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Five side populations isolated from rat bone marrow-derived mesenchymal stem cells

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Abstract. Over the past decade, there has been marked enthusiasm surrounding the potential application of mesenchymal stem cells (MSCs) as universally compatible donor cells in the field of regenerative medicine. Due to their distinctive immune-tolerant characteristics, these entities hold promise for potential applications in cell replacement, gene therapy and immunomodulatory therapy. MSCs have been defined and characterized based on adhesion properties and different surface markers that are negative for hematopoietic stem cells and positive for MSCs, and their capacity for differentiation into osteocyte, chondrocyte and adipocyte is observed under suitable circumstances. However, this protocol is costly and time-consuming and cannot confirm the homogeneity of the isolated population of stem cells. Since no unique cell surface marker for prospective MSC isolation has yet been reported, at least to the best of our knowledge, the study, evaluation and characterization of MSCs greatly depend on their capacity to attach to, and subsequently undergo proliferation on a plastic surface. In the present study, MSCs were isolated from rat bone marrow, and these cells were divided into five groups according to their morphology, colony formation and proliferation rate. The expression levels of four pluripotency genes responsible for the differentiation, proliferation and self-renewal of MSCs were examined in each group with the aim of identifying an alternative protocol for stem cell characterization which can be used instead of the current protocols (trilineage differentiation and flow cytometry), which are costly and time-consuming. The molecular characterization of the MSCs revealed different expression levels of each gene, suggesting that the adherence isolation protocol led to the isolation of heterogenous populations that had different molecular patterns.

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Introduction

Stem cells are undifferentiated cells that possess the ability to undergo self-renewal through cell division and to differentiate into specialized cell types. As a result, individuals develop a more specialized phenotype through the adoption of a distinct genetic expression profile. Ultimately, these cells differentiate into particular cell types that exhibit distinct and defining characteristics (1).

Stem cells can be classified according to their capacity for self-renewal and potency. Self-renewal refers to the inherent capability of cells to undergo multiple rounds of cellular division while preserving their undifferentiated form. On the other hand, potency denotes the capacity of cells to differentiate into distinct different types of cells (2). Stem cells can be categorized into four distinct categories based on their potency. The initial category consists of totipotent stem cells. The aforementioned cells are generated from the union of a female egg and a male sperm cell, and possess the ability to undergo differentiation into both embryonic and extraembryonic cell lineages. The second category encompasses pluripotent stem cells, which possess the capacity to differentiate into cell types derived from all three primary germ layers (endoderm, mesoderm and ectoderm), but do not possess the ability to generate placental or extraembryonic cells. The third category comprises multipotent stem cells, which possess the capacity to undergo differentiation into a finite range of cell types that are confined to a specific germinal layer. The fourth category comprises unipotent cells, which undergo differentiation leading to the formation of a singular cell type. These cells appear in adult organisms (3).

According to Barky *et al* (4), stem cells can be categorized into numerous types, including embryonic, fetal, adult,

amniotic-derived, cord blood-derived and induced pluripotent stem cells. Embryonic stem cells are derived from embryos, as their name suggests. Pluripotent cells possess the ability to remain in an undifferentiated state or undergo differentiation into any cell type found inside the human body (5).

The derivation of the initial embryonic line in 1998 marked the commencement of a prominent, impassioned and persistent discourse within the realm of research ethics (5). In order to obtain embryonic stem cells, it is imperative to terminate the preimplantation embryo at the age of 5 days (6). Another barrier to their potential medical use is the need for human leukocyte antigen (HLA)-compatible human embryonic stem cell lines (7). The limited understanding of their biological characteristics serves as a constraining element that diminishes their prospects for use. One further limitation that poses a significant concern for their utilization in clinical practice is the potential formation of teratomas and teratocarcinomas (8).

Mesenchymal stem cells (MSCs) are currently undergoing experimental testing in numerous biological systems and clinical contexts in order to investigate potential therapeutic effects for a wide range of reasons (9,10). MSCs are distinguished and described by a number of key features. Firstly, they exhibit the capacity to adhere to plastic culture flasks. Additionally, they exhibit the positive expression of specific membrane antigens, namely CD105, CD73, CD90 and CD44. Conversely, they do not express certain antigens, such as CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR. Furthermore, they possess the capacity to differentiate into osteoblasts, adipocytes and chondroblasts under suitable conditions *in vitro* (11). One of the major hurdles for improving MSC transplantation efficacy is the lack of molecular characterization (12).

This molecular characterization can be used to replace current expensive and time-consuming characterization protocols such as flow cytometry. The molecular characterization of MSCs may provide a promising solution for easier and improved characterization of MSCs, in order to make full use of stem cells in regenerative medicine.

