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Surface Marker Expression and Morphological Alterations in Umbilical Cord-Derived MSCs Over Passages 6 to 9: A Flow Cytometry and Microscopic Analysis

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) possess strong regenerative and immunomodulatory potential, making them promising candidates for therapeutic applications. However, prolonged in vitro expansion may alter their phenotypic and functional characteristics, potentially affecting their efficacy. Methods: Umbilical cord-derived MSCs (UC-MSCs) were cultured and analyzed at passages 6, 7, 8, and 9 to evaluate morphological consistency and surface marker expression. Morphological assessment was performed using an inverted microscope, while flow cytometry analysis was conducted to determine the expression of MSC-positive markers (CD90, CD105, CD73) and negative hematopoietic markers (CD34, CD45, CD11b, CD19, HLA-DR). Results: UC-MSCs from passages 6 to 9 displayed a consistent spindle-shaped, fibroblast-like morphology characteristic of mesenchymal cells. Flow cytometry analysis confirmed positive expression for CD90, CD105, and CD73, and negative expression for CD34, CD45, CD11b, CD19, and HLA-DR. A reduction in CD90 expression was observed at passages 8 and 9 (67.3% and 64.3%, respectively), while CD105 and CD73 expression levels remained stable, indicating the maintenance of core MSC identity despite prolonged passaging. Conclusion: UC-MSCs maintained their characteristic morphology and immunophenotypic stability through passage 9, with only minor decreases in CD90 expression at later passages. These findings demonstrate that UC-MSCs preserve essential MSC traits during extended culture, supporting their potential for cell-based therapeutic applications while underscoring the importance of continuous phenotypic monitoring to ensure clinical quality and safety.

Keywords: Non-Communicable Disease, Risk Factor, Family Approach

INTRODUCTION

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) have emerged as a valuable source of stem cells for therapeutic applications due to their multilineage differentiation capacity, low immunogenicity, and non-invasive collection procedure[1,2]. UC-MSCs have better proliferation rates and more accessibility than MSCs from other sources such bone marrow or adipose tissue, making them promising candidates for cell-based therapeutics, tissue engineering, and immunomodulatory treatments[3]. MSCs are defined by the International Society for Cellular Therapy (ISCT) as adhering to plastic under standard culture conditions, being able to differentiate into osteoblasts, adipocytes, and chondroblasts, and expressing specific surface markers, namely CD73, CD90, and CD105, while lacking hematopoietic markers such as CD34 and CD45[3-5]. These markers are critical for determining

MSC identification and functioning in both research and therapeutic settings. MSCs, on the other hand, can undergo phenotypic and morphological alterations as they expand in vitro[6]. These alterations might include decreased proliferation, cellular senescence, altered morphology, and variations in the expression of identifying surface markers[7,8]. These variances may have an influence on the therapeutic efficacy and safety of MSC-based therapies.

Understanding how UC-MSCs respond in different passages is critical for enhancing their therapeutic use. Identifying the passage range in which UC-MSCs retain surface marker expression and morphological integrity might help determine the best conditions for growth and application.[9,10] The purpose of this work is to investigate surface marker expression (CD73, CD90, and CD105) and morphological alterations in UC-MSCs from passage 6 to passage 9 using flow cytometry and microscopic examination. By assessing both phenotypic and morphological properties over multiple passages, this study hopes to shed light on the stability and quality of UC-MSCs during protracted culture, therefore helping to the creation of standardized methods for their therapeutic use.

MATERIALS AND METHODS

Cell Culture and Preparation

UC-MSCs were cultured in 6-well plates at a seeding density of approximately 5×10^4 cells per well, using complete culture medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 until they reached 70–90% confluency.

Sample Preparation

Initially, UC-MSCs at passages 6 through 9 were harvested using Trypsin-EDTA and subsequently centrifuged at 300 × g for 5 minutes. The resulting cell pellets were then resuspended in 1X phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) and counted using a hemocytometer to determine cell concentration. Following this, approximately 1×10^5 cells were aliquoted into individual flow cytometry tubes for further staining and analysis.

