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qPCR ANALYSIS OF MESENCHYMAL STEM CELL MARKER EXPRESSION DURING THE LONG-TERM CULTURE OF CANINE ADIPOCYTE DERIVED STEM CELLS

Rut Bryl[1], Claudia Dompe[2], Maurycy Jankowski[1], Katarzyna Stefańska[3], Afsaneh Golkar Narenji[4], Jakub Kulus[5], Magdalena Kulus[6], Maria Wieczorkiewicz[7], Grzegorz Wąsiatycz[6], Jędrzej M. Jaśkowski[5], Mariusz Kaczmarek[8], James N. Petitte[4], Paul Mozdziak[4,9], Paweł Antosik[6], Dorota Bukowska[5]

Abstract

Due to its availability and accessibility, adipose tissue has been the subject of various studies in many different medical fields and is believed to be a useful source of stem cells. The ability of ASCs to differentiate towards different cell lineages, with possibility of directing this differentiation, increases their possible clinical applications, and they have been widely employed in multiple therapies and treatment of different pathologies. However, a deeper understanding of the molecular mechanisms underlying the ASCs osteoblastic and chondrocyte differentiation may lead to novel applications treating a multitude of different bone-related diseases through techniques more likely meeting worldwide consensus. In this study, the RT-qPCR method was used to determine the changes in expression of ASC specific markers (CD105, CD73, CD14, CD34, CD90 and CD45) before and after long-term (14-day) *in vitro* cultures. To confirm the identity of the investigated cells, flow cytometry was used to evaluate the presence of positive (CD44, CD90) and negative (CD45, CD34) ASC markers. Overall, the results of the PCR analysis showed a significant change in expression of most of the marker genes, indicating significant changes in the cultured cells caused by their long-term culture, potentially altering their original stem-like characteristics.

Running title: ASC marker expression during long-term *in vitro* culture

Keywords: adipose, stem cells, long-term, culture, markers

[1]Department of Anatomy, Poznań University of Medical Sciences, Poznań, Poland
[2]School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK
[3]Department of Histology and Embryology, Poznań University of Medical Sciences, Poznań, Poland
[4]Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC, USA
[5]Department of Diagnostics and Clinical Sciences, Institute of Veterinary Medicine, Nicolaus Copernicus University in Toruń, Toruń, Poland.
[6]Department of Veterinary Surgery, Institute of Veterinary Medicine, Nicolaus Copernicus University in Toruń, Toruń, Poland.
[7]Department of Basic and Preclinical Sciences, Institute of Veterinary Medicine, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, Toruń, Poland.
[8]Department of Cancer Immunology, Poznań University of Medical Sciences, Poznań, Poland.
[9]Physiology Graduate Program, North Carolina State University, Raleigh, NC, USA
*** Correspondence:** dbukowska@umk.pl
Full list of author information is available at the end of article

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Introduction

First described in 2001, ASCs are an effective source of mesenchymal progenitors [1]. In fact, they have mesenchymal stem cells-like characteristics and show great proliferation and differentiation potential and self-renewal ability, granting multiple and diverse clinical application and serving as a promising tool for gene-based therapies, including tissue engineering and regenerative medicine[2]. ASCs are multipotent and can differentiate into different cells types of the tri-germ lineages, as well as exhibit paracrine activity impacting immune responses [3]. They show ability to differentiate into many different cell types such as osteocytes, adipocytes, neural cells, vascular endothelial cells, cardiomyocytes, pancreatic cells, muscle cells and hepatocytes [4–8].

ASCs potential and qualities aside, this type of cells is readily accessible and is widely available. In fact, they are obtained through a minimally invasive procedure from adipose depots that are found at different locations throughout the body serving a variety of functions, including energy homeostasis of an organism. They can be isolated from tissues usually discarded upon surgery, for example excision of adipose tissue or liposuction, considered as the best method for storage preparation [9]. The analysis of samples obtained from liposuction do not show any significant loss of preadipocytes and no significant stroma damage. Since it is possible to obtain many ASCs from the adipose tissue, in vitro proliferation can be performed resulting in cells showing more predictable results [10].

Due to its availability and accessibility, adipose tissue has been the subject of various studies in many different medical fields and is believed to be a useful source of stem cells. The ability of ASCs to differentiate towards different cell lineages, with possibility of directing this differentiation, increases their possible clinical applications, and they have been widely employed in multiple therapies and treatment of different pathologies [11]. However, a deeper understanding of the molecular mechanisms underlying the ASCs osteoblastic and chondrocyte differentiation may lead to novel applications treating a multitude of different bone-related diseases through techniques more likely meeting worldwide consensus.

In this study, the RT-qPCR method was used to determine the changes in expression of ASC specific markers before and after long-term *in vitro* cultures. The results should aid the understanding of the influence of *ex vivo* conditions, similar to those employed during the usual procedures of ASC extraction and application, on the stemness and plasticity of the adipose progenitor cells.