Materials and methods

Isolation of rat bone marrow-derived MSCs (BM-MSCs). BM-MSCs were isolated from the femurs and tibiae of 6-8-week-old female Sprague-Dawley rats. A total of 15 rats (weighing 150-180 g) were obtained from the Medical Experimental Research Center (Mansoura, Egypt) as previously described by Meurer *et al* (13), with some modifications. In the present study, rats were housed at a temperature of 20-25˚C and a humidity of 30-50% in the animal house of the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University. They were conditioned in standard metallic cages (6 rats per cage) with regular dark/light cycles. They were acclimatized to the laboratory conditions, fed standard rat chow and water was available *ad libitum*. The health status of the rats their welfare condition were observed daily by a veterinarian. The experimental protocol of the present study was approved by the Local Ethics Committee, Faculty of Medicine, Mansoura University in accordance with the Ethics Committee of the National Research Center, Egypt with registration number (09/189), which has been accepted by MU-ACUC.

Briefly, the rats were euthanized using an overdose (5%) of halothane; the death of the rats was confirmed by the absence of a corneal reflex, failure to detect respiration, and the absence of a heart beat for a period >5 min. Following the confirmation of death, the rats were soaked in 70% (v/v) alcohol for 2 min and transferred to a surgery table in a class II biological safety cabinet. The femur and tibia were dissected out. The bilateral connection parts around the ankle, hip and knee joints were cut to isolate the tibiae and femurs gently and carefully. Any remaining muscle or tissue on the bones was removed using sterile lint-free tissue paper (Kleenex; Kimberly-Clark Worldwide, Inc.) and the bones were sprayed with 70% ethanol. The proximal end of the femur and the distal end of the tibia were cut using fine scissors. The needle of the syringe was filled with proliferation medium (DMEM/F12, 10% FBS and 1% penicillin/streptomycin; HyClone; Cytiva) and inserted into the bone diaphysis, and all bone marrow was flushed into a sterile 50-ml conical tube. These flushed bone marrow cells were plated in 75-cm^2 tissue culture flasks and the flasks were incubated in a 5% CO_2 incubator with a humidified atmosphere containing 95% air at 37˚C.

The cells were allowed to adhere for 24 h, and the non-adherent cells were transferred to another flask for further culture. The culture medium was changed every 3 days. The adherent cells were observed under an inverted microscope (Olympus Corporation) and detached using 0.25% trypsin-EDTA when they became 70-90% confluent. At passage 3, the cells were divided into five groups (A, B, C, D and E) according to their morphology.

Flow cytometry. Since there is no well-defined basis for determining MSCs using their cellular surface markers, it was confirmed that they were not of hematopoietic origin ($CD45^-$), and subsequently two selected markers that have been defined (11) as MSC markers ($CD29^+$/Entegrin β1 and $CD105^+$/Endoglin; cat. no. FMC003; R&D Systems, Inc.) were detected.

Following bone marrow extraction, the cells were cultured in a T-75 flask as aforementioned and sub-cultured until passage 3. Once the cells reached 80-90% confluency, the cells were harvested using a trypsin/EDTA solution, and then washed twice with PBS and resuspended in staining buffer at a concentration of $1x10^6$ cells/ml. Each group was stained first for CD45/CD29 and then for CD45/CD105. In brief, the cells were labelled according to the manufacturer's instructions with conjugated monoclonal antibodies: Peridinin-chlorophyll-protein-conjugated mouse anti-rat CD45, phycoerythrin-conjugated mouse anti-rat CD29/integrin β1 and carboxyfluorescein-conjugated mouse Endoglin/CD105 (included with the kit). The stained cells were then fixed with formaldehyde buffer in PBS and analyzed using a flow cytometer (Invitrogen). The results were analyzed using Attune NxT software v3,1,2 (Invitrogen; Thermo Fisher Scientific, Inc.).

Gene expression analysis using reverse transcription-quantitative PCR (RT-qPCR). SOX-2, octamer-binding transcription factor 4 (Oct-4), Nanog and ES cell expressed Ras (Eras) mRNA expression was assessed in the five categorized groups by total RNA extraction followed by RT-qPCR. Briefly, RNA was extracted from the cells in the five categorized

Table I. Primer sequences of the studied genes.

Gene	Sequence	Product size	Gene ID
Oct-4	F: CGAGAACCTTCAGGAGATATGC R: TACAGAACCACACTCGAACC	197	NM_001009178.2
Nanog	F: CCTGAGCTATAAGCAGGTGAAGA R: CTGCAATGGATGCTGGGATAC	144	NM_001100781.1
SOX-2	F: AACCGTGATGCCGACTAGAA R: CGCCTAACGTACCACTAGAAC	93	NM_001109181.1
Eras	F: CATCCTAACCCCCAACTGTCC R: TGGCTCTCCTCTGGCGATCT	186	NM_001109375.1

F, forward; R, reverse; Oct-4, octamer-binding transcription factor 4; Eras, ES cell expressed Ras.

groups using an RNeasy® Mini Kit (Qiagen, Inc.). The isolated RNA was reverse-transcribed into cDNA using the Maxima First Strand cDNA kit, and it was then kept at -20˚C for further analysis. The qPCR reaction mixture mainly contained 20 μl (total volume) triple-step SYBR-Green PCR master mix (Thermo Fisher Scientific, Inc.), 2 μl (10 pmol/μl) forward and reverse primers for the genes studied, and 2 μl cDNA template. The amplification process for each reaction was conducted over a span of 40 cycles. The primer designing tool [Primer designing tool (nih.gov); https://www.ncbi.nlm.nih.gov/tools/primer-blast/] was used to design primer sequences for the studied genes listed in Table I. The cycle threshold (Cq) value of the control gene, GAPDH, was used to normalize the Cq values for each target gene. The determination of the expression of several target genes was conducted using the comparative ΔCq approach (14).