Staining Procedure

Each sample tube was incubated with the corresponding antibody cocktail provided in the BD Stemflow™ Human MSC Analysis Kit. Tube A contained antibodies specific to the positive MSC surface markers CD73-APC, CD90-FITC, and CD105-PE while Tube B included a cocktail of antibodies targeting negative lineage markers CD34, CD45, CD11b, CD19, and HLA-DR all conjugated with PerCP-Cy5.5. The samples were incubated for 30 minutes at 4°C in the dark to ensure optimal antibody binding. Following incubation, 2 mL of BD Lyse Solution (diluted 1:10 in distilled water) was added to each tube to facilitate red blood cell lysis and fixation of nucleated cells. The samples were then centrifuged at 300×g for 5 minutes, and the supernatant was carefully removed. The resulting cell pellets were washed with 1x phosphate-buffered saline (PBS) and resuspended in 500 µL of PBS for flow cytometric analysis.

CD Marker Analysis

The expression profile of CD markers in UC-MSCs was evaluated by flow cytometry using the BD Stemflow™ Human MSC Analysis Kit in conjunction with BD Lyse Solution, following the manufacturer's protocol. This analysis kit is comprised of fluorochrome-conjugated monoclonal antibodies specific for established positive mesenchymal stem cell surface markers (CD73, CD90, and CD105), as well as a panel of negative lineage markers (CD34, CD45, CD11b, CD19, and HLA-DR), enabling comprehensive immunophenotypic characterization of MSCs in accordance with the criteria defined by the International Society for Cellular Therapy (ISCT).

Data Acquisition and Analysis

To guarantee statistical robustness, flow cytometric analysis was performed using a BD Accuri™ C6 Plus flow cytometer with at least 10,000 events per sample. Data was processed and analyzed with FlowJo™ software. Cell populations were detected and gated using forward and side scatter parameters to remove cellular debris and doublets. The percentage of cells that expressed the positive MSC markers CD73, CD90, and CD105 were measured, and comparative studies were done throughout passages to determine possible phenotypic change during in vitro growth.

Cell Morphology Analysis

The morphological characteristics of UC-MSCs at passages 6 to 9 were assessed using an inverted phase-contrast microscope (Zeiss Primovert) to observe structural changes during cell culture and expansion.

RESULT AND DISCUSSION

Morphology of UC-MSCs

Umbilical cord–derived mesenchymal stem cells (UC-MSCs) were successfully isolated and expanded in culture up to passage 9. Throughout passages 6 to 9, UC-MSCs exhibited a consistent spindle-shaped, fibroblast-like morphology typical of mesenchymal stem cells (Figure 1). The cells formed a uniform monolayer with parallel alignment and maintained their adherence to the culture surface. No significant morphological alterations, such as enlargement, irregularity, or vacuolation, were observed during serial passaging, indicating stable proliferation and phenotype maintenance during in vitro expansion.

Immunophenotypic Characterization by Flow Cytometry

To confirm the mesenchymal identity of the cultured cells, flow cytometric analysis was performed to assess the expression of characteristic MSC surface markers at passages 6, 7, 8, and 9. Cells were stained with antibodies against CD73 (APC), CD90 (FITC), and CD105 (PE), representing positive MSC markers, and with antibodies against CD34, CD45, CD11b, CD19, and HLA-DR as negative markers. Flow cytometry profiles demonstrated that UC-MSCs were consistently positive for CD73, CD90, and CD105, while negative for CD34, CD45, CD11b, CD19, and HLA-DR, in accordance with the minimal criteria for MSCs established by the International Society for Cellular Therapy (ISCT). These results confirm the mesenchymal nature of the isolated UC-MSCs (Figure 2 and 3).