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.

Flow cytometry analysis

5 µL of antibodies were added to the bottom of flow cytometry tubes (following producer's protocol), with around 100 µL of stained cell suspension.

After 30 min of incubation, the mix was washed twice in 5 mL of PBS and centrifuged at 200 x g for 6 min, removing the remnant unbound antibodies. The antibodies used were Anti-Dog CD44, Anti-Dog CD90, and negative isotype controls, Anti-Dog, Anti-Dog CD45.

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 [12]. 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

Morphological changes

The morphology of the cultured ASCs was evaluated under a light microscope at the beginning (after the initial 3 day period of adhesion) and endpoint of the primary culture. The results were compiled and presented in the form of **figure 1.**

As can be seen, the initial fibroblast-like morphology changed to epithelial-like after the period of 14 day *in vitro*. Furthermore, due to extensive cell

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

proliferation, the cell density increased, possibly inducing the change in cell shape.

Flow cytometry

The cultured cells were subjected to a flow cytometry assay using available canine antibodies targeting the known ASC markers. CD44 and CD90 expression was evaluated as positive, and lack of CD45 and CD34 as a negative marker. All of the antibodies were ran along their respective isotype control to correct for non-specific binding. The results were presented in the form of Mean Fluorescence Intensity graph (**Fig. 2**).

As can be seen, CD44 and CD90 peaks present no significant overlap with their respective isotype controls, indicating the presence of these surface proteins on the analysed cells. In turn, CD45 and 34 show a major overlap with the isotype control, proving their expected lack of expression on the cells' surface.

RT-qPCR

As the main aim of the study, the expression of MSC specific markers was evaluated in the cultured ASCs before and after the period of long-term *in vitro* cultures. According to literature, three positive (CD105, CD73 and CD90) and three negative (CD34, CD14 and 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 (**Fig. 3**).

As can be seen, the analysed ASC markers showed differing changes of expression during long-term *in vitro* culture. One of the positive markers (CD105) showed a minor downregulation, while two others were majorly upregulated (CD73 and CD90). In the same time, two of the negative markers exhibited significant upregulation, more notable for CD34 than for CD14. CD45 was not presented on the graph, as its expression was not detected in any of the analysed time periods.

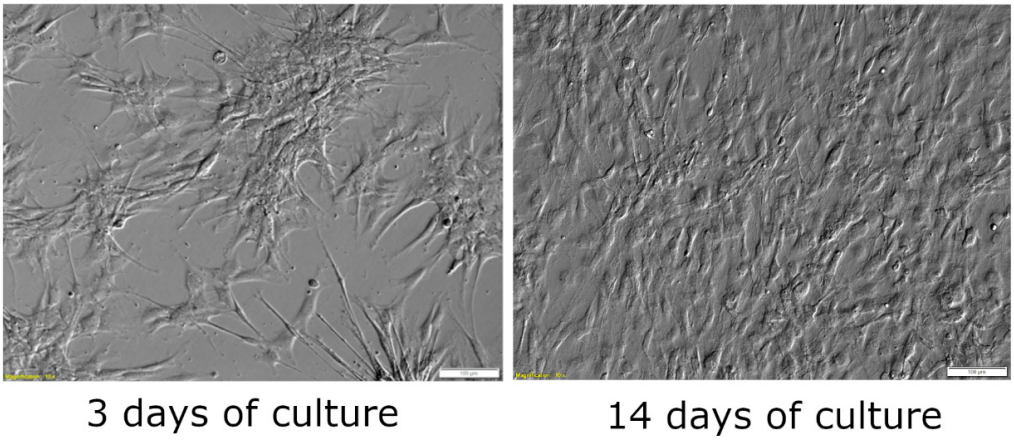


FIGURE 1. Photographs of morphological changes between the beginning and end-point of *in vitro* culture. Taken using an inverted microscope, using a relief contrast and 10x magnification.