Statistical analysis. The results were analyzed using one-way ANOVA with GraphPad Prism 8.0 software (Dotmatics). To examine significant differences between groups, Tukey's multiple comparison post hoc test was applied following ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphology of MSCs. The morphology of the adhered BM-MSCs was examined under an inverted microscope at passage 3, and then, according to the shape of the cells and colonies, the cells were divided into five groups (A, B, C, D and E). In group A, the cells had a spindle fibroblast-like shape and formed a monolayer sheet, with cells arranged in arrays without gaps between cells as shown in Fig. 1A. In group B, the cells still had a spindle fibroblast-like shape, forming colonies with gaps between cells, and cells could not form a monolayer sheet, as shown in Fig. 1B. In group C, cells had the ability to form colonies, but the cells in the colonies had different shapes (ranging between spindle-shaped cells or flattened cells), as shown in Fig. 1C. In group D, the cells lost their ability to form colonies, and the cells were fattened and large, as shown in Fig. 1D. In group E, the cells in the monolayer sheet began to aggregate to form multilayer sheets, as shown in Fig. 2A. Over time, the cells collapsed, formed spheres and began to become enlarged, as shown in Fig. 2B and C.

Flow cytometry. Once arrived at passage 3, the cells from the five groups were harvested and stained as aforementioned. Flow cytometric analysis of the five groups revealed that they met the criteria of MSCs. They were all negative for CD45, and positive for both mesenchymal markers, CD29 and CD105, as shown in Figs. 3 and 4. No differences in these markers were observed among all groups, although they had different morphologies and different self-renewal abilities.

Gene expression. The present study examined four genes (Nanog, Oct-4, SOX-2 and Eras) that play a role in the pluripotency state of stem cells, as previously reported by Takahashi and Yamanaka (15). In order to examine the expression of different pluripotency genes in groups A, B, C, D and E, RT-qPCR was performed on the cDNA of transcripts extracted from each group. The highest expression levels of Nanog were observed in group E and the lowest expression levels were observed in group A, while the other groups exhibited different expression levels within the range of those in groups A and E, as shown in Figs. 5 and 6. As regards Oct-4 and Eras expression, the highest expression levels were also observed in group E, while the lowest expression levels were observed in group A, as shown in Figs. 5 and 6. The expression levels of SOX-2 were significantly increased in group E compared with group A ($P=0.0221$) and group C ($P=0.0451$), as shown in Figs. 5 and 6.

Discussion

In the present study, a total of 12 Sprague-Dawley rats were used for breeding, and only the female rats aged 2 months were selected for MSC isolation. The utilization of this particular age criterion serves the purpose of mitigating the potential impact of age-related disparities. It has been documented that the functional attributes of BM-MSCs (16) and adipose-derived stem cells, including yield, proliferation and differentiation capabilities, are detrimentally influenced by an advancing age (17), which has also been confirmed at a later date by another group (18).

