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THE EFFICIENCY OF IMMUNOMAGNETIC SORTING OF RABBIT BONE MARROW CELLS FOR THE ESTABLISHMENT OF MESENCHYMAL STEM CELL CULTURE

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ABSTRACT

The aim of this study was to assess if immunomagnetic sorting of SSEA-4 or MSCA-1 positive bone marrow cells could facilitate and expedite the establishment of rabbit MSCs culture. Briefly, rabbit bone marrow cells were enriched for SSEA-4$^+$ and MSCA-1$^+$ cells using two clones of SSEA-4 antibodies (REA101 and MC-813-70) and MSCA-1 antibody (clone W8B2) via magnetic-activated cell sorting. Flow-cytometric analysis revealed a high level of sorting efficiency, since the SSEA-4$^+$ cells were significantly ($P<0.001$) enriched from 17 to 95% and MSCA-1$^+$ cells from 8 to 74%. According to morphological changes, both sorted fractions (positive and negative) successfully initiate rabbit MSCs culture, but not expedite the proliferation rate as compared to standard MSCs culture initiated from unsorted cells. Moreover, all samples (sorted and unsorted) expressed high levels of the typical MSCs markers (CD29 and CD44) and low expression of hematopoietic marker CD45. In conclusion, although this study revealed the possibility to enrich SSEA-4$^+$ and MSCA-1$^+$ positive cells from the rabbit bone marrow, the standard method of MSCs isolation using the plastic adherent ability of the unsorted bone marrow cells seems to be still useful and less cost effective.

Keywords: rabbit, MSCs, MACS, SSEA-4, MSCA-1, plastic adherence

INTRODUCTION

The method of plastic adherence became a standard technique for the isolation of human (**Pittenger *et al.*, 1999**), mouse (**da Silva Meirelles *et al.*, 2006**) or rabbit mesenchymal stem cells (MSCs) from the different biological sources such as bone marrow (**Sahoo *et al.*, 2010**), adipose tissue (**Sun *et al.*, 2016**) or amniotic fluid (**Kovac *et al.*, 2017**). However, in the recent years new technique has been established to facilitate the isolation of mesenchymal stromal or stem cells via magnetic-activated cell sorting (MACS) or fluorescence-activated cell sorting (FACS). This method allows to obtain enriched human clonogenic (CFU-F) MSCs subsets directly from the heterogeneous cell populations based on the expression of specific antigen such as STRO-1 (**Gronthos and Simmons, 1995**), MSCA-1 (**Batulla *et al.*, 2009**) or CD271 (**Quirici *et al.*, 2002**). The CFU-F fraction of bone marrow is considered to be the precursor or stem cell that resides in the bone marrow (primary MSCs) from which culture-expanded MSCs originate (**Rozemuller *et al.*, 2010**). Recently, SSEA-4 was also found to be expressed by human MSCs (**Gang *et al.*, 2007**) that can be as well used to enrich the bone marrow CFU-F cell fraction (**Rozemuller *et al.*, 2010**). MSCA-1, mesenchymal stem cell antigen-1, was shown to be identical to tissue-nonspecific alkaline phosphatase, an ectoenzyme known to be expressed at high levels in liver, bone, and kidney as well as in embryonic stem cells (**Sobiesiak *et al.*, 2009**). SSEA-4, stage specific embryonic antigen-4, previously found specifically on human embryonic stem cells and very early cleavage to blastocyst stage embryos (**Thomson *et al.*, 1998**), was found also on induced pluripotent cells (**Valamehr *et al.*, 2012**), embryonal carcinoma cells (**Kannagi *et al.*, 1983**), and embryonic germ cells (**Shamblott *et al.*, 1998**) as well as a variety of somatic stem cells, such as dental pulp stem cells, umbilical cord blood-derived very small embryonic like stem cells (**Bhartiya *et al.*, 2012**) and as mentioned above also MSCs (**Gang *et al.*, 2007**). The objective of this study was to assess if immunomagnetic sorting of SSEA-4 or MSCA-1 positive bone marrow cells could facilitate and expedite the establishment of rabbit MSCs culture.

