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The influence of osteogenic differentiation on the stem-like properties of adipose derived stem cells – an RT-qPCR study

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Abstract

ADSCs are readily accessible and widely available. Isolated through a minimally invasive procedure from adipose depots, they can be found at diverse body location, where they served various functions, including energy homeostasis. They can be obtained upon surgeries from otherwise waste tissues, like after excision of fat tissue or liposuction. In addition, due to the possibility to isolate many ADSCs, in vitro proliferation can be performed in a short time period, resulting in cells showing more predictable results[1]. For this study ADSCs were obtained from waste material following routing sterilization procedures of dogs. This study aimed to analyse the expression of MSC specific markers before and after in vitro differentiation of ASCs. Three positive and three negative markers were analysed, *CD105, CD73, CD90, CD34*, *CD14* and *CD45*. There were significant differences detected in the expression of all of the genes, with most of them exhibiting notable downregulation. The only exception, *CD14* showed major upregulation after the process of differentiation. These changes confirm the success of differentiation, as well as suggest that this process significantly lowers the stem-like ability of ASCs. This knowledge should serve as a reference for further molecular and clinical studies, possibly aiding the understanding of the internal mechanisms governing the differentiation and stemness of ASCs, to enable their widespread and safe application in regenerative medicine.

Running title: Mesenchymal markers during ASC osteogenic differentiation

Keywords: adipose, stem cells, differentiation, osteogenic, markers

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Introduction

Adipose Derived Stem Cells are an effective source of mesenchymal progenitors and were first described in 2001 [2]. This class of cells presents mesenchymal stem cells-like characteristics and present proliferation and differentiation potential, and self-renewal ability. These attributes grant them multiple and diverse clinical application, serving as a promising tool for gene-based therapies, such as tissue engineering and regenerative medicine [3]. Not only ADSCs are multipotent and can differentiate into the tri-germ cell lineages, but also show paracrine activity impacting immune responses [4]. They can differentiate into various cell types, specifically osteocytes, but also adipocytes, neural cells, vascular endothelial cells, cardiomyocytes, pancreatic cells, muscle cells and hepatocytes [5–8]

ADSCs are readily accessible and widely available. Isolated through a minimally invasive procedure from adipose depots, they can be found at diverse body location, where they served various functions, including energy homeostasis. They can be obtained upon surgeries from otherwise waste tissues, like after excision of fat tissue or liposuction. [9]. In addition, due to the possibility to isolate many ADSCs, *in vitro* proliferation can be performed in a short time period, resulting in cells showing more predictable results [1]. For this study ADSCs were obtained from waste material following routing sterilization procedures of dogs.

Due to its qualities, adipose tissue has been studied in many different medical fields and is thought to be a powerful source of stem cells. The capability of ADSCs to differentiate towards various cell lineages and the opportunity of directing this differentiation, increases their possible clinical applications and their employability in different therapies and treatments of diseases [10].

ADSCs show great potential in tissue regeneration, specifically bone reconstruction upon trauma, poor bone formation or the removal of cancer tissue, and more generally in situations when bone cells lack regenerative capacity. Although many techniques already exist for bone tissue augmentation and repair, they are usually costly and present high risk of morbidity at donor site and risk of rejection [11]. The osteogenic potential of ADSCs, together with their ability to regulate the release of growth factor and cytokines with anti-inflammatory and angiogenic properties, has opened new prospects for bone tissue engineering and regeneration [12]. Different cases of implementation of bone regeneration therapies with ADSCs delivered positive results, showing ossification with little or no side effects [13]. As the potential of ADCS in orthopedic application is investigated, a deeper understanding of the mechanism underlying the osteoblastic differentiation of ADSC and its influence on the expression of mesenchymal stem cell markers could result in novel techniques and applications in tackling a diverse range of bone-related diseases.

Materials and Methods

Animals

Fat samples were obtained from adult dogs subjected to the routine sterilisation surgery at a commercial veterinary clinic. The process of material collection did not involve any additional medical procedures, as it was based on a usually discarded remnant tissue. Hence, the study did not necessitate obtaining of a bioethical committee approval.

