

Features of Lipid Metabolism along Differentiation Pathway of Human Mesenchymal Stem Cells towards Mature Adipocytes

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CARMEN S. TATU¹, FLORINA M. BOJIN^{1*}, GRUIA T. ALEXANDRA²,
VALENTIN L. ORDODI¹, FELIX A. MIC², IMAN VLAD¹, ADA CEAN³,
OANA I. GAVRILIUC¹, VIRGIL PAUNESCU¹

¹ Department of Functional Sciences, University of Medicine and Pharmacy "Victor Babes" Timisoara, Pta. E. Murgu No.2, RO-300041 Timisoara, Romania

² Center of Immunophysiology and Biotechnologies, Emergency Clinical County Hospital Timisoara, Blvd. Iosif Bulbuca No. 10, RO-300736 Timisoara, Romania.

³ Banat's University of Agricultural Sciences and Veterinary Medicine from Timisoara

*Corresponding author: Florina Bojin, MD, PhD, Phone: 0040-729-580609, Phone: 0040-729-580609, E-mail: florinabojin@umft.ro

Abstract

Human mesenchymal stem cells (MSCs) obtained from adult bone marrow are characterized by *in vitro* ability to differentiate towards at least three cellular lineages: adipocytes, osteocytes, and chondrocytes. When considering the adipogenic potential of MSCs, 21 days after induction with specific media, these cells are capable of accumulating cytoplasmic lipid vacuoles, similar to native adipose tissue-isolated adipocytes. There is scarce data regarding the composition of fatty acids within the *in vitro*-differentiated adipocytes and the regulation of the metabolic functions.

We used gas chromatography/mass spectrometry to identify fatty acid (FA) species in MSC-derived preadipocytes/adipocytes and compared them to the FA profile found in adipocytes isolated from normal-weight adult adipose tissue. We found that differentiation media did not contain essential fatty acids, and MSCs-differentiated preadipocytes and mature adipocytes contain significantly reduced amounts of arachidonic acid (C20:4) and docosahexaenoic acid (C22:6) compared to native adipose tissue. MSCs, adipocytic precursors and mature adipocytes lacked linolenic acid (C18:3) and contained almost insignificant amounts of linoleic acid (C18:2) compared to native adipose tissue-derived adipocytes. To investigate the gene expression-related causes in the dysregulation of lipid metabolic pathways, we compared gene expression profiles of undifferentiated MSCs, preadipocytes/adipocytes to the reference adipose tissue, and microarray analysis showed different expression profiles in genes along the adipocytic differentiation pathway, mainly in desaturase and elongases, genes involved in polyunsaturated fatty acids (PUFA) synthesis from essential fatty acids.

The composition of FAs in adipocytic differentiation media, together with MSCs inability to functionally convert these acids into appropriate lipidic end-products required for normal functioning of mature adipocytes makes the MSCs poor candidates of *in vitro* adipogenesis process, while the differentiation media requires supplements of essential fatty acids, which may improve cellular function.

Keywords: fatty acids, GC-MS, microarray, gene expression profile, differentiation media

Introduction

Adipogenesis is a complex process involving proliferation of precursor cells, commitment towards the adipogenic lineage and terminal differentiation into mature adipocytes (CONGET & MINGUELL, [1]; ENTENMANN & HAUNER, [2]). Although the terminal differentiation of adipocytes is intensively studied and described using murine cell

lines, key information about the commitment of precursor cells to the adipogenic lineage is still lacking.

Human pre-adipocytes derived from adipose tissue through collagenase digestion are widely used to study human adipogenesis; however, these cells are already committed to the adipogenic lineage (PITTINGER & al., [3]). Moreover, human pre-adipocytes have reduced proliferative ability, unpredictable variability based on different donors and anatomical sites, and limited availability (PITTINGER & al., [3]).

Committed preadipocytes have to withdraw from the cell cycle before undergoing adipose conversion. For preadipose cell lines as well as for primary preadipocytes, growth arrest is required for adipocyte differentiation and is normally achieved through contact inhibition. However, contact inhibition *per se* is not a prerequisite for adipogenesis since cells plated at low density in serum-free medium or kept in a methylcellulose suspension still undergo differentiation (DELORME & al., [4]). Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps, leading to the progressive acquisition of the morphological and biochemical characteristics of the mature adipocytes. The nature of the induction depends on the specific cell culture model used because the responsiveness to inducing agents may vary considerably between preadipose cell lines and primary preadipocytes (DELORME & al., [4]). Overall, in serum-containing medium, the standard adipogenic cocktail contains supraphysiological concentrations of insulin, dexamethasone, and isobutylmethylxanthine, highlighting the involvement of the insulin/IGF-1, glucocorticoid, and cAMP signaling pathways in the adipocyte differentiation process (MENSSEN & al., [5]).

Bone marrow-derived mesenchymal stem cells (MSCs) represent an alternative to this model (VIDAL & LOPEZ, [6]), because they are multipotent precursors of several cell types, including adipocytes, osteocytes, and chondrocytes (GIMBLE & al., [7]), they can be isolated from donor bone marrow and separated from other cell types by centrifugation on a density gradient. The functional variability and differentiation flexibility among MSCs from different subjects was reported to be low, while MSCs proliferate under *in vitro* conditions and retain their trilineage differentiation potential (GIMBLE & al., [7]).