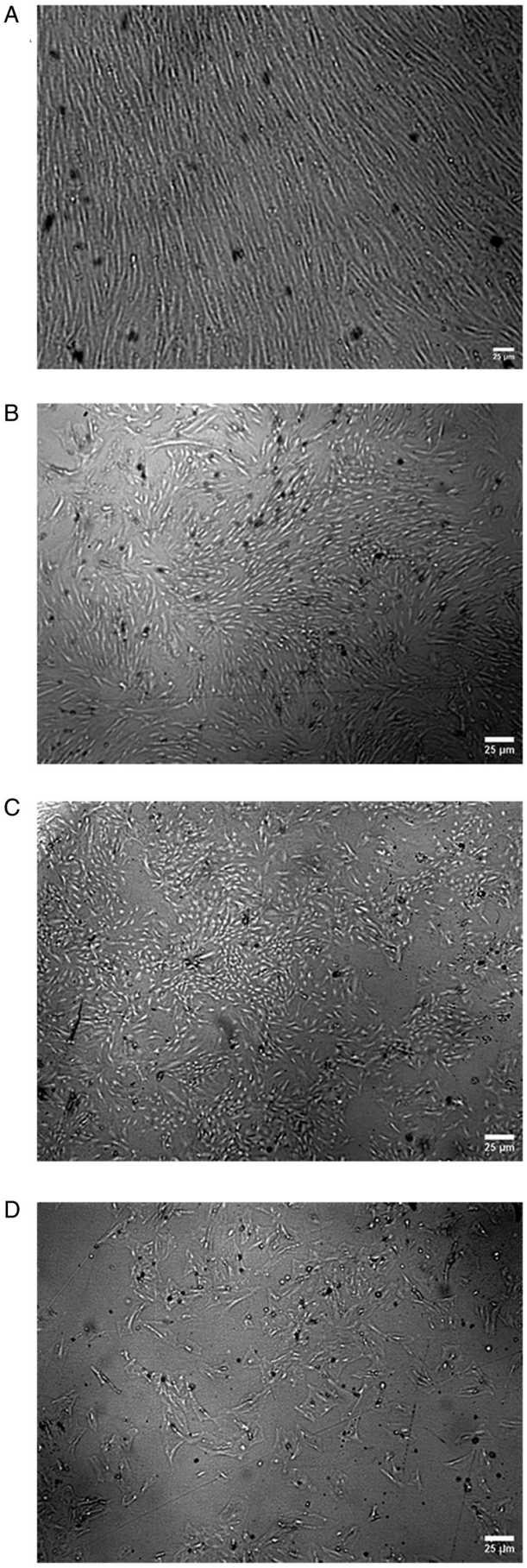

Figure 1. Morphology of the groups of bone marrow-derived mesenchymal stem cells. (A) represents group A, (B) represents group B, (C) represents group C, and (D) represents group D.

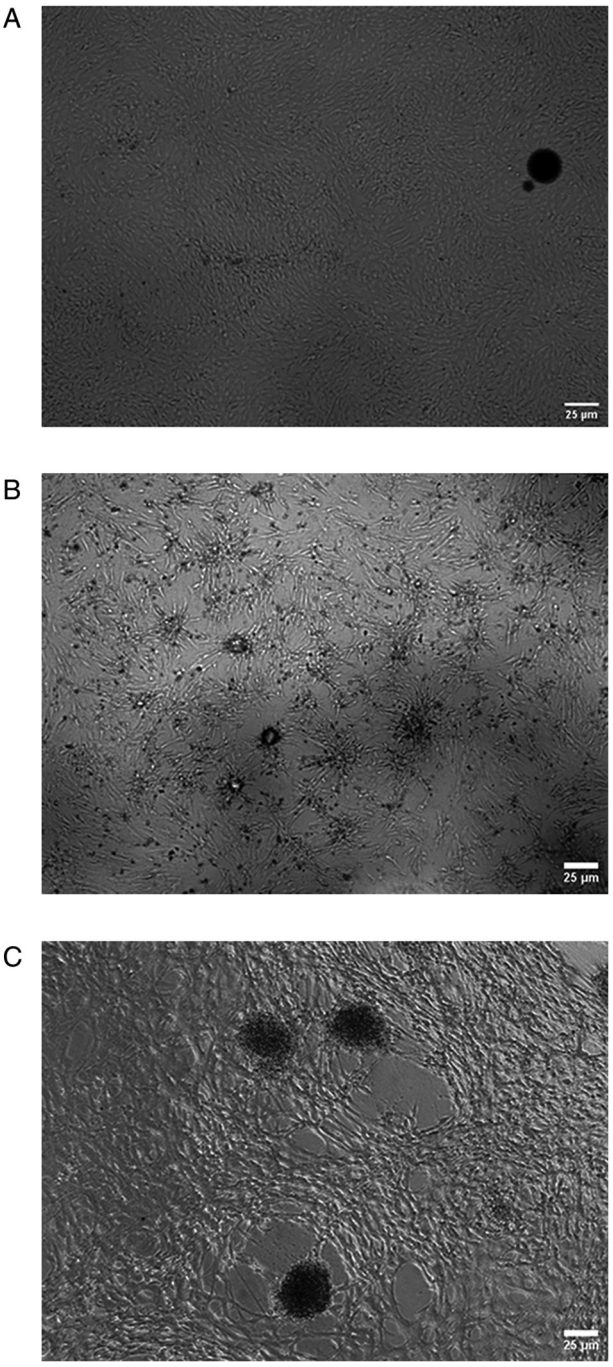

Figure 2. Morphology of bone marrow-derived mesenchymal stem cells from group E; (A) illustrates the appearance of cell aggregation; (B) illustrates cells beginning to form spheres; and (C) illustrates the enlargement of spheres.

In the present study, different shapes and morphologies of MSCs were observed using the same methodology of isolation and the same conditions in culture, including seeding density, type of medium, percentage of serum and tissue culture flasks.

In another study, different morphologies of cells were observed when using six different types of culture media and different seeding densities. Based on this, five different morphologies were identified: i) Cells cultured in Rooster Nourish-MSC XF media were spindle-shaped and elongated, and aggregated; ii) cells cultured in Stem MACS-MSC XF and MSC NutriStem XF were spindle-shaped and slender, and had a mat-like appearance