Initial material preparation

After collection, the obtained tissue samples were placed in a DPBS solution supplemented with an antibiotic-antimycotic mix (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, they were stored at 4°C until being transported to the laboratory for further processing no longer than 6h after collection. Upon arrival, the samples were subjected to a triple wash in ice-cold DPBS (to ensure the removal of remnant blood), minced with sterile surgical blades and placed in an 1mg/ml Collagenase I (solution in DMEM (Sigma-Aldrich, St. Louis, MO, USA) for the period of 40 minutes at 37°C. During that time, the sample was regularly vortexed to ensure maximum enzyme exposure. After the incubation period, the samples were centrifuged at 1200 x g for 20 mins. Furtherly, the resulting upper layer of mature adipocytes was discarded, while the pellet located at the bottom of the tube was resuspended in DMEM and centrifuged once more at 400 x g for 10 mins to ensure proper washing. Then, a fraction of the obtained cell solution was collected for identification analyses, while the remaining cells were seeded onto a 25cm^3 culture flask in 4mL of DMEM.

Cell culture

The cells were cultured for a period of 14 days, with the culture media changed every 72h. The pictures of cells were taken every day to analyse the culture-induced changes in morphology. After the culture period, the culture media were removed. The cells were then double washed with DPBS, with 1mL of trypsin added to facilitate their detachment. After 5 min of incubation, the culture flasks were analysed under a 10x magnification to confirm full detachment, after which the cell suspension was transferred to a conical tube. FBS was added to neutralise the remaining enzymes, after which the sample was centrifuged again and suspended in 1mL of TRI Reagent for RNA isolation or PBS for flow cytometry analysis.

Differentiation

A 6-well tissue culture plate was coated with 10 µg/ml bovine fibronectin and ADSCs were plated at 1x105 cells per well using DMEM low-glucose with

2 mM L-glutamine and 10% fetal calf serum, each sample was plated in two wells. The cells were allowed to grow until at least 100% confluency was reach, which took around 48 to 72 hours. One of each of the duplicates of ADSCs were then induce with MSC Osteogenic Differentiation Medium (C-28013). Growth medium was used for the remaining wells as a negative control. The induced cells were incubated for 12–14 days. The media was changed every third day, without disturbing the cell monolayer.

RNA isolation

Each of the analyzed groups was processed in three independent samples. Total RNA was extracted from the samples using the TRI Reagent® (Sigma, St Louis, MO, USA), following the Chomczyński-Sacchi method [14]. After being collected from the cultures, the samples were suspended in 1ml of TRI Reagent (Sigma-Aldrich, Saint Louis, MO, USA), a mix of guanidine thiocyanate and phenol in monophasic solution. After the addition of chloroform, the samples were centrifuged to achieve separation of the 3 phases. The upper aqueous phase containing the RNA was collected with little or no contaminating DNA and proteins. The RNA was then stripped with 2-propanol (Sigma-Aldrich, Saint Louis, MO, USA) and washed with 75% ethanol. RNA, prepared in such way, was used for further analyses. Total mRNA was measured using the optical density at 260 nm, with the purity evaluated based on the 260/280 nm absorption ratio (above 1.8) (NanoDrop spectrophotometer, Thermo Scientific, ALAB, Poland). RNA was diluted to a 100 ng/L concentration with an OD260 / OD280 ratio of 1.8 / 2.0.

RT-qPCR analysis

To perform the RT-qPCR validation of the results, sequence specific primers for the genes of interest were designed using Primer 3 Software (Whitehead Institute, Cambridge, MA, USA. Gene sequences were obtained from Ensembl database, with the common parts of different transcript variants extracted by Clustal Omega Software (both EMBL, Heidelberg, Germany). All primers were designed as intron-intron border spanning to avoid genomic cDNA contamination (**Tab. 1**). The reaction mix was prepared on a dedicated 96-well plate and contained: 1 µL of cDNA matrix, 1 µL of forward + reverse primer mix, 5 µL of Sybr Green qPCR mix (Qiagen, Hilden, Germany) and 3 µL of PCR grade water (Roche Diagnostics, Manheim, Germany). The reactions were performed according to reagent producers' guidelines, in primer specific temperatures, using LightCycler 96 Real-Time PCR System (Roche Diagnostics, Manheim, Germany). The outcomes of reactions were analysed using manufacturer provided LightCycler 96 Software 1.1 (Roche Diagnostics, Manheim, Germany). Specificity of the 15 reactions was confirmed based on the Tm calling, graphs generated by the software. The relative quantification was calculated based on the double delta Ct methods, with HPRT and ACTB used as housekeeping genes.