The first hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cells are converted from fibroblastic to spherical shape. These morphological modifications are paralleled by changes in the level and type of extracellular matrix (ECM) components and the level of cytoskeletal components (BOONE & al., [8]; KHAN & al., [9]). Recent findings indicate that these are key events for regulating adipogenesis as they may promote expression of critical adipogenic transcription factors, including CCAAT/enhancer binding protein(C/EBP) and/or peroxisome proliferator-activated receptor (PPAR) (MUTCH & al., [10]; RANGWALA & LAZAR, [11]; TREMAIN & al., [12]). Mediation of the proteolytic degradation of the stromal ECM of pre-adipocytes by the plasminogen cascade is required for cell-shape change, adipocyte-specific gene expression, and lipid accumulation (MORRISON and FARMER, [13]; SELVARAJAN & al, [14]). Several reports have attempted to schematize the stages of adipocyte differentiation into a simple hierarchy of molecular events. Genes differentially regulated during adipogenesis have been categorized into early, intermediate, and late mRNA/protein markers (MENSSEN & al., [5]; NTAMBI & YOUNG, [15]; ROSEN and SPIEGELMAN, [16]). However, adult adipocytes require fatty acids (FA) to perform some of their specific functions, mainly polyunsaturated fatty acids (PUFA), which were shown to regulate lipogenic genes, dependent upon chain length and degree of unsaturation (JUMP, [17]; NAKAMURA & al., [18]; ROSEN & MacDOUGALD, [19]).

The overall goal of our study was to investigate the pattern of fatty acids accumulating within the MSCs-derived preadipocytes and adipocytes during the differentiation process and

to correlate data with FA existent in native human adipose tissue. We were particularly interested in differentially expressed transcription factors corresponding to adipogenesis process and we report genome-wide gene expression profiling of 5 human MSCs primary lines on adipogenic differentiation at 3 time points (day 0, 5-7, and 21) in reference to normal human fat tissue.

Materials and methods

Isolation and expansion of human BM-derived MSCs

Bone marrow was obtained using iliac crest aspirates from five patients undergoing orthopedic surgery, and further tested for exclusion of any hematological disorder. Approximately 5 ml of bone marrow was mixed with culture medium and normal human MSCs were isolated by adherence of the fibroblast-like cells to the culture plate's surface and following two consecutive passages. MSCs culture and expansion media contained alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 1ng/ml fibroblast growth factor (FGF; R&D Systems, Minneapolis, USA) and 1% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell) and cells were maintained at 37°C in 5% CO₂ atmosphere. When reaching 80% to 90% confluence, the cells were passed using 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company, Ayrshire, UK) followed by washing step (10 minutes centrifugation, 300×g) and were replated in T75 culture flasks at a density of 10,000 cells/cm² to ensure optimal proliferation. Starting with passage 3, part of the cells was used for further phenotypical analyses, whereas the other part of MSCs was employed for further adipocytic differentiation assays.

Isolation of native fat tissue

Fat tissue samples were harvested from normal subcutaneous areas of consenting patients undergoing orthopedic surgery. The patients were age and sex-matched to the patients involved in MSC harvesting and isolation. Donation of fat tissue was approved by the ethical committee and was performed in sterile conditions, without endangering the patients' health or the surgery outcomes.

All tissue and bone marrow samples were obtained after signing the written informed consent elaborated under an approved protocol by the Ethics Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara, according to the World Medical Association Declaration of Helsinki.

Adipocytic differentiation

Adipogenic differentiation assays were conducted for MSCs seeded in appropriate culture flasks at a cellular density of 20,000 cells/cm², being stimulated to differentiate in adipogenic medium containing Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), 10% FCS (PromoCell), 45 mM 3-isobutyl-1-methylxanthine (IBMX), 50 M indomethacin, 10 µg/ml insulin, 1 µM dexamethasone, and 1% Penicillin/Streptomycin (PromoCell). Alternative adipogenic differentiation media consisted of ready-to-use Nonhematopoietic stem cell medium for generation of adipocytes (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 1% Penicillin/Streptomycin (PromoCell). All chemical substances were purchased from Sigma-Aldrich Company, unless otherwise specified.

The process of differentiation towards fully developed *in vitro* adipocytes (mature adipocytes) requires 21 days, during which the MSCs pass through a period of pre-adipocytes

(days 3-7 upon adipogenic induction). Along the differentiation pathway, we identified as check-points for further analyses D0 (MSCs at passage 3 and 4), D5 and D7 (MSC-derived pre-adipocytes), and D21 (MSC-derived mature adipocytes).

Histochemical and immune staining

MSCs, pre-adipocytes and mature adipocytes obtained in *in vitro* cultures were stained for Oil Red O following the manufacturer protocol. Briefly, cells were washed twice with PBS, fixed in 10% formaldehyde solution in aqueous phosphate buffer for 30 minutes at room temperature, washed with 60% isopropanol, and stained with filtered Oil Red O (Sigma-Aldrich Company) working solution for 15 minutes. After repeated washing steps with tap water, the nuclei were counterstained with hematoxylin solution (Hematoxylin, Mayer's Lillie's Modification, Dako, Glostrup, Denmark).

Approximately 5 mm blocks of adipose tissue were snap-frozen into Killik-cryostat embedding medium (Bio-Optica, Milano, Italy) and liquid nitrogen and sectioned (3-5 μ m thick) using Microm HM 505 E cryostat (Microm GmbH, Walldorf, Germany). Sections, mounted on Superfrost Plus Fisher (Thermo Fisher Scientific Inc., Hennigsdorf, Germany) and air dried for 30 minutes were fixed in 10% formaldehyde for 30 minutes and Oil Red O stained as previously described.

Adult adipocytes were isolated by enzymatic digestion of adipose tissue with collagenase type IV-S (from *Clostridium histolyticum*; Sigma-Aldrich Company) for 30 minutes at 37° C, washed with Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich Company) and cytospin slides were obtained by 6 minutes centrifugation at 600 rpm in Shandon Cytospin 4 (Thermo Fisher Scientific). Slides were air-dried for 10 minutes and then used for Oil Red O staining.