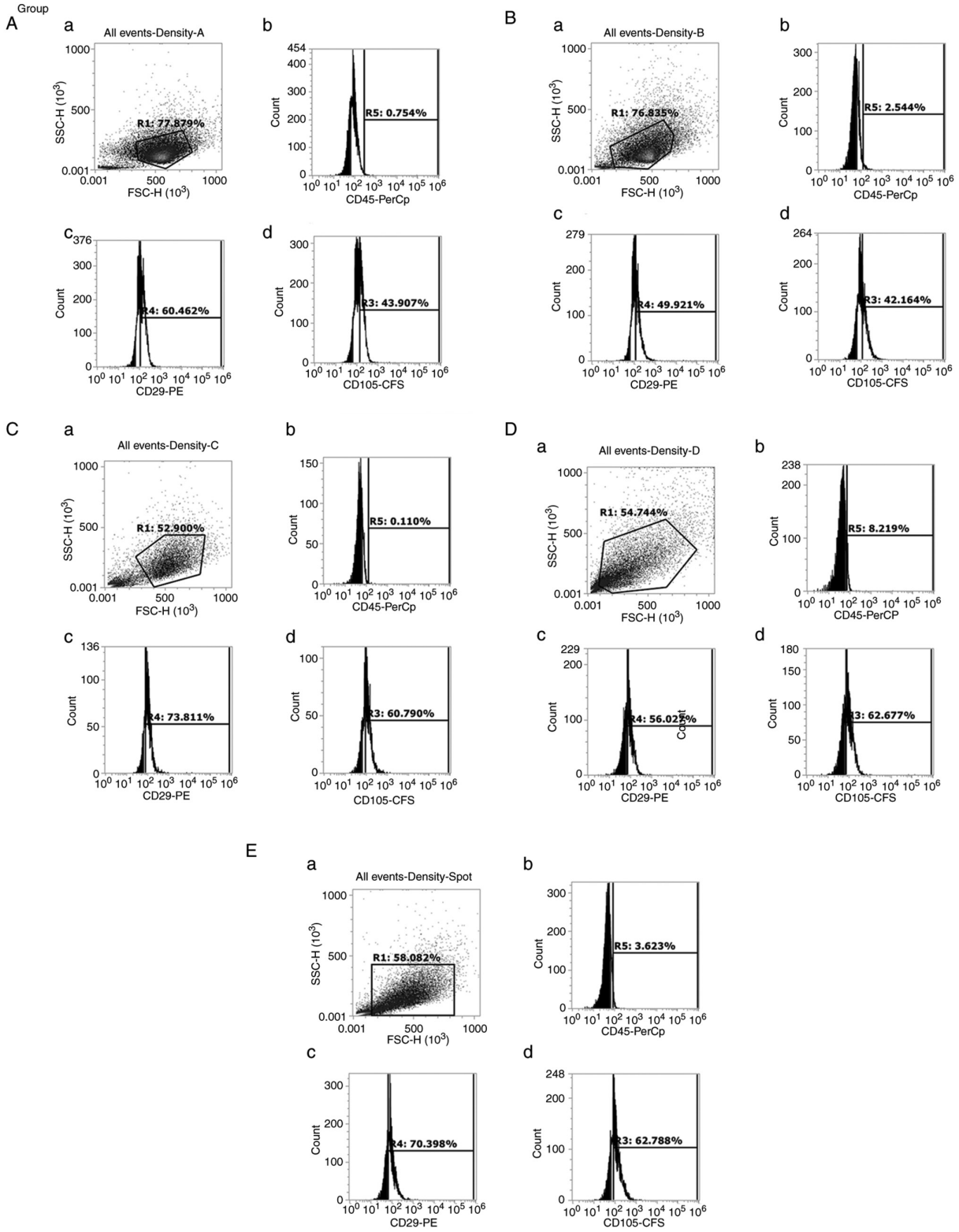

Figure 3. Flow cytometry histogram of bone marrow-derived mesenchymal stem cells from the five groups, A, B, C, D and E (A-E, respectively), labeled with antibodies against CD45, CD29, CD105. (A-a) Represents the total events in group A and the selected population for analysis (gating); (A-b) the histogram of cells harvested and labeled with antibodies against CD45; (A-c) the histogram of cells harvested and labeled with antibodies against CD29; (A-d) the histogram of cells harvested and labeled with antibodies against CD105. (B-a) Represents the total events in group B and the selected population for analysis (gating); (B-b) the histogram of cells harvested and labeled with antibodies against CD45; (B-c) the histogram of cells harvested and labeled with antibodies against CD29; (B-d) the histogram of cells harvested and labeled with antibodies against CD105. (C-a) Represents the total events in group C and the selected population for analysis (gating); (C-b) the histogram of Cells harvested and labeled with antibodies against CD45; (C-c) the histogram of Cells harvested and labeled with antibodies against CD29; (C-d) the histogram of Cells harvested and labeled with antibodies against CD105. (D-a) Represents the total events in group D and the selected population for analysis (gating). (D-b) the histogram of Cells harvested and labeled with antibodies against CD45; (D-c) the histogram of Cells harvested and labeled with antibodies against CD29; (D-d) the histogram of Cells that were harvested and labeled with antibodies against CD105. (E-a) Represents the total events in group E and the selected population for analysis (gating); (E-b) the histogram of cells harvested and labeled with antibodies against CD45; (E-c) the histogram of cells harvested and labeled with antibodies against CD29; (E-d) the histogram of cells harvested and labeled with antibodies against CD105.