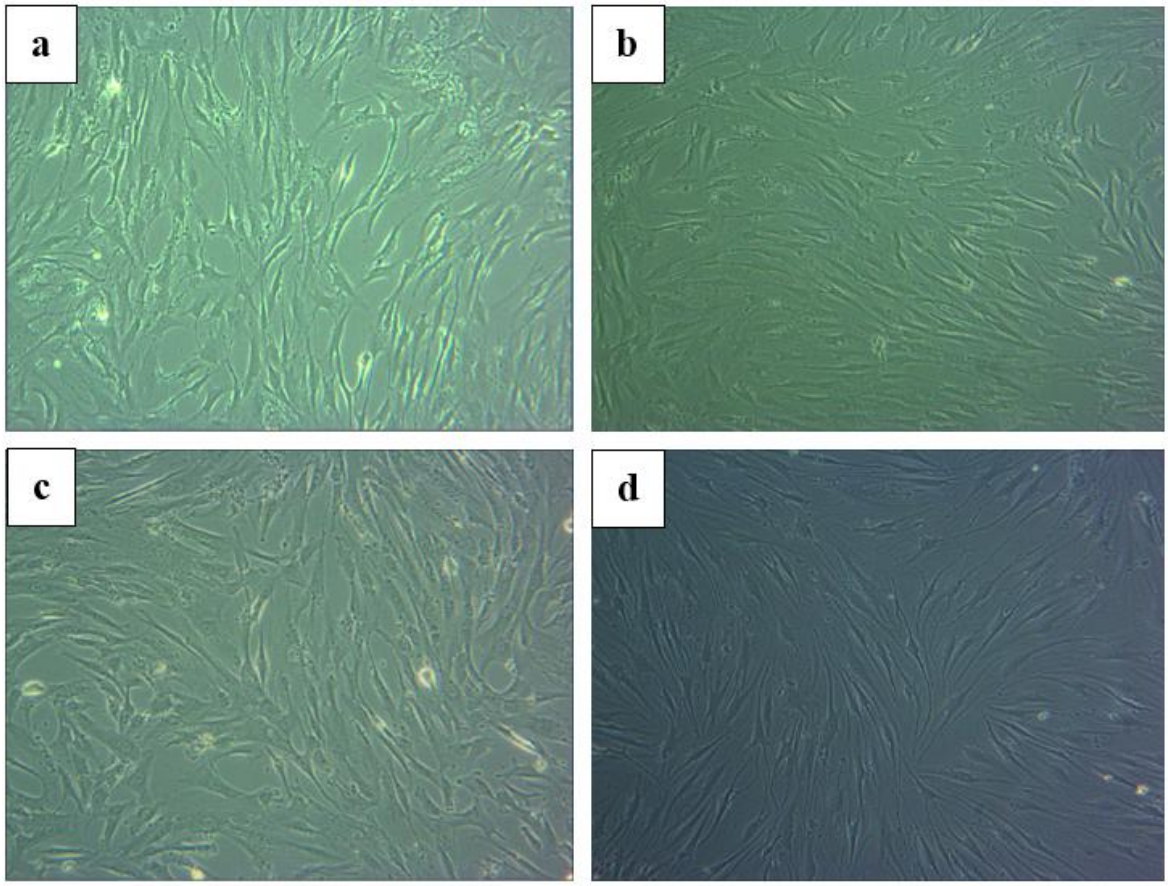

Figure 1. Morphology of mesenchymal stem cells (MSCs) at different passages. Representative phase-contrast images showing MSCs at (a) passage 6, (b) passage 7, (c) passage 8, and (d) passage 9. The cells maintained a typical fibroblast-like, spindle-shaped morphology throughout the serial passages, indicating stable growth and phenotypic characteristics.

Quantitative analysis revealed slight variations in the percentage of positive cells among the passages. The expression of CD105 and CD73 remained relatively stable from passage 6 through passage 9, showing no statistically significant differences. In contrast, a reduction in CD90 expression was observed at later passages. Specifically, CD90-positive cells decreased from high levels at early passages to 67.3% and 64.3% at passages 8 and 9, respectively (Figure 2 and 3). Despite this decline, all cell populations continued to meet MSC immunophenotypic criteria, suggesting that extended in vitro expansion did not compromise the overall mesenchymal identity of UC-MSCs, though it may influence specific surface marker expression associated with stemness.