Statistical analysis

Moderated t-statistics from the empirical Bayes method were preformed to determine the statistical significance of the genes analysed. The resulting p-value was corrected for multiple comparisons, using Benjamini and Hochberg's false discovery rate. Genes were deemed to be significantly altered if they had a p-value below 0.05.

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. As the study was based on a remnant waste material, the Bioethical Committee approval was not necessary.

Results

RT-qPCR

As the main aim of the study, the expression of MSC specific markers was evaluated in the cultured ASCs before and after osteogenic differentiation. According to literature, three positive (*CD105, CD73* and *CD90*) and three negative (*CD34, CD14* and

TABLE 1. The sequences of primers used in the analysis

GENE NAME	FORWARD PRIMER	REVERSE PRIMER
CD105	CTCAGGTCCCCAATGCTACC	GGTTGAAGGCCAGGTAGAGT
CD73	CCCATTGACGAACGGAACAA	TATACCACGTGAATTCCGCC
CD14	CACTAGAGCCCTGCGAAGTA	CGACGGCAATCATACACTGG
CD34	ATGAGACCTCCAGCTGTGAG	AGGTCAGACTGGTGCTTTCT
CD90	CGAGAATGCTACCACCTTGC	AGCCGGAGTTCACATGTGTA
CD45	ACCTAGGCAAACATGTGAGGA	CTTCCAGATCAAAATTTCCACGA
HPRT	CCATCACATCGTAGCCCTC	ACTTTTATATCGCCCGTTGAC
ACTB	CCCTTGCCGCTCCGCCTTC	GCAGCAATATCGGTCATCCAT

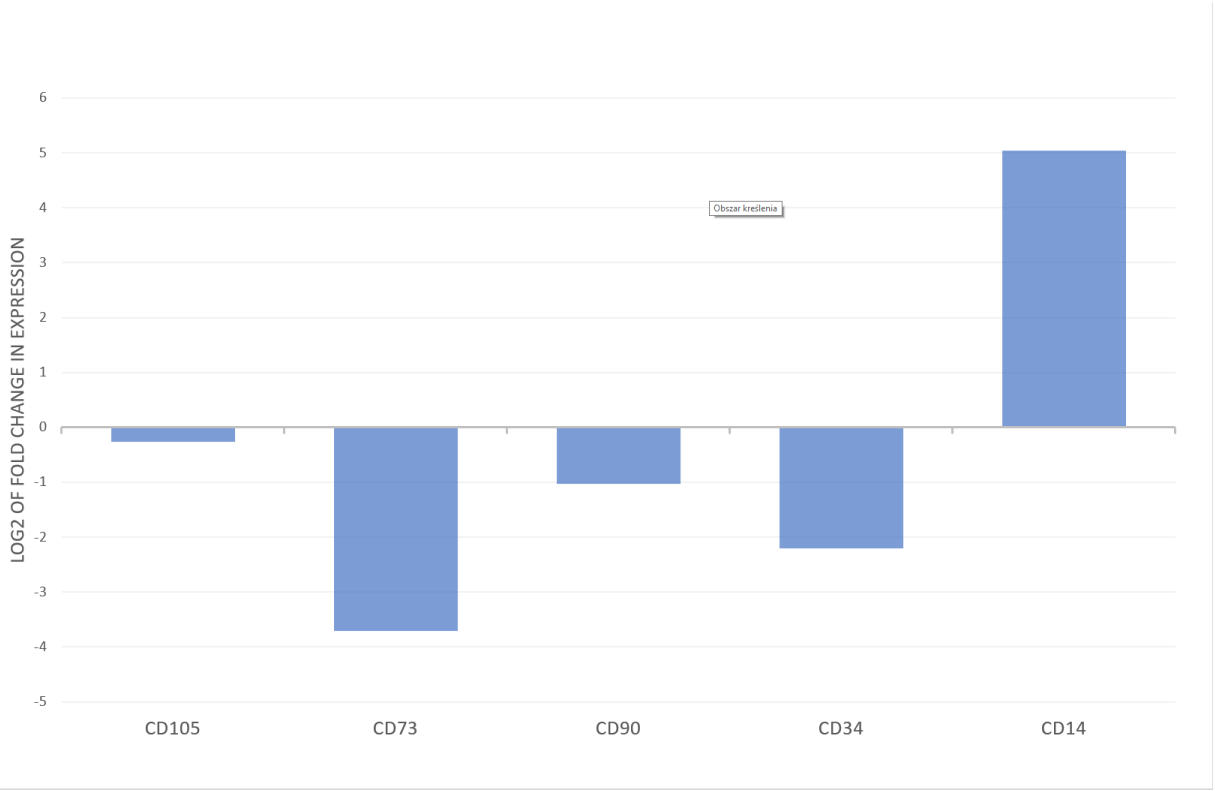

FIGURE 1. The results of the RT-qPCR analysis of the change in expression of ASC specific markers before and after osteogenic differentiation. All data was presented as a log2 of fold change

CD45) markers were evaluated. The results were presented on a bar graph in the form of logFC, to allow for easy determination of the direction and scale of expression change.