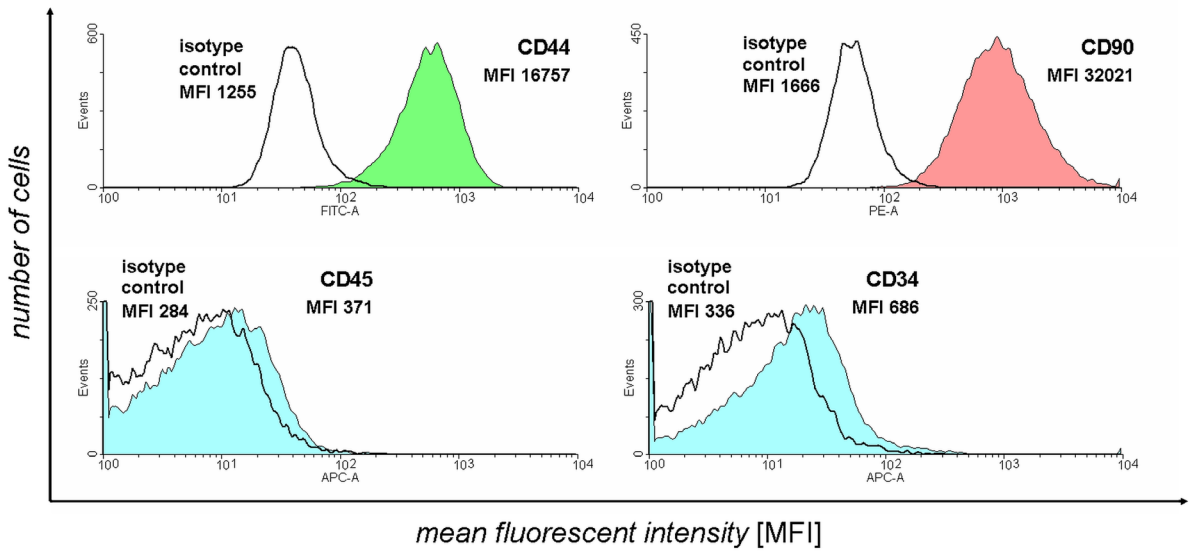


FIGURE 2 The results of the flow cytometry analysis of the available canine ASC markers. The coloured peaks represent the cells stained by the specific antibodies, while the transparent peaks correspond to isotype controls.

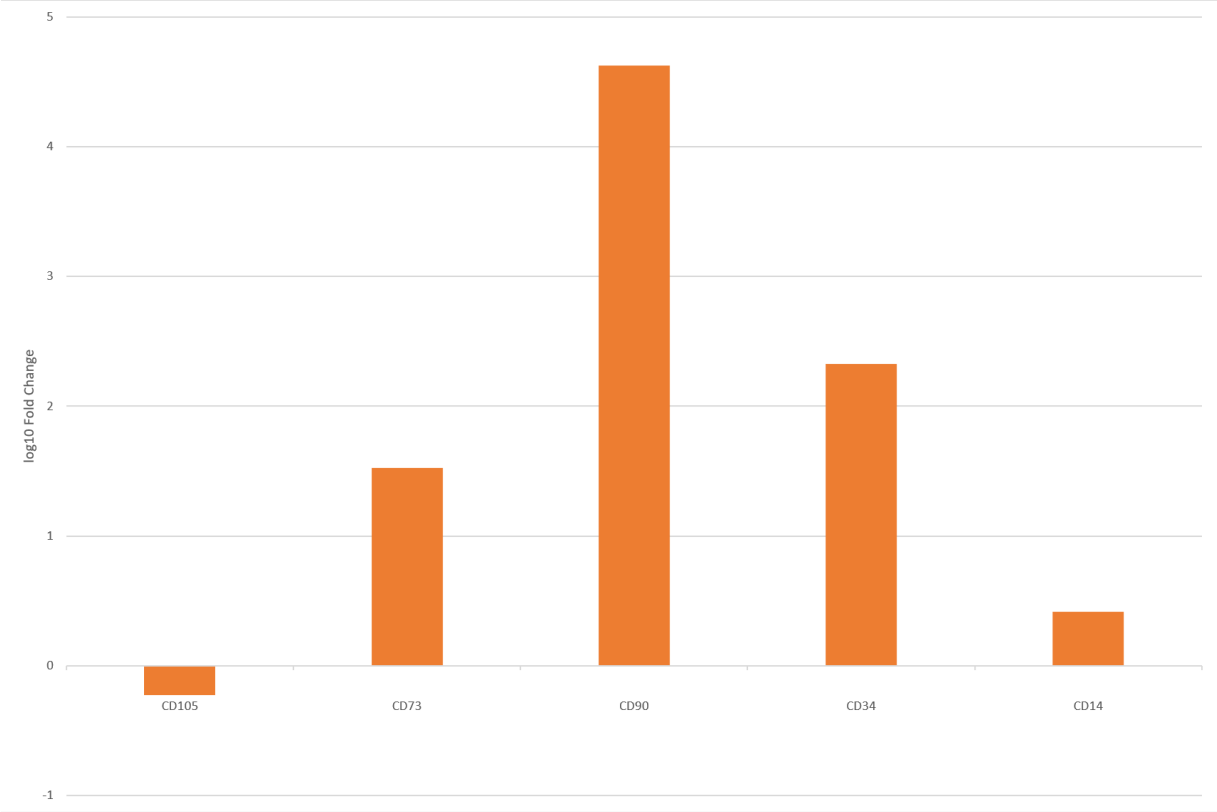


FIGURE 3 The results of the RT-qPCR analysis of the change in expression of ASC specific markers during a 14-day primary culture of adipocyte derived stem cells. All data was presented as a log10 of fold change.