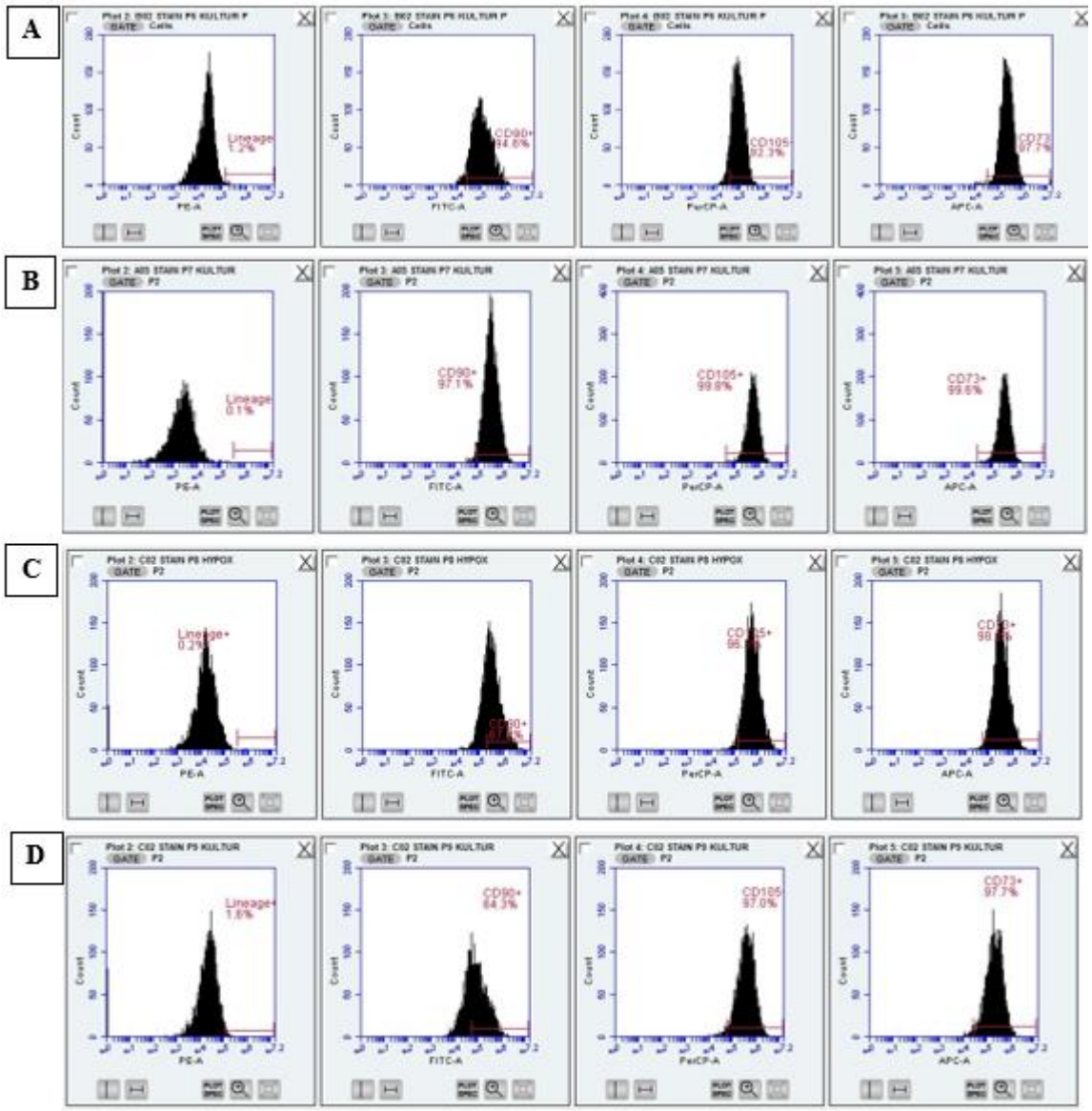

Figure 2. Characterization of mesenchymal stem cells (MSCs) by flow cytometry. Representative histograms showing the expression of MSC surface markers in different experimental conditions. The cells were analyzed for negative hematopoietic marker (Lineage cocktail) and positive MSC markers CD90, CD105, and CD73. (A) Bone marrow-derived MSCs (BMSC) before culture. (B) Adipose-derived MSCs (ADSC) after culture. (C) Cord blood-derived MSCs (CBMSC) under hypoxic conditions. (D) Cord blood-derived MSCs (CBMSC) after culture under normoxic conditions. All cell populations showed negative expression for hematopoietic lineage markers (<2%) and strong positive expression for CD90, CD105, and CD73 (>95%), confirming MSC identity.