MATERIAL AND METHODS

Isolation and immunomagnetic sorting of rabbit bone marrow mononuclear cells (BMMCs)

Rabbit BMMCs were isolated from 16 young (5 months-old) and clinically health rabbits of NZW line as described previously (**Vašíček *et al.*, 2016**). BMMCs were divided into control (fresh unsorted) cells and cells intended for MACS sorting as described below. Briefly, BMMCs were incubated with two different clones of monoclonal PE-conjugated SSEA-4 antibodies (REA101 from Miltenyi Biotec, Germany; and MC-813-70 from eBioscience, Austria) and PE-conjugated MSCA-1 antibody (clone W8B2 from Miltenyi Biotec, Germany) according to the producer's manual. After washing, samples were incubated with Anti-PE MicroBeads (Miltenyi Biotec, Germany) and then sorted using AutoMACS Pro Separator (Miltenyi Biotec, Germany) according to the producer's manual. Positive selection in sensitive mode "POSSEL_S" was applied in order to sort rare cells with a low antigen expression

Culturing of fresh rabbit BMMCs and sorted MSCs

Briefly, fresh BMMCs or sorted cells from negative (SSEA-4$^-$ and MSCA-1$^-$) and positive (SSEA-4$^+$ and MSCA-1$^+$) fractions were resuspended in 20% MEM Alpha medium (Thermo Fisher Scientific, USA) with supplements and then plated at density of 1.2 - 1.5 x 10^6 cells/cm^2 (fresh BMMCs) or at density of 0.3 - 0.5 x 10^5 cells/cm^2 (sorted cells) on appropriate tissue culture flasks. Morphology of cultured cells was visualized by Zeiss Primovert phase-contrast microscopy (Carl Zeiss Slovakia, Slovakia). The rate of cell proliferation of each sample was assessed as the length of culture (days) when cells achieve first confluency (about 80-90%). Cells were cultured approximately 3-4 weeks till the passage 3. MSCs were then dissociated using 0.05% Trypsin-EDTA (Thermo Fisher Scientific, USA) and analysed for the MSCs phenotype using flow cytometry.

Flow cytometry analysis

Control samples of BMMCs were analysed for initial SSEA-4 and MSCA-1 expression using above mentioned antibodies, but not sorted. Aliquots of sorted

samples before seeding were analysed for the purity and efficiency of MACS sorting in terms of the percentage of SSEA-4$^+$ and MSCA-1$^+$ cells in both fractions. MSCs phenotype of cultured cells at passage 3 was confirmed by incubation with following monoclonal antibodies: FITC-conjugated CD29 (clone P4G11 from Merck, Slovak Republic), unconjugated CD44 (IgG1, clone W4/86) and CD45 (IgG1, clone L12/201, both from Bio-Rad, UK) and appropriate FITC-conjugated anti-mouse IgG1 secondary antibody (clone M1-14D12 from eBioscience, Austria). 7-AAD (eBioscience, Austria) was used in order to exclude debris and dead cells from the analysis. Labelled cells before and after sorting were evaluated using the FACSCalibur flow cytometer (BD Biosciences, USA). At least 50,000 events (cells) were analysed in each sample.

Statistical analysis

The experiments were replicated three times. Observed results were evaluated statistically using one-way ANOVA (Holm-Sidak method) by SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