As can be seen on the graph, four of the analysed genes were downregulated after the process of differentiation. *CD73* and *CD34* exhibited the biggest decrease in expression, while the change of *CD90* and *CD105* genes was less notable. In turn, *CD14* was significantly upregulated after osteogenic differentiation. *CD45* was not presented on the graph, as its expression was not detected in any of the analysed groups.

Discussion

Regarding the osteogenic differentiation ability of ADSCs, multiple studies have reported the pathways, genes, proteins, and hormones involved in the process. Some factors influencing the commitment of ADSCs have been identified, such as the parathyroid hormone, PTH1-34, and the Notch proteins, a family of key regulator ligands usually involved in osteogenesis [15,16]. Moreover, the evaluation of osteogenic marker expression during ADSCs cultures was performed, resulting in the identification of genes such as CBFA1. However, since stem cells grow *in vivo* in a multifactorial environment presenting diverse biochemical and mechanical signals affecting their functioning and properties, which are subject to constant changes, it is very complicated to elucidate the mechanisms underlying niche cues and the connection between them. Moreover, their osteogenic potential has been investigated for other possible clinical utilizations, such as in dental placement strictly upon tooth extraction in healthy dogs [17]. In fact, the proliferation and osteogenic potential of ADSCs are expected to show great capacity to implement bone formation upon diseases or trauma, providing a resourceful alternative in reconstructive surgeries. As the results obtained so far appear ambiguous, the need for the understanding of their basic functioning is clear [18–21]. However, promising results were obtained from the treatment of dogs with a combination of ASC-derived cell sheets and platelet-rich fibrin membranes transplantation, presenting elevated re-osseointegration percentage and increased new bone formation [22]. Although, in a different study, the application of ADSCs for dental implants did not present any substantial improvements [23]. This study aimed to analyse the expression of MSC specific markers before and after *in vitro* differentiation of ASCs. Three positive and three negative markers were analysed, *CD105, CD73, CD90, CD34, CD14* and *CD45* [24]. There were significant differences detected in the expression of all of the genes, with most of them exhibiting notable downregulation. CD45, was not detected in any of the analysed sample groups.

CD14 (Cluster of differentiation 14) is gene encoding a protein mostly produced by macrophag-

es, playing a role in the proper functioning of the immune system. It was proven to aid the organism in detection of bacterial pathogens, through the binding of lipopolysaccharides (LPS), especially the pathogen-associated molecular protein (PAMP) [25]. In the human body, it can be found in two forms, soluble and attached to the cellular membrane. During LPS detection, this protein acts as a co-receptor with Toll-like receptor 4 and MD-2 [26]. It was also found to be able to bind other molecules associated with pathogens, aiding the recognition process [27]. In the context of stem cells, this protein was found to be a regulator of differentiation in porcine spermatogonia [28]. Furthermore, $CD14^+$ macrophages were found to participate in chondrogenesis of synovium-associated stem cells in osteoarthritis [29]. Finally, single-nucleotide polymorphisms of CD14 were associated with outcomes of bone marrow stem cell transplantations [30].

Summarising, in this study, the subset of genes defined in literature as MSC markers underwent significant alterations in expression after osteogenic differentiation of ASCs. Downregulation of most of the genes was most probably associated with loss of stem-like properties over the time of *in vitro* culture and due to lineage commitment. In turn, a significant increase in the expression of CD14 could be an indicator of culture and differentiation stress on the occurrence of immune-response-like symptoms in the cultured cells.

Conclusions

Overall, the above described changes confirm the success of differentiation, as well as suggest that this process significantly lowers the stem-like ability of ASCs. This knowledge should serve as a reference for further molecular and clinical studies, possibly aiding the understanding of the internal mechanisms governing the differentiation and stemness of ASCs, to enable their widespread and safe application in regenerative medicine.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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