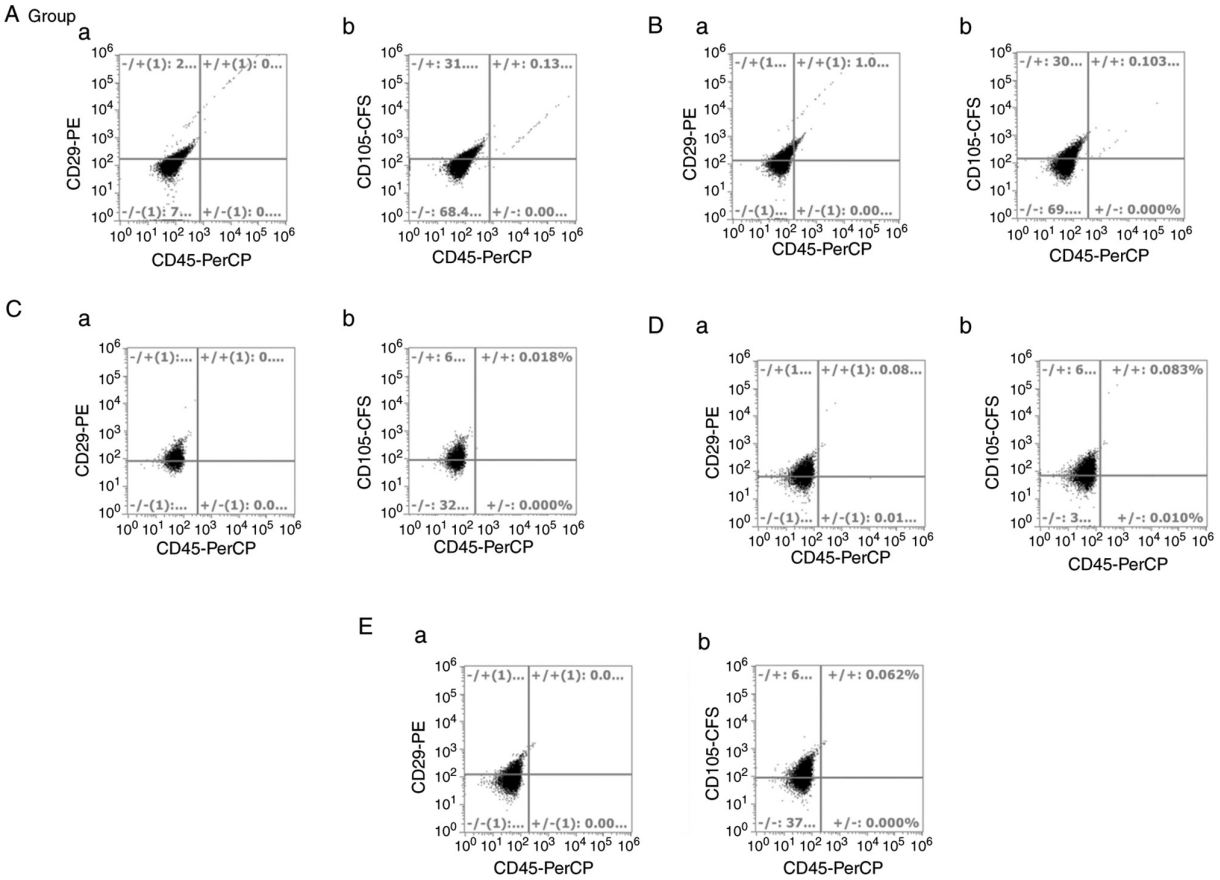

Figure 4. Flow cytometric analysis of surface protein markers of mesenchymal bone marrow-derived stem cells from the five groups, A, B, C, D and E (A-E, respectively) for the combination of CD29, CD45, and the combination with CD105. (A-a) Co-expression of CD29-CD45; (A-b) co-expression of CD105-CD45; (B-a) co-expression of CD29-CD45; (B-b) co-expression of CD105-CD45; (C-a) co-expression of CD29-CD45; (C-b) co-expression of CD105-CD45; (D-a) co-expression of CD29-CD45; (D-b) co-expression of CD105-CD45; (E-a) co-expression of CD29-CD45; (E-b) co-expression of CD105-CD45.

at higher confluency, as well as being shorter and thicker; iii) cells cultured in PLTMax comparable to DMEM-KO, which was used for control cultures, were spindle-shaped, elongated and bright with tapering ends; and iv) cells cultured in StemXVivo MSC SFM medium were highly elongated, with tapering ends at passage 4, while at passage 5, few cells were aggregated and there was a change in shape of the cells. However, the aforementioned morphologies were medium-dependent and reflected the response of cells to different environments rather than different types of stem cells (19).

Another group from the University of Munich isolated BM-MSCs, and then classified the cells into three sub-populations according to their morphology: Rapidly self-renewing cells, elongated fibroblast-like spindle-shaped cells and slowly replicating, large, cuboidal or flattened cells. These sub-populations exhibited distinct morphologies, proliferation rates and differentiation properties (20).

In the present study, it was possible to easily and clearly differentiate between five distinct groups of cells based on the morphology and colony forming ability only after 1 week of isolation. These different shapes were observed using the same conditions, medium and methodology.