MSCs and pre-adipocytes were also grown in 4-well glass chamber slides, washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then investigated for expression of cytoskeleton protein using the primary monoclonal mouse anti-swine vimentin (clone V9) (DakoCytomation, Dako), AlexaFluor488 conjugated secondary antibody (Molecular Probes, Invitrogen) and nuclear counterstaining with DAPI (Sigma-Aldrich Company). All incubation steps were accomplished at room temperature, using antibody solutions in PBS containing 2% Bovine Serum Albumin (BSA; Sigma-Aldrich Company).

Microscopy analysis was performed on a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with adequate fluorescence filters.

Lipid extraction and analysis of fatty acids

For lipid extraction and subsequent analysis by GC/MS cells were washed three times with ice-cold PBS, and the pellet was resuspended in 50 μ L PBS. Further, the cells were mixed with 400 μ L chloroform/methanol 2/1, centrifuged at 4000 rpm for 5 min, and the chloroform phase was transferred in a clean tube. 200 μ L of water and 100 μ L of chloroform were added to the previous chloroform phase, admixed and centrifuged at 4000 rpm for 10 min. The chloroform phase was transferred, evaporated to dryness in a stream of nitrogen, and resuspended in 100 μ L of chloroform/methanol 1/2. 50 μ L of the previous mixture was saponified 1 h at 65°C, neutralized with HCl, and extracted with hexane containing acetic acid 2%, according to a previously described method (Jump, 2009). The organic phase was dried and resuspended in 200 μ L methanol, followed by derivatization and GC/MS injection.

Gas chromatograph-mass spectrometry of free fatty acids

The MSCs, pre-adipocytes, and mature adipocytes were trypsinized, washed twice in PBS, and the resulting pellet was resuspended in 200 μ l cold lysis buffer (150 mM NaCl, 20mM Tris-HCl, 0.2% Triton-X 100 pH=7.4). Fragments of human adipose tissue (~200 mg) were homogenized in 200 μ l cold PBS. All cellular lysates and the homogenate were derivatized in order to convert free fatty acids (FFAs) to fatty acid methyl esters (FAMES), as previously described (WANG & al., [20]). Briefly, a 150 μ l aliquot of each cell lysate or homogenate was added to a 5 ml mixture of 50:1 v/v methanol: acetyl chloride in a screw-top glass tube. The tubes were vortexed at room temperature in the dark for 45 min, and the methylation was stopped by adding 3 ml of 0.25 M potassium carbonate. 1 ml hexane was added, and after 1 h of vortexing in the dark at room temperature, the hexane layer, which contained FAMES, was collected, and evaporated under nitrogen flow. The dry residue was redissolved in 100 μ l of hexane, and the samples were analyzed with a HP6890 Series Gas Chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled with a Hewlett Packard 5973 Mass Selective Detector. The gas chromatograph was equipped with a split-splitless injector and a Factor Four™ Capillary Column VF-35ms (Varian, Middelburg, The Netherlands) 30 m long and 0.25 mm inner diameter, with a film thickness of 0.25 μ m. For gas chromatography, the temperature ranged from 100 to 300 °C, with a 6°C/min gradient, and the solvent delay was set up at 7 min. The injector was maintained at 250°C, and helium was the GC carrier gas. Gas flow was set up to 1.0 ml/min, and 2 μ l of sample were injected in the splitless mode. The mass spectrometry conditions were the following: ionization energy, 70 eV; electronic impact ion source temperature, 200°C; quadrupole temperature, 100°C; scan rate, 1.6 scans/s; mass, 40-500 amu. For the identification of compounds, the mass spectra of the samples were compared to mass spectra in the Mass Spectral Library 2.0 developed by the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health (NIST/EPA/NIH). The retention times for derivatized FFAs were as follows: palmitic acid (C16:0) 18.28 min; oleic acid (C18:1) 21.31 min; stearic acid (C18:0) 21.39 min; linoleic acid (C18:2) 21.46 min; linolenic acid (C18:3) 21.71 min; arachidonic acid (C20:4) 24.10 min.

Manual integration of each individual peak was performed for area calculation. The peak areas of FFAs summed to 100%, and the percentage of area that corresponded to each FFA was plotted. Data represent the mean of all five experiments performed for each individual bone marrow sample harvested from donors and further differentiated, as well as the native adipose tissue samples.

Microarray analysis

Human MSCs from 5 different donors, at passages 3 and 4, were subjected to *in vitro* induced adipogenesis for up to 21 days, as previously described. At days 0 (undifferentiated MSCs), 5, 7 (preadipocytes) and 21 (adipocytes), total RNA was isolated using the GenElute Mammalian Total RNA Miniprep (Sigma, St. Louis, MO, USA) and its concentration was determined on the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA isolated from human native fat tissue served as positive control. The RNA was checked for integrity and purity with the Agilent Bioanalyzer 2100 (Agilent Technologies, Boblingen, Germany). Total RNA from each individual donor and sample was used for genome-wide gene expression profiling. Hybridization on Human GE 4x44K v2 oligonucleotide microarrays was performed according to standards supplied by the manufacturer (Agilent Technologies). In brief, cDNA was synthesized from 50 ng of total RNA and submitted to *in vitro* transcription to generate Cy3-labelled complementary RNA. 1.65 μ g of the fragmented complementary RNA were hybridized to the microarray chips for 17 hours at 65°C. Arrays were washed and scanned on a GenePix4000B scanner.