Discussion

The recent literature places ASCs at the forefront of the potential candidates for stem cells based therapies for a variety of current diseases. This sparks a significant amount of *in vivo* and *in vitro* research, as well as a significant number of clinical trials [13]. However, there are still certain risks associated with stem cell application, mostly due to their property that is also considered their most notable advantage- the extraordinary plasticity [14]. While using the adult stem cells brings a significantly lower risk of malignancy compared pluripotent stem cells, a large number of their characteristics depend on their placement in a specific microenvironment and can potentially undergo significant alterations when subjected to *ex vivo* conditions [15]. This occurrence, resulting in gain of new stem-like properties has been demonstrated by a number of studies, focused on a large amount of different somatic cells, e.g. the ovarian granulosa [16]. This brings concern that the extraction and later application of stem cells can lead to the development of malignancies, especially if said cells were subjected to significant *ex vivo* modification, such as in the case of their induction towards a specific lineage e.g. osteogenic or chondrogenic. This proves to be problematic, as several approaches looking to employ ASCs in treatment of diseases such as osteoarthritis or osteoporosis often base on their *in vitro* differentiation into non-adipose related cells [17]. Hence, there is a large need for basic molecular studies focused on the mechanisms associated with *in vitro* cultures of ASCs, to enable full understanding of the processes that drive the potential changes in these cells induced by the absence of their usual environment.

In this study, six MSC markers were analysed, as suggested by Dominici et al. [18]. The first one, CD105 is a well-known receptor for TGF-β [19]. It has been widely researched in the topic of cancer, especially tumour angiogenesis [20]. However, while it is also expressed in stem cells, its role is currently not fully elucidates, as it was demonstrated that its expression has no effect on differentiation ability and does not allow to distinguish a population of MSCs able to progress towards a chondrogenic lineage fate [21]. In this analysis, CD105 was the only positive marker that decreased in expression. The second surface protein, CD73, facilitates the conversion of mononucleotides to nucleosides, playing an important role in the maintenance of immune system homeostasis [22]. It has also been investigated in the context of cancer and was found to be a suppressor of antitumor immune response [23]. Hence, it has been a subject of studies targeting this surface protein in antitumor therapy [24]. In our study, this marker was notably upregulated in ASC primary *in vitro* culture. The final positive marker, CD90 was found to be majorly upregulated

in this analysis. While the function of this protein has not been yet fully elucidated, it has been previously implicated in mechanisms associated with cell-cell and cell-matrix interactions and is, hence, associated with a range of physiological, e.g. nerve growth and regeneration, as well as pathological processes, e.g. fibrosis or tumour metastasis. Because of the latter, it has been largely investigated in the processes associated with the progression of a range of cancers [25]. While its levels in the primary cell cultures were are not fully established, it has been proposed as a biomarker that could be used to purify the cultures of fibroblast contamination [26]. Furthermore, it has been reported that its increased expression is linked to the expanded differentiation potential of MSCs, which is especially interesting in the context of this study [27].

When it comes to negative markers, two of them were upregulated during the course of primary *in vitro* culture. First of them, CD34, was first identified in hematopoietic cells and is still used as a method of stem cell enrichment for bone marrow transplants. It is an important cell adhesion molecule, that has important roles in immune response, mostly through its ability to facilitate cell migration [28]. It needs to be noted that while the majority of scientific community considers lack of its expression as a characteristic MSC marker, there are some reports stating the contrary and even indicating it as a surface protein characteristic for progenitor populations [29]. The second upregulated negative marker, CD14, plays a major role in the immune system, mediating the response to bacterial infections through its lipopolysaccharide receptor action [30]. It has been suggested as a marker for cancer stem cells, specifically in breast cancer stem lines [31]. Furthermore, it has been found to increase the response of certain types of stem cells to Toll-Like Receptor 2 Agonists [32]. The last negative marker, CD45 was, as expected, not detected in the initial time of culture, as well as during its course.

Conclusions

Overall, the change in the expression of MSC markers during long-term primary culture suggests at least a partial loss of ASC characteristics, most probably caused by the influence of *ex vivo* conditions. Furthermore, highly elevated expression of CD90 might suggest a gain of expanded plasticity, possibly connecting extended periods of *in vitro* culture to a gain of new stem-like properties by ASCs. This brings further attention to the fact that there is a need for a large amount of molecular studies analysing the internal mechanisms of stem cells before a widespread application can be introduced to a clinical setting.

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Corresponding author

Dorota Bukowska,Department of Diagnostics and Clinical Sciences, Institute of Veterinary Medicine, Nicolaus Copernicus University in Torun, Torun, Poland, email: dbukowska@umk.pl

Conflict of interest statement

The authors declare they have no conflict of interest.

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