MACS sorting

In this study, we found out that about 20% of fresh rabbit bone marrow cells showed the positivity for SSEA-4 antigen and less than 10% positivity for MSCA-1 antigen (Tab 1). The small proportion of SSEA-4$^+$ cells have been previously reported in human bone marrow (2-4%, **Gang *et al.*, 2007**). Moreover, SSEA-4 expression was found on the human and murine (**Gang *et al.*, 2007**), monkey and goat (**Rozemuller *et al.*, 2010**) as well as on the rabbit MSCs (**Ding and Huang, 2015**). Similarly, MSCA-1 antigen was previously detected on human (0.9 ± 0.4%), monkey (9.4 ± 4.9%), goat (2.2 ± 1.2%), dog (5.5 ± 4.2%), pig (3.9 ± 6.8%) and sheep (19.2 ± 15.4%) BMMCs as well as on the MSCs of mentioned species (**Rozemuller *et al.*, 2010**). Furthermore, the MACS technique revealed a high level of sorting efficiency, since this method significantly ($P<0.001$) enriched the population of SSEA-4$^+$ cells (from 17 to 95%, Tab 1). Also population of MSCA-1$^+$ cells was significantly enriched by the positive immunomagnetic selection (from 8 to 74%, Tab 1). Thus, we could assume that enrichment and seeding of SSEA-4$^+$ or MSCA-1$^+$ BMMCs could give rise to whole rabbit clonogenic MSCs culture. To confirm this assumption, sorted cells from both fractions and unsorted fresh rabbit BMMCs (served as control) were seeded and cultured as described above or elsewhere.

Table 1 Sorting efficiency of MACS enriched (SSEA-4$^+$ and MSCA-1$^+$) bone marrow cells assessed by flow cytometry

Marker	Before MACS (%)	MACS Negative Fraction (%)	MACS Positive Fraction (%)
SSEA-4 (REA101)	17.74 ± 1.64^b	1.52 ± 0.05^b	90.31 ± 4.07^a
SSEA-4 (MC-813-70)	25.64 ± 0.56^b	1.97 ± 0.08^b	95.67 ± 0.01^a
MSCA-1 (W8B2)	8.58 ± 2.08^b	2.10 ± 0.20^b	73.63 ± 7.45^a

Legend: Results are expressed as mean values ± SEM. Different letters indicate significant differences (^{a} vs ^{b} at $P<0.001$) among the means in each line.

Cell morphology and growth rate

Morphological changes of cultured unsorted or sorted cells confirmed successful initiation of rabbit MSC culture. However, the growth rate of sorted (nor negative neither positive) cells did not significantly differ from the proliferation of fresh unsorted BMMCs, since all samples required approximately 14-19 days in order to reach 80-90% cell confluency after initial seeding (Tab 2). Moreover, cells in all samples showed typical fibroblastic-like morphology with a spindle shape (Fig 1A-D). On the contrary, **Gang *et al.* (2007)** noticed that human SSEA-4$^-$ cells failed to grow, whereas SSEA-4$^+$CD45$^-$ cells adhered to the plastic and produced a homogeneous cell-monolayer characteristic of MSCs. Moreover, they observed that an equivalent number of unsorted bone marrow cells plated at the same cell density did not successfully initiate MSC cultures. Such cultures could only be derived from higher densities of unsorted cells as was confirmed by higher seeding density of fresh rabbit BMMCs in our study (Fig 1D). On the other hand, **Batulla *et al.* (2009)** successfully initiate the human MSC culture also from the MSCA-1$^+$ and CD271$^+$ sorted bone marrow cells. Furthermore, culture of CD271$^+$ (LNGFR$^+$) sorted bone marrow cells could by more effective in the terms of growth rate of MSCs than the culture of unsorted heterogeneous cell population isolated from bone marrow (**Quirici *et al.*, 2002**).

Table 2 Rate of cell proliferation assessed as the time period required for the achieving of first confluency (80 – 90%)

Sample	SSEA-4 (REA101)		SSEA-4 (MC-813-70)		MSCA-1 (W8B2)		Unsorted BMMCs
	Negative fraction	Positive fraction	Negative fraction	Positive fraction	Negative fraction	Positive fraction	
Time period (days)	17 ± 3	17 ± 1	18 ± 1	17 ± 1	14 ± 1	19 ± 1	15 ± 4

Legend: Results are expressed as mean values ± SEM.