Quantitative RT-PCR

RNA samples used in this study were isolated from MSC, pre-adipocytes and mature adipocytes, as well as from adipose tissue. Total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Company). Concentration of each sample was measured using Nanodrop ND-1000 spectrophotometer. Sample purity, as indicated by the 260/280 absorbance ratio, was 1.9-2.02. We used 1 µg total RNA for every 20 µl reverse transcription reaction performed with the AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Palo Alto, CA). cDNA samples were analyzed by quantitative real-time PCR, using the LightCycler 480 SYBR Green I Master (Roche, Florence, SC, USA) and the following primers: PPAR γ forward 3'-AAGACCACTCCCCTCTTTG-5' and reverse 3'-GTCAGCGGACTCTGGATTCA-5'; C/EBP α forward 3'-TCGACATCAGCGCCTACATC-5' and reverse 3'-CTTGTCACCGACTTCTTGG-5'; LPL forward 3'-CTGAAGACACAGCTGAGGAC-5' and reverse 3'-CTGGTGAATGTGTGTAAGAC-5'; FABP4 forward 3'-ATGCTTTTGTAGGTACCTGG-5' and reverse 3'-CTCTCTCATAAACTCTCGTG-5'; leptin forward 3'-CCAAGATGGACCAGACACTG-5' and reverse 3'-GCCACCCTCTGTGGAGTA-5'; huOB-R forward: 3'-TTGTGCCAGTAATTATTCCTCTT-5' and reverse 3'-ATAGCTTTTTCATTCTTTGGTGTG-5'; huB219.1, huB219.2, and huB219.3 forward 3'-TTGTGCCAGTAATTATTCCTCTT-5' and reverse 3'-CTGTGGCCTTCCGCAGTG-5', 3'-ACCTCCACCCAGTAGTTCCTT-5', and 3'-AGTTGGCACATTGGGTTCAT-5', respectively. HPRT1 (hypoxanthine phosphoribosyltransferase 1) was chosen as a suitable reference gene; the primers were HPRT1 forward 5'-CCTGGCGTTCGTGATTAGTGAT-3' and reverse 5'-AGACGTTCA-GTCCTGTCCATAA-3' We performed a relative basic quantitation based on the $\Delta\Delta C_t$ method with the LightCycler480 Software.

Statistical analysis

All statistical tests and values were calculated with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean \pm s.e.m. Groups were compared using either two-way ANOVA or the Mann-Whitney U test for nonparametric values. $P < 0.05$ was considered to be significant.

Results and discussion

Microscopy of MSCs-derived cells

Bone marrow-derived MSCs and pre-adipocytes stained with Oil Red O did not reveal lipid accumulation. Compared to MSCs, pre-adipocytes presented a clear morphological change, becoming round-shaped and losing intercellular contacts, while undifferentiated MSCs retained their fibroblast-like, spindle-shape morphology. Both cellular types were positive for cytoskeleton protein vimentin, which is usually abundant in stromal cells (Figure 1).

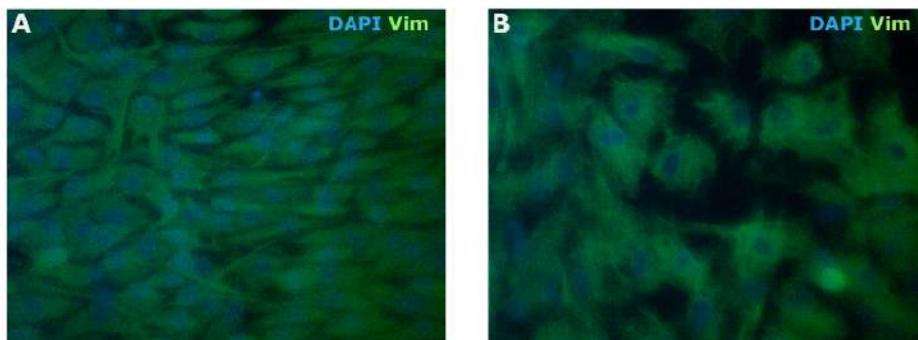


Figure 1. Morphological variation and vimentin expression in undifferentiated MSCs (A) and MSC derived preadipocytes (B). Immunofluorescence shows the fibroblast-like morphology of MSC converging to spindle shape organization, and highly expressing vimentin. Preadipocytes become round-shaped, with disorganized distribution on the culture plate, but maintain increased expression of vimentin within the cytoplasm. AlexaFluor 488-conjugated antibody is used for fluorescence visualization and DAPI as nuclear counterstaining (Ob. 40x).

Most of the MSC-derived mature adipocytes were positive for Oil Red O histochemical staining, showing agglomeration of red lipid vacuoles within the cytoplasm, while the nuclei were pushed aside. The morphological changes are similar to the shape described for preadipocytes (Figure 2C). The differentiation ratio from MSCs to mature adipocytes, based on lipid droplets intracytoplasmic assemblage, was determined as approximately 60% after counting the Oil Red O positive cells on 5 microscopic fields. Cryotome adipose tissue 3 μ thick sections stained for lipid accumulation revealed typical Oil Red O positivity, indicating that the lipids are present within the adult adipocytes (Figure 2B). When isolated from the native adipose tissue, adult adipocytes showed presence of various sized lipid vacuoles, also staining red for the elected histochemical dye (Figure 2A).