These different sub-populations of bone marrow stem cells exhibited the same profile in flow cytometry analysis, suggesting that they met the criteria of stem cells introduced by the International Society for Cell & Gene Therapy. However, these cells were not the same in terms of morphology, ability to form colonies and doubling rate. Thus, it was determined which sub-population was closer to the criteria of stem cells and the pluripotency state in particular. Additionally, the results of flow cytometry highlighted the inaccuracy of using cellular makers and cytoplasmic markers to define and characterize stem cells.

The present study subsequently aimed to approach this issue from another angle. Takahashi and Yamanaka (15) determined transcription factors that controlled the internal cellular system and changed cells from the quiescent state of being a fibroblast to the pluripotent state. Thus, these factors should serve a crucial role in stem cells in adult somatic tissues.

To better understand the heterogeneity among these different morphologies of stem cells derived from the bone marrow, the expression of four pluripotency genes was analyzed. This investigation was motivated by the recognition that the functionality of MSCs is regulated by distinct molecular profiles. In the present study, the expression of SOX-2 was observed in the isolated sub-populations, especially in group E, and this is a factor implicated in the self-renewal of pluripotent stem cells and the multipotency of BM-MSCs. This finding suggests that BM-MSCs possess a more primitive state, which has been previously acknowledged (21) and is in

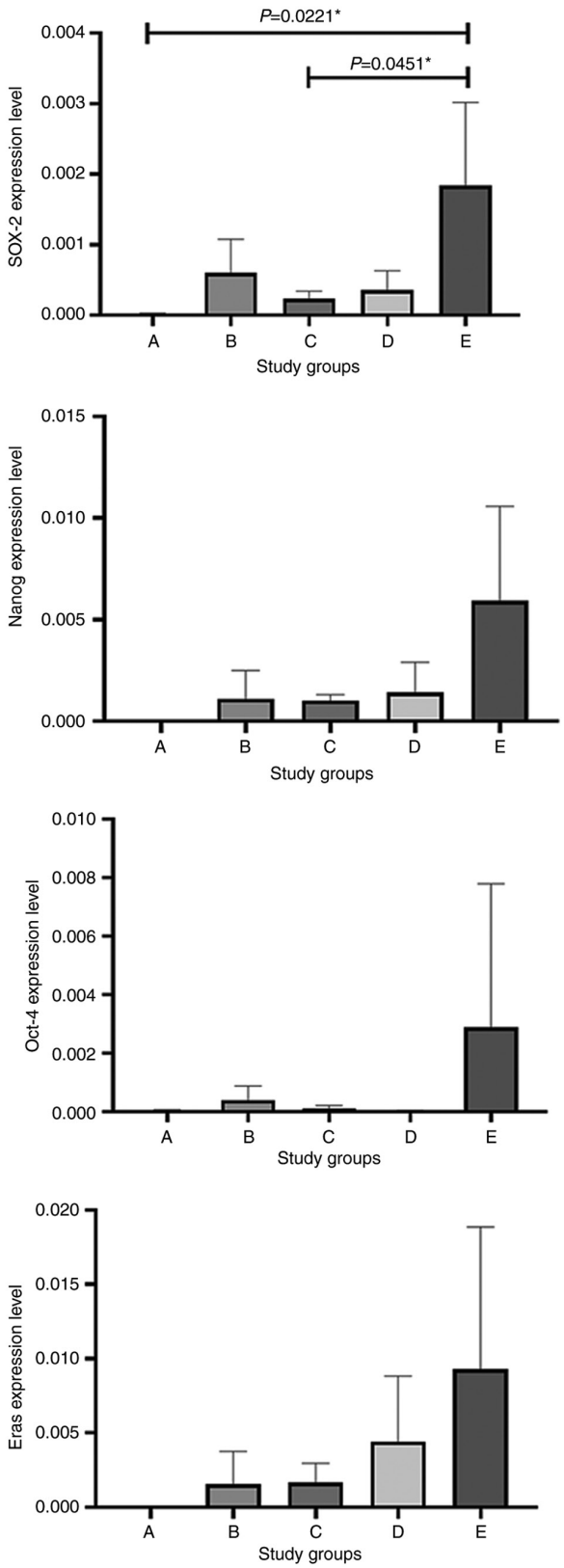

Figure 5. Gene expression levels of Nanog, Oct-4, SOX-2 and Eras in the different study groups. The figure represents each individual gene in the five groups exhibiting SOX-2, Nanog, Oct-4 and Eras expression. Oct-4, octamer-binding transcription factor 4; Eras, ES cell expressed Ras.

accordance with the study by Heo *et al* (22), which revealed that SOX-2 expression was higher in BM-MSCs than in adipose tissue-derived MSCs.

