63	0,01	27,46	0,01	30,37	0,06
BM78 p4 day 14 AD	23,64	0,05	23,67	0,01	21,59	0,04	24,92	0,01	24,85	0,04	28,58	0,02
BM78 p4 day 2 C	-	-	22,92	0,04	21,98	0,01	-	-	29,84	0,07	29,49	0,03
BM78 p4 day 5 C	34,45	0,14	23,13	0,02	20,88	0,03	33,93	0,09	27,85	0,00	29,31	0,08
BM78 p4 day 7 C	-	-	23,89	0,13	22,36	0,04	-	-	31,01	0,10	29,37	0,22
BM78 p4 day 14 C	37,99	0,22	23,68	0,01	21,86	0,03	36,87	0,17	30,77	0,23	28,64	0,12

Genes	ADPQ		B2M		GAPDH		perilipin		PPAR γ		TBP	
<i>Samples</i> \ <i>Values</i>	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
LA47 p3 day 0	-	-	23,82	0,01	22,82	0,02	-	-	29,05	0,02	28,89	0,05
LA47 p3 day 2 AD	30,79	0,01	23,10	0,01	22,88	0,02	27,31	0,05	25,04	0,02	28,73	0,04
LA47 p3 day 5 AD	30,24	0,03	23,56	0,01	22,90	0,04	28,41	0,06	26,47	0,05	28,99	0,11
LA47 p3 day 14 AD	25,87	0,17	23,54	0,02	21,84	0,13	27,07	0,01	25,25	0,03	29,36	0,07
LA47 p3 day 2 C	-	-	23,25	0,01	22,68	0,04	-	-	28,26	0,04	28,72	0,09
LA47 p3 day 5 C	-	-	23,66	0,02	22,77	0,09	37,63	0,01	28,67	0,01	29,31	0,03
LA47 p3 day 14 C	-	-	23,38	0,07	22,64	0,01	-	-	28,91	0,06	29,01	0,06
Genes	ADPQ		B2M		GAPDH		perilipin		PPAR γ		TBP	
<i>Samples</i> \ <i>Values</i>	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
DF24 p24 day 0	-	-	21,15	0,04	24,08	0,07	-	-	30,31	0,05	29,54	0,48
DF24 p24 day 2 AD	-	-	20,07	0,67	24,02	0,17	-	-	28,79	0,13	30,58	0,40
DF24 p24 day 5 AD	-	-	20,85	0,02	23,48	0,03	-	-	28,63	0,06	30,36	0,19
DF24 p24 day 7 AD	-	-	20,60	0,32	23,42	0,10	36,93	0,14	27,50	0,29	29,94	0,01
DF24 p24 day 14 AD	-	-	20,78	0,11	23,06	0,03	37,34	0,20	27,37	0,09	29,08	0,01
DF24 p24 day 2 C	-	-	20,27	0,01	23,52	0,14	-	-	29,39	0,21	29,83	0,04
DF24 p24 day 5 C	-	-	20,28	0,03	23,84	0,00	-	-	29,84	0,09	29,75	0,04
DF24 p24 day 7 C	-	-	19,79	0,32	23,12	0,00	-	-	29,06	0,01	28,90	0,04
DF24 p24 day 14 C	-	-	19,76	0,02	23,22	0,01	-	-	29,21	0,04	29,91	0,07

Genes	ADPQ		B2M		GAPDH		perilipin		PPARγ		TBP	
<i>Samples \ Values</i>	Ct	sd	Ct	Sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
BM72 p6 d0 PBS	-	-	22,05	0	21,92	0,09	-	-	31,81	0,04	28,20	0,10
BM72 p6 d2 AD PBS	35,41	0,34	21,31	0,44	21,24	0,01	26,63	0,09	26,57	0,02	28,05	0,00
BM72 p6 d5 AD PBS	34,73	0,03	23,22	0,17	22,72	0,07	31,90	0,12	28,45	0,05	29,86	0,11
BM72 p6 d7 AD PBS	32,50	0,11	22,09	0,60	22,06	0,03	28,43	0,30	26,62	0,16	29,56	0,04
BM72 p6 d14 AD PBS	30,12	0,10	23,12	0,06	21,65	0,45	28,59	0,10	27,07	0,10	30,39	0,10
BM72 p6 d0 AD Col I	-	-	22,22	0,06	22,74	0,00	36,66	0,13	30,52	0,37	29,35	0,01
BM72 p6 d2 AD Col I	35,90	0,56	21,35	0,13	19,94	0,03	29,12	0,39	26,69	0,26	28,48	0,07
BM72 p6 d5 AD Col I	35,51	0,36	23,59	0,10	22,14	0,03	32,16	0,26	28,05	0,16	29,74	0,02
BM72 p6 d7 AD Col I	30,07	0,11	22,10	0,13	21,71	0,17	27,05	0,01	25,62	0,02	28,17	0,39
BM72 p6 d14 AD Col I	25,39	0,01	21,11	0,14	21,11	0,02	24,72	0,03	24,02	0,01	27,55	0,30
BM72 p6 d0 AD Col IV	-	-	22,23	0,19	22,49	0,04	-	-	32,01	0,29	29,15	0,05
BM72 p6 d2 AD Col IV	35,09	0,33	21,17	0,16	21,23	0,01	27,09	0,03	26,37	0,08	27,71	0,02
BM72 p6 d5 AD Col IV	33,89	0,08	22,05	0,01	21,45	0,20	30,73	0,24	27,06	0,18	29,28	0,01
BM72 p6 d7 AD Col IV	31,84	0,02	21,46	0,25	20,94	0,27	28,19	0,02	25,87	0,33	28,57	0,03
BM72 p6 d14 AD Col IV	30,54	0,08	23,29	0,20	22,28	0,38	29,27	0,20	26,18	0,43	30,33	0,25
BM72 p6 d0 FN	-	-	22,40	0,64	22,87	0,04	36,89	0,14	31,01	0,42	29,35	0,10
BM72 p6 d2 AD FN	36,34	0,13	21,56	0,25	20,39	0,01	29,72	0,04	26,90	0,13	28,56	0,12
BM72 p6 d5 AD FN	34,65	0,81	23,64	0,03	23,31	0,25	32,04	0,35	28,16	0,77	30,61	0,32
BM72 p6 d7 AD FN	28,57	0,07	21,36	0,02	20,93	0,01	25,82	0,12	24,86	0,01	27,74	0,01
BM72 p6 d14 AD FN	24,40	0,01	21,29	0,33	21,05	0,04	24,02	0,02	23,53	0,06	27,64	0,02

Genes	ADPQ		B2M		GAPDH		perilipin		PPARγ		TBP	
<i>Samples \ Values</i>	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd	Ct	sd
BM72 p6 d0 LN	-	-	21,92	0,19	21,66	0,04	-	-	31,72	0,14	28,28	0,01
BM72 p6 d2 AD LN	36,17	0,07	20,71	0,20	21,07	0,03	27,48	0,04	26,42	0,03	27,94	0,07
BM72 p6 d5 AD LN	35,84	0,01	24,01	0,54	23,29	0,03	33,34	0,14	28,77	0,10	31,34	0,06
BM72 p6 d7 AD LN	30,12	0,04	21,93	0,14	21,19	0,03	27,16	0,08	25,65	0,06	28,00	0,01
BM72 p6 d14 AD LN	29,32	0,05	22,50	0,01	21,86	0,04	28,18	0,17	25,90	0,20	29,59	0,03

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