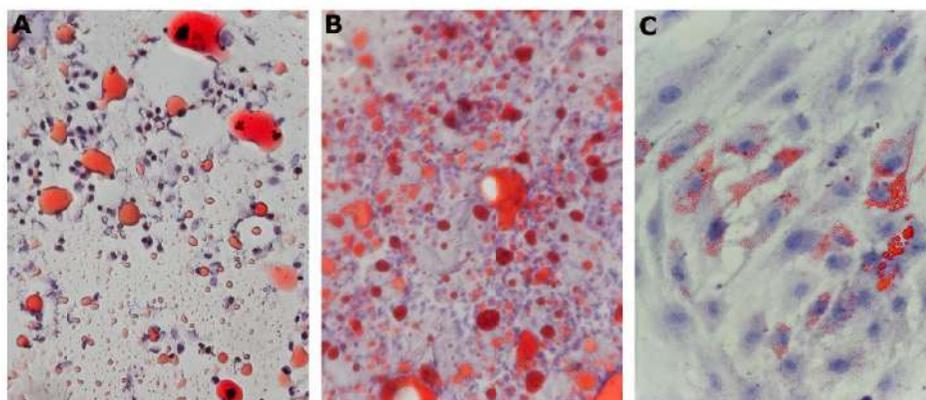


Figure 2. Histochemical Oil Red O staining of adipocytes accumulating lipid droplets within the cytoplasm. Adult adipocytes isolated from adipose tissue by enzymatic digestion and cytospun on a slide (A) present numerous lipid vacuoles with inhomogeneous distribution compared to adipose tissue sections (B), due to enzymatic disruption of cellular membrane and further release of lipid content. Only 60% of adipocyte-induced MSCs were able to differentiate into mature adipocytes (C) presenting distinct pattern of lipid accumulation and pushing the nucleus aside. Hematoxylin nuclear counterstain is visible in all panels.

Fatty acids composition is different between MSC-derived adipocytes and native adipose tissue

Fatty acids composition was determined using two methods, which can identify either free fatty acids (FFA) in cellular lysate or fatty acids fraction bound to phospholipids and triglycerides within the cells. Samples used for analysis were cultured MSCs, MSC-derived pre-adipocytes and mature adipocytes, as well as native adipose tissue (adult adipocytes). The derivatization method for obtaining the FFA composition from cellular lysates was previously described by the authors to provide similar results in rat models (BOJIN & al., [21]). The same methods were applied for identification of FA in culture media – MSCs culture media and adipogenic differentiation media.

We were not able to identify linolenic, linoleic and arachidonic acids in culture media (both MSC and adipocyte culture media) using the FFA detection method. The composition of these media in respect to FA consisted of palmitic (16:0), oleic (C18:1), and stearic (18:0) acids in almost similar proportions (approximately 30%). The amount of these acids did not present significant variations along the differentiation pathway from MSCs to pre-adipocytes and mature adipocytes. However, stearic acid determined from native adipose tissue represented only 6.5% from total FFA, which is 5 times less than the value found in differentiated adipocytes. Same method provided data about linoleic, linolenic and arachidonic acids in cellular lysates of differentiated cells, and we were not able to identify the presence of linolenic acid in either MSCs, pre-adipocytes, or mature adipocytes, while the adult adipocytes counterparts contained 1.6% of total determined FFA. Linoleic acid amount presented a progressive decrease in all differentiation stages, from MSC to mature adipocytes, but the value was 20 times lower than the proportion found in cellular lysate of adipose tissue. Arachidonic acid was found in increased proportion in MSCs (10% of total FFA), but its amount decreases in pre-adipocytes to almost undetectable values, increasing again within mature adipocytes to values representing 2% of total FFA. In adipose tissue lysate, the proportion of arachidonic acid is around 1% of total free fatty acids (Figure 3G).