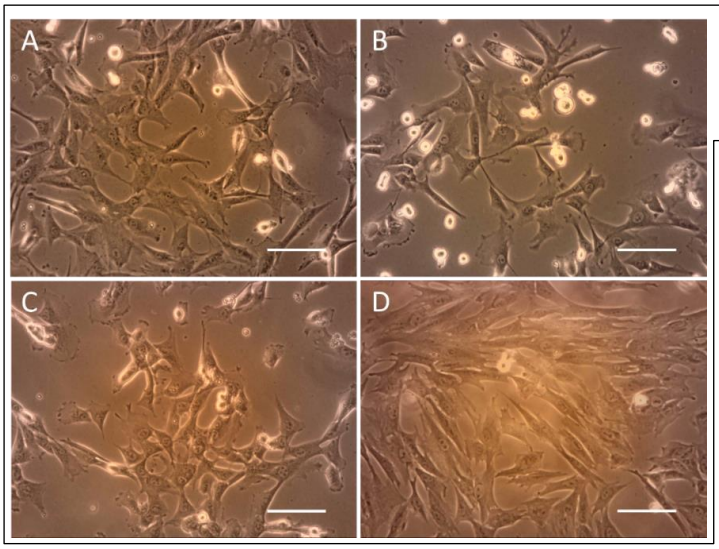

Figure 1 Illustrative morphology images of the cultured unsorted and MACS enriched (SSEA-4$^+$ and MSCA-1$^+$) bone marrow cells. A) Cells sorted using SSEA-4 (REA101); B) Cells sorted using SSEA-4 (MC-813-70); C) Cells sorted using MSCA-1 (W8B2); D) Unsorted cells seeded immediately after isolation; 100 × magnification; Scale bars = 200 µm

MSCs phenotyping

Flow-cytometric analyses (Tab 3) confirm the typical MSCs phenotype of all samples (sorted or unsorted), since cells showed high positivity for CD29 and CD44, and were negative for hematopoietic lineage marker CD45. Although in some samples (SSEA-4: MC-813-70$^+$ and MC-813-70$^+$, and MSCA-1$^+$) the purity of MSCs were significantly decreased ($P<0.001$, Tab 3) by the contamination with hematopoietic (CD45$^+$) cells in comparison to other analysed samples, the majority of these cells still expressed typical rabbit MSCs markers and morphology as observed by other studies (**Tan *et al.*, 2013; Lee *et al.*, 2014**).

Table 3 MSCs phenotype of cultured unsorted and MACS enriched (SSEA-4$^+$ and MSCA-1$^+$) bone marrow cells assessed by flow cytometry

Sample/ Marker (%)	SSEA-4 (REA101)		SSEA-4 (MC-813-70)		MSCA-1 (W8B2)		Unsorted BMMCs
	Negative fraction	Positive fraction	Negative fraction	Positive fraction	Negative fraction	Positive fraction	
CD29	72.13 ± 6.54	74.82 ± 12.45	82.76 ± 8.94	72.54 ± 1.89	86.96 ± 0.87	78.62 ± 1.09	94.36 ± 4.05
CD44	89.15 ± 2.14	95.86 ± 1.47	78.84 ± 12.87	78.77 ± 2.98	96.74 ± 1.18	89.07 ± 1.52	92.12 ± 2.16
CD45	2.23 ± 0.36^b	1.7 ± 0.10^b	10.03 ± 0.03^a	10.03 ± 0.51^a	3.90 ± 1.95^b	9.35 ± 0.23^a	3.79 ± 1.73^b

Legend: Results are expressed as mean values ± SEM. Different letters indicate significant differences (^{a} vs ^{b} at $P<0.001$) among the means in each line.

CONCLUSION

The present study revealed the possibility to enrich SSEA-4$^+$ and MSCA-1$^+$ positive cells from the rabbit bone marrow. These cells successfully initiated the MSCs culture after seeding as confirmed by the typical morphology and phenotype of mesenchymal stem cells. However, these technique was not more efficient in the terms of MSCs proliferation rate in comparison to the culture initiated from the fresh isolated heterogeneous bone marrow cells. Thus, the standard method of MSCs isolation using the plastic adherent ability of these cells seems to be still useful and less cost effective than the immunomagnetic sorting of small bone marrow fractions. On the other hand, further experiments are required in order to examine if more specific sorting of bone marrow cells by the depleting of CD45$^+$ cells from the SSEA-4$^+$ and MSCA-1$^+$ enriched fractions, could bring really pure populations of rabbit MSCs.

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