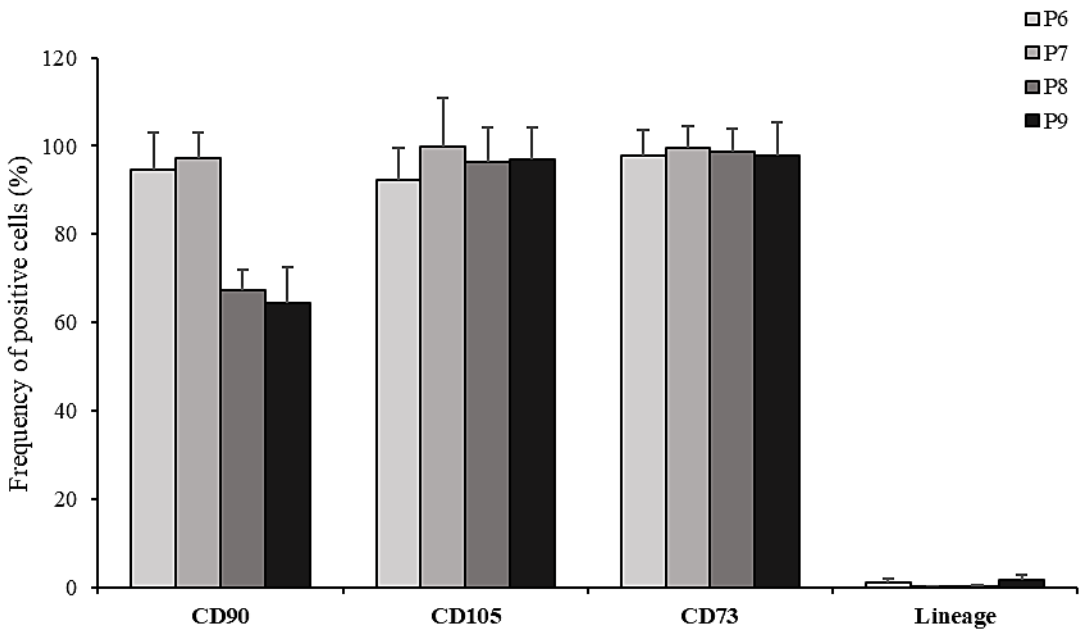

Figure 3. Expression of MSC surface markers during serial passages. Bar graph showing the frequency of positive cells expressing mesenchymal stem cell (MSC) markers CD90, CD105, and CD73, and the absence of hematopoietic lineage markers during passages 6 to 9 (P6–P9). The cells maintained high expression of CD105 and CD73 (>95%) across passages, while CD90 expression showed a slight decline at later passages. Lineage marker expression remained below 2%, confirming the stable MSC phenotype throughout subcultures. Data are presented as mean ± SD from three independent experiments.

DISCUSSION

Mesenchymal stem cells (MSCs) have emerged as promising candidates for regenerative and immunomodulatory therapies, supported by a growing body of preclinical and clinical evidence. [9-11] The therapeutic application of mesenchymal stem cells (MSCs) has garnered substantial attention due to their regenerative potential, immunomodulatory capabilities, and relative ease of isolation and expansion. [12,13] In line with the minimal criteria established by the International Society for Cellular Therapy (ISCT), MSCs must exhibit plastic adherence, express CD105, CD73, and CD90, lack hematopoietic and endothelial markers such as CD45, CD34, CD11b, CD19, and HLA-DR, and demonstrate trilineage differentiation capacity in vitro[6]. However, it is well recognized that prolonged in vitro expansion can alter the phenotypic and functional properties of MSCs. [12] Changes may include downregulation of surface markers characteristic, reduced differentiation potential, senescence-associated features, and shifts in their immunomodulatory secretome. [13] These alterations underscore the importance of stringent quality control and characterization at multiple stages of culture, especially prior to clinical application. [14] Our findings highlight both the therapeutic potential and the biological variability of MSCs during expansion, emphasizing the need for standardized protocols and thorough batch validation to ensure efficacy and safety[15,16].