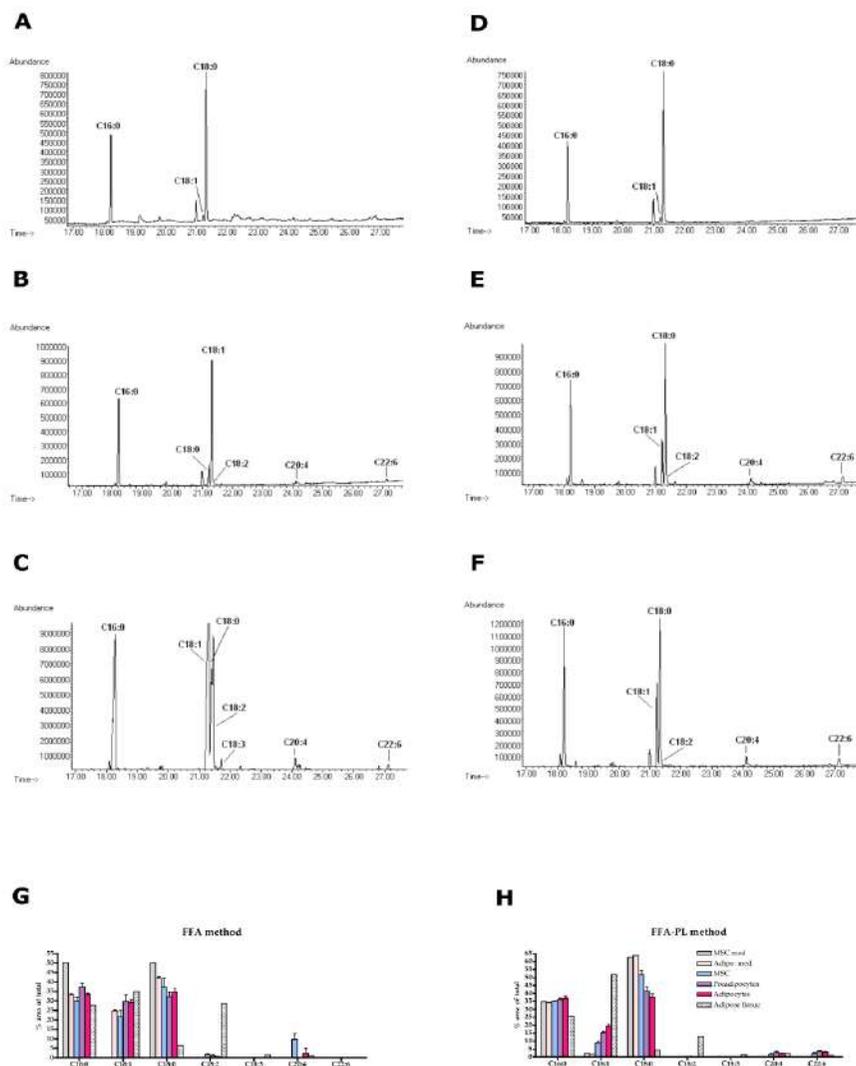


Figure 3. Distribution of fatty acids determined by GC/MS method. Chromatograms show presence of bound fatty acids in mesenchymal stem cells culture media (A), mesenchymal stem cells (B), adipose tissue (C), adipocytic induction media (D), differentiated pre-adipocytes (E), differentiated mature adipocytes (F). Graphs showing percentage distribution of saturated and unsaturated FA comparatively determined in all samples using FFA method (G) and total lipids extraction of FA method (H).

Lipid extraction method revealed additional fatty acid previously undetected - docosahexaenoic acid (DHA, C22:6), n-3 family member long chain poly unsaturated fatty acid, resulting from linolenic acid metabolism. Consequently, it can be assumed that this compound is present in the cells in bound form. This FA is detected in constant amount in all *in vitro* cultured samples, being almost double compared to the amount within the adipose tissue. This method was not helpful in determining essential linolenic acid (C18:3) in MSCs or MSCs-derived pre-adipocytes and mature adipocytes, while the amounts of bound linolenic acid detected in adipose tissue were comparable to that of free form, suggesting that this acid

present a balance between free and bound forms in adipose tissue (Figures 3B, 3C, 3E, and 3F).

Analyzing the percentage area for saturated (palmitic and stearic) and monounsaturated (oleic) fatty acids we found that they maintain a constant percentage of 95% in MSCs, pre-adipocytes and mature adipocytes, oleic acid increasing progressively along the differentiation pathway from 10% to 15%, and 20% respectively, while stearic acid decreases its amount proportionally. However, the native adipose tissue has only a total amount of 80% of these acids, but the ratio between oleic and stearic acid is 13:1 (52% oleic acid, and 4% stearic acid) (Figure 3C). When extracting percentage area of total FA amount, palmitic acid (C16:0) has a constant area percentage in all samples, using both methods, with the exception of native adipose tissue, which has a non-significant smaller area (Figures 3G and 3H).

Only traces of linoleic acid (C18:2) were found using lipid extraction method, in all the *in vitro* cultured and differentiated cells, representing 0.2% of total fatty acids, compared to the adipose tissue in which the proportion of linoleic acid was around 13%. Arachidonic acid (C20:4) was detected using this method in MSC, pre-adipocytes and mature adipocytes having a value of approximately 2%, which is similar to the value in adipose tissue (2.34%).

We were not able to identify linoleic, linolenic, arachidonic or docosahexaenoic acids within MSCs culture media and adipogenic induction media. Both media types contained palmitic acid (35%), oleic acid (2%), and stearic acid (63%) (Figures 3A and 3D).

Differential gene expression profiling

We documented mRNA expression of genes well established for their involvement in adipocytes metabolism and lipid transport by quantitative RT-PCR, considering as candidate genes LPL, PPAR gamma, C/EBP alpha, FABP4, Leptin and Leptin receptors (huB219.1, huB219.2, huB219.3, and huOB-R). Compared to MSCs, upregulation of the genes was progressive, from preadipocytes (D5-7) towards mature adipocytes (D21), but no statistical significance was found except for LPL gene. When compared to adipose tissue, FABP4, Leptin and Leptin receptors were significantly lower expressed in MSCs-differentiated adipocytes (Figure 4).

Initially, one-color data obtained from our microarray experiment were extracted using Agilent Feature Extraction Software and normalized to the 75th percentile signal value. Further downstream analysis was performed with the Genespring GX 11.0 program.

To investigate the gene expression-related causes in the dysregulation of lipid metabolic pathways, we compared the gene expression profiles of undifferentiated MSCs to the reference adipose tissue and identified a number of 148 candidate genes which were differentially expressed by $FC \geq 10$ in adipose tissue and changed by less than $FC = 2$ during *in vitro* differentiation of MSCs into mature adipocytes. These candidate genes were clustered hierarchically by both entities and conditions, resulting in 15 distinct clusters of gene expression. We hypothesized that the defect in the induction of these genes might be correlated to the incomplete maturing of adipocytes during *in vitro* protocols of differentiation when compared to their *in vivo* tissue counterparts. Of these 148 genes, 3 genes are involved in cell proliferation ($p=0.043$) and 35 are connected to extracellular components ($p=0.0006$).

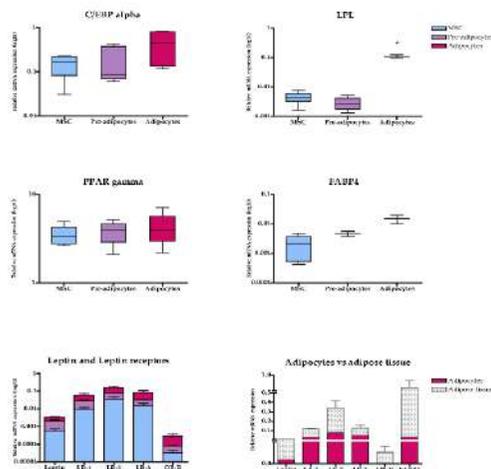
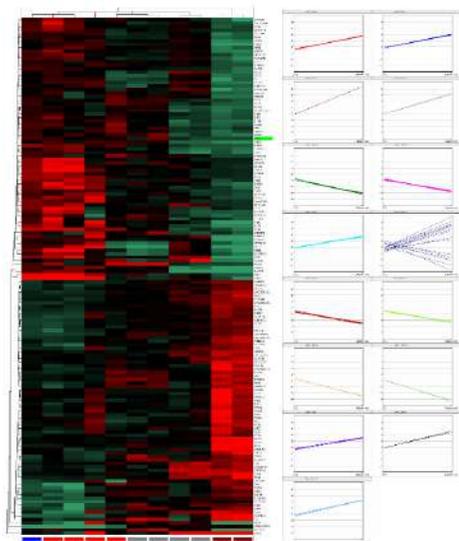