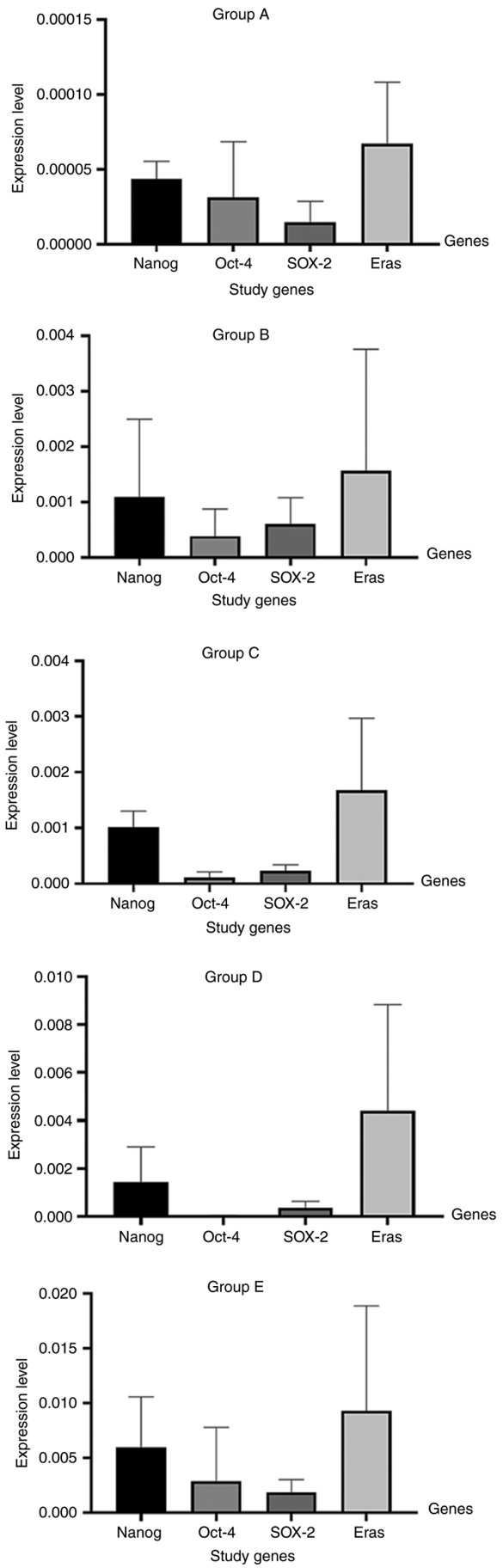

Figure 6. Gene expression levels of Nanog, Oct-4, SOX-2 and Eras in the five groups. The figure represents the four genes in each group, A, B, C, D and E. Oct-4, octamer-binding transcription factor 4; Eras, ES cell expressed Ras.

In the present study, the BM-MSCs also exhibited the presence of considerable levels of a number of essential transcription factors, including Oct-4 and Nanog. This was confirmed by RT-qPCR, even in the absence of external stimuli. The findings of the novel study by Labedz-Maslowska *et al* (23) indicated a notable increase in the mRNA concentration of two key transcription factors, Oct-4 and Nanog, in a specific subset of rat bone marrow cells. Furthermore, it revealed a higher mRNA concentration of both transcription factors regulating cell pluripotency (Oct-4 and Nanog) in a purified $CD45^-/Lin^-/CD106^+$ population of rat bone marrow cells compared with unfractionated bone marrow cells (23). On the other hand, the present study revealed low expression levels of Oct-4 in groups A, C and D, and high expression levels in group E. Nanog expression was higher than Oct-4 expression, but was still low in group A, and different expression levels were observed in groups B, C and D, while high expression levels were observed in group E.

In their novel study, Takahashi and Yamanaka (15) reported that a number of genes, including Stat3, Eras, c-myc, KLF transcription factor 4 and β-catenin, which are commonly observed to be upregulated in malignancies, play a role in sustaining the embryonic stem cell phenotype over an extended period and promote the fast proliferation of embryonic stem cells in a controlled environment. This concurs with the results of the present study, which demonstrated that Eras was highly expressed in group E rather than other study groups.

In conclusion, the bone marrow is a rich source of multiple types of cells that have adhesiveness properties as well as a considerable ability to divide multiple times and differentiate in some cases. Observations of the morphology and colony-forming ability are more effective for the identification of stem cells than flow cytometry and adhesiveness protocols. It would be of great value to deeply study these 10 factors (Fbxo15, Nanog, Eras, Dppa2, Oct3/4 (Pou5f1), Sox2, Tcl1, Klf4, β-catenin AND c-Myc) mentioned in the study by Takahashi and Yamanaka (15) in adult stem cells, and to identify the connection between pluripotent stem cells and stem cells in adult tissues.

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Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

DASMA proposed the main hypothesis for the study, designed the main experiments and participated in the writing of the manuscript. MES proposed the main hypothesis for the study, designed the main experiments, carried out many stages in the experiments and participated in the writing of the manuscript. AAE carried out many stages of the research protocol and prepared the introduction section of the manuscript. SMF carried out many stages of the research protocol, and prepared the figures and graphs for the study, and participated in the writing of the manuscript. BHO carried out the animal breading, animal care and bone marrow extraction. SHH carried out the animal breading, animal care and bone marrow extraction. All authors have read and approved the final manuscript. MMS proposed the main hypothesis for the study, designed the main experiments and participated in the writing of the manuscript. DASMA and MES confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal care during the experimental procedures was carried out according to the recommendations and following the approval of the Mansoura University Animal Care and Use Committee (MU-ACUC) (Decision no. MED. RP.23.06.02).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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