This study evaluates MSCs CD markers from early passage (P6) until P9. UC-MSCs maintained the characteristic spindle-shaped, fibroblast-like morphology through passages 6 to 9, which suggests robust proliferative capacity without overt morphological signs of senescence or differentiation. This consistency in morphology is aligned with many reports of MSCs from varied

sources that retain fibroblast-like shape over early to moderate passages. [17-19] Flow cytometry data showed that the standard mesenchymal markers CD73**,** CD105**,** and CD90 were expressed in all passages tested. [6,19] Negative markers (CD34, CD45, CD11b, CD19, HLA-DR) were also suitably absent, which meets the minimal criteria for human MSC identity established by the International Society for Cellular Therapy (ISCT) in 2006[6]. According to Dominici et al. (2006), MSCs should express CD105, CD73, CD90, and lack expression of hematopoietic and endothelial markers such as CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. [6]

However, we observed a decline in CD90 expression at passages 8 and 9 (to ~67.3% and ~64.3%, respectively), while the expression levels of CD73 and CD105 remained stable. This selective decrease in CD90 is meaningful because CD90 (Thy-1) is implicated not only in defining MSC identity but also in maintaining aspects of their stemness and immunomodulatory function. [21] Moraes et al. showed that reducing CD90 expression via shRNA increased MSC differentiation (both adipogenic and osteogenic) and affected expression of other markers like CD44 and CD166. [22] Shall et al. [10] reported P4 MSCs expressed higher levels of the MSC marker, CD90, and neuronal markers (TUJ1 and Nestin), while P40 MSCs had a higher expression of the MSC marker, CD105. High expression of CD90 related to undifferentiated MSCs, while decrease in CD90 expression can be correlated with MSCs differentiation into mesodermic lineage[11,23].

Similarly, in studies of bone marrow–derived MSCs, marker heterogeneity has been observed with increasing passage number, including decreases in CD105 and CD90 in some studies (for example, in bone marrow MSCs from patients with hematologic disease, changes in CD90 and CD105 correlated with microenvironment changes)[12]. Also, proteomic profiling has noted that CD90 expression can diverge, with some populations showing a $CD90^{high}$ vs $CD90^{low}$ subpopulation over later passages[24]. The fact that CD73 and CD105 remained stable in your passages suggests that while some stemness-associated features (such as related to CD90) may decline, the core identity and adherence to ISCT criteria are maintained. [19] This stability is important, as CD105 (endoglin) is involved in TGF-β signalling, which plays a role in MSC multipotency and immunomodulation, and CD73 contributes to the MSC role in immunosuppression via adenosine generation. [22] The decline in CD90 at higher passages might reflect early signs of phenotypic drift or partial loss of stemness, which may precede functional declines. [18] Decreased CD90 has been associated elsewhere with reduced immunosuppressive capacity; in one study, human MSCs with lower CD90 expression elicited different interactions with T cells and had reduced inhibition of lymphocyte proliferation[25]. From a practical standpoint, these findings suggest that although passages 6–9 are still relatively moderate, researchers should monitor CD90 expression when expanding UC-MSCs in vitro. For therapeutic applications where immunomodulation or differentiation potential is critical, using lower-passage cells may preserve functionality. Further, CD90 could be considered a sentinel marker of drift in long-term MSC culture.

CONCLUSION

Prolonged culture of MSCs from passage 6 to 9 resulted in a notable decline in CD90 expression at passages 8 and 9 (67.3 % and 64.3%), while CD105 and CD73 remained stable. These findings indicate that extended passaging may partially affect stemness-related markers without altering the core mesenchymal phenotype, emphasizing the importance of using early-passage MSCs to maintain quality and functionality.

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Competing Interests

The authors declare that there is no conflict of interest.

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