Figure 4. Graphic representation of qRT-PCR for well established genes of adipocyte metabolism. All genes investigated presented an increased expression along the adipocytic differentiation pathway, comparative to originating MSCs. Only LPL gene confirmed a significant increase ($p < 0.05$) in reference to MSC. When compared to adipose tissue, differentiated mature adipocytes demonstrated a low expression level for leptin, leptin receptors, and FABP4.

Pathway analysis revealed 2 genes (PDK4 and PLTP) associated with lipid transport (Figure 5 and 6).



- Cluster 0: SLC40A1, TMEM150C, CTTNBP2, ST8SIA4, LASSA, CLYBL, SERPINA5, STON1-GTF2A1L, ELANE, LNX1
- Cluster 1: LOC729083, TSKS, PCSK6, CADM3, CXCR7, ABCG1, SPATA22, SOX5, NRCAM, PRG2, ADCY1, ADAMTSL3, PPM1L
- Cluster 2: ANGPTL7, ZBED3, CITED1, CD40, LAMA2, RIMKLA, SHC2, GLUL, TMEM178, RNF144A, C1QTNF4, ZIC4, PCBP3, C17orf14, FRAS1, TRIM50, MOGAT1, C1QTNF1
- Cluster 3: PLTP, RASD1, PRPH, IMPA2, MKX, SPINK1, ITPRIPL1, METTL7A, GRIN2C, HPSE, PELI2, BDH1, C13orf18
- Cluster 6: ELMO1, PDGFD, NGFR, FOXO1
- Cluster 7: KCNK5, EDNRB, CXCL2, FAM134B
- Cluster 11: ADRA2A, SLC7A2
- Cluster 16: CNR1, RERG, PRELP
- Cluster 18: KDELR3, DSP, ALPK2, KRT83, IL6, PLAT, C1orf110, LOC100129171, SNORA28, CNN1, SLC20A1, SNAR-A3, B3GALT2, NT5E, LOC100129619, Cyorf15A, VEGFC
- Cluster 19: MCFD2, ITGA8, PLAU, C15orf52, AGMAT, HHIPL1, IGFBP3, TGM2, LOXL2, LOC100128242, KIF23, UBASH3B, LOC100134259, PLEKHN1, ALS2CR11, TM4SF4, RHBDL2, LOC388849, PIF1, TMEM35, GATA6
- Cluster 20: MMP7, ESM1, PBK, SMOCC, OGN, NR0B1, JAKMIP2, EPGN, PSG3, APCDD1, KRTAP1-1, DEPDC1, ZBTB16, NTN1, PDK4, F3
- Cluster 21: GRAMD1B, SOST, KIF20A, KIF14, LOC152225, MPP4, ERCC6, RAGE, FOXM1, C1orf135, NDP, GLYATL1, PTTG1, ANLN, LOC283484
- Cluster 22: CKAP2L, CASC5, TOP2A, ASPM, SKA3, FOLR3, NUF2, KIFC1
- Cluster 26: ERMN, PADI1
- Cluster 28: LYPD1, NCRNA00185

Figure 5. Heat map of selected candidate genes potentially correlated with adipocytes characteristics. The number and names of the corresponding genes are listed in Figure 6. Graphic result of the hierarchical clusterization of adipose tissue-related genes

Figure 6. Cluster analysis with corresponding differentially expressed genes. Expression profiles of different cluster genes, some unknown for adipogenesis, showing differences between MSCs and adipose tissue.

From data analysis we extracted a number of 45 genes involved in lipid metabolism and regulation of adipogenic differentiation, more than 2 FC upregulated in preadipocytes compared to corresponding MSCs considering these genes as important in triggering adipogenesis. Some of the

genes (GCHFR, ALOXB15, GAS, FADS1, FADS2) will be further down regulated in mature adipocytes, while others will be upregulated, reaching similar expression level to that found in native human adipose tissue (Table 1).

Table 1. Expression level of lipid metabolism-involved genes in MSCs derived pre-adipocytes and mature adipocytes compared to originating mesenchymal stem cells.

Gene symbol	Gene name	Gene expression level		
		Preadipocytes (D5)	Preadipocytes (D7)	Mature adipocytes (D21)
MMP7	matrix metalloproteinase 7 (matrilysin, uterine)	7.147	6.740	7.476
APOD	apolipoprotein D	6.364	6.659	7.838
HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	6.336	5.277	5.363
GCHFR	GTP cyclohydrolase 1 feedback regulator	6.270	6.161	3.122
MMP13	matrix metalloproteinase 13 (collagenase 3)	5.931	6.231	4.858
ALDH1L1	aldehyde dehydrogenase 1 family, member L1	5.506	4.054	5.981
ALOX15B	arachidonate 15-lipoxygenase, type B	5.421	5.069	3.056
FMO3	flavin containing monooxygenase 3	5.417	5.607	3.980
OLAH	oleoyl-ACP hydrolase	5.078	5.531	5.009
CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	4.960	4.333	4.594
TIMP4	TIMP metalloproteinase inhibitor 4	4.919	4.822	7.250
NOX4	NADPH oxidase 4	4.617	4.839	4.318
MAOA	monoamine oxidase A	4.598	4.071	5.090
HPD	4-hydroxyphenylpyruvate dioxygenase	4.467	6.310	6.838
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	4.367	4.320	5.727
AOC3	amine oxidase, copper containing 3	4.315	3.939	7.785
GAS1	growth arrest-specific 1	4.042	4.132	3.897
FADS1	fatty acid desaturase 1	3.822	3.308	1.959
APOE	apolipoprotein E	3.580	3.171	4.186
FADS2	fatty acid desaturase 2	3.570	3.323	2.788
GCH1	GTP cyclohydrolase 1	3.551	2.581	2.716
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	3.483	3.197	5.781
PDK4	pyruvate dehydrogenase kinase, isozyme 4	3.449	2.519	7.644
MME	membrane metallo-endopeptidase	3.300	3.654	3.501
ADHFE1	alcohol dehydrogenase, iron containing, 1	3.194	1.887	3.470
GAS2	growth arrest-specific 2	3.194	2.271	1.966
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	3.193	3.547	3.160
G0S2	G0/G1 switch 2	3.116	2.909	5.374
DHCR24	24-dehydrocholesterol reductase	3.115	2.782	3.411
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	3.072	2.930	2.488
OSBPL5	oxysterol binding protein-like 5	2.991	2.704	3.331
LBP	lipopolysaccharide binding protein	2.982	2.609	1.585
PLB1	phospholipase B1	2.938	2.606	1.783
CPT1B	carnitine palmitoyltransferase 1B (muscle)	2.534	2.248	2.615
PPARG	peroxisome proliferator-activated receptor gamma	2.389	1.994	4.319
ACSL1	acyl-CoA synthetase long-chain family member 1	2.385	2.004	2.719
LEPR	leptin receptor	2.288	2.408	0.526
MMP15	matrix metalloproteinase 15 (membrane-inserted)	2.235	2.341	1.831
ACACB	acetyl-Coenzyme A carboxylase beta	2.213	1.913	4.238
PLCL1	phospholipase C-like 1	2.192	1.824	1.270
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	2.190	1.657	4.974
MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	2.160	0.630	2.228
LPA	lipoprotein, Lp(a)	2.113	2.330	1.524
ECM2	extracellular matrix protein 2, adipocyte specific	2.095	2.481	0.772
ALOX5AP	arachidonate 5-lipoxygenase-activating protein	2.037	2.399	0.747

Humans lack the ability to introduce double bonds in the fatty acids beyond C9 and C10, missing the desaturase enzymes required for production of some fatty acids (GREGOIRE & al., [22]; MADSEN & al., [23]). Consequently, the linolenic (C18:3) and linoleic (C18:2) acids are considered essential short chain polyunsaturated fatty acids (SC-PUFA). Under the action of desaturase and elongase enzymes, both acids would be metabolized resulting in long chain polyunsaturated fatty acids (LC-PUFA) – eicosapentaenoic acid (EPA, C20:5) and DHA (C20:6), gamma linolenic acid (GLA, C18:3), dihomo gamma linolenic acid (DGLA, C20:3), and arachidonic acid (AA, C20:4), respectively (BELURY, [24]). Arachidonic acid is not one of the essential fatty acids. However, it does become essential if there is a deficiency in linoleic acid or if there is an inability to convert linoleic acid to arachidonic acid, which is required by most mammals (AZAIN, [25]; ZHONG & al., [26]). Microarray analysis revealed an overexpression of desaturase enzymes (FADS1, FADS2, and SCD) in preadipocytes and mature adipocytes comparative to progenitor mesenchymal stem cells, but expression level was similar to adipose tissue. Moreover, expression profile for the other enzymes involved in conversion of essential fatty acids to final products disclosed a dysregulation in MSCs, preadipocytes and mature adipocytes compared to native adipose tissue. Except for ELOVL4 (family member 4, elongation of very long chain fatty acids) which is upregulated FC > 3, ELOVL6 decreases its expression along the differentiation pathway. ELOVL7 is not well characterized in humans, being associated with prostate cancer and is dramatically downregulated in MSCs and differentiated cells. MSCs, preadipocytes and mature adipocytes lack elongases specific for C20-22 PUFA, and C16-22 elongation, such as ELOVL2 and ELOVL5, respectively, which are responsible for endogenous PUFA synthesis (Table 2). Since ELOVL4 elongate a broad array of fatty acids (<C26) we may assume that the product of this gene is the only responsible for generation of DHA detected in bound form, and AA detected both as free and bound fatty acids.

Table 2. Expression profile of ELOVL family members found in MSCs, preadipocytes at 2 check points and mature adipocytes. Expression level is reported to native adipose tissue.

Gene symbol	Gene name	Gene expression level			
		MSCs	Preadipocytes D5	Preadipocytes D7	Mature adipocytes
ELOVL4	ELOVL family member 4, elongation of very long chain fatty acids	3.66	4.209	4.286	4.316
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids	-1.826	-	-	-
ELOVL6	ELOVL family member 6, elongation of long chain fatty acids	2.499	1.835	1.661	1.778
ELOVL7	ELOVL family member 7, elongation of long chain fatty acids	-5.441	-4.815	-5.067	-4.730

Lack of essential fatty acids within the differentiation media, required for normal adipogenesis, is a possible cause of bone marrow-derived mesenchymal stem cells failing to transform into fully-grown and functional mature adipocytes. Moreover, combined with MSCs inability to upregulate the key genes required for endogenous PUFA synthesis, we prove that the current adipocytic differentiation media is not appropriate for generating mature functional adipocytes, while the MSCs are not the optimal model of in vitro induced

adipogenesis, lacking essential specific enzymatic components, as result of gene expression profile.

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