

CD45[−]CD14⁺CD34⁺ Murine Bone Marrow Low-Adherent Mesenchymal Primitive Cells Preserve Multilineage Differentiation Potential in Long-Term *In Vitro* Culture

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Bone marrow-derived cells have been postulated as a source of multipotent mesenchymal stem cells (MSC). However, the whole fraction of MSC remains heterogeneous and the expansion of primitive subset of these cells is still not well established. Here, we optimized the protocol for propagating the low-adherent subfraction of MSC which results in long-term expansion of population characterized by CD45[−]CD14⁺CD34⁺ phenotype along with expression of common MSC markers. We established that the expanded MSC are capable of differentiating into endothelial cells highly expressing angiogenic markers and exhibiting functional properties of endothelium. Moreover, we found these cells to be multipotent and capable of giving rise into cells from neuronal lineages. Interestingly, the expanded MSC form characteristic cellular spheres *in vitro* indicating primitive features of these cells. In sum, we isolated the novel multipotent subpopulation of CD45[−]CD14⁺CD34⁺ bone marrow-derived cells that could be maintained in long-term culture without losing this potential.

INTRODUCTION

Bone marrow (BM) has been well described as a place harboring not only hematopoietic stem and progenitor cells (HSPC), but also heterogeneous fraction of non-hematopoietic stem/primitive cells able to differentiate into several cellular lineages (Grove et al., 2004). Apart from the HSPC, which are well-characterized and the most abundant stem cells residing in the bone marrow, the non-hematopoietic compartment contains also neuronal and endothelial progenitors (Kabos et al., 2002; Quirici et al., 2001) as well as primitive cells exhibiting multilineage differentiation potential such as mesenchymal stem/stromal cells (MSC), multipotent adult progenitors cells (MAPC), marrow-isolated adult multilineage inducible cells (MIAMI) and very small embryonic-like stem cells (VSEL) (D'Ippolito et al., 2004; Jiang et al., 2002; Kucia et al., 2006; 2008; Pittenger et al., 1999). Recently, potential therapeutic applications of bone mar-

row-derived stem/primitive cells are extensively investigated in animal models as well as in patients with several types of diseases (Abdel-Latif et al., 2007; Dawn et al., 2008; 2009; Hofstetter et al., 2002; Houlihan and Newsome, 2008; Karussis et al., 2008; Lim et al., 2006; Matoba et al., 2008; Martin-Rendon et al., 2008; Torrente and Polli, 2008).

Mesenchymal stem/stromal cells (MSC) belong to the adherent fraction of BM cells and have been characterized by their wide capacity for differentiation into osteogenic, chondrogenic, adipogenic, myogenic, neural, endothelial and other non-hematopoietic lineages (Jiang et al., 2002; Phinney and Prockop, 2007; Pittenger et al., 1999). This multilineage differentiation potential makes them interesting for regenerative applications. MSC have been described as cells relatively easy to expand in simple culture media, which may also be an advantage for their applications (Girdlestone et al., 2009). However, the expanded population of MSC remains heterogeneous and contains subpopulations of morphologically and antigenically distinct cells (Phinney and Prockop, 2007). Still little is known about the subpopulations of MSC which are able to differentiate into cells of specific lineages. Moreover, the culture conditions selectively propagating some fractions of MSC also have not been established.

The aim of the study was to identify MSC fraction that show broad plasticity and can be expanded in long-term culture. We found that low-adherent CD45[−]CD14⁺CD34⁺ subpopulation of MSC could be expanded for at least 10 passages without losing the primitive morphology and possessed the differentiation potential toward endothelial and neuronal phenotype.

MATERIALS AND METHODS

Bone marrow isolation and culture of adherent fractions

Bone marrow (BM) cells were collected from tibias and femurs of C57Bl/6 adult mice. The cavities of the bones were flushed with low glucose (LG) DMEM (PAA, Austria) with 20% serum (FBS GOLD, PAA, Austria). The bone marrow suspension was filtered through 40 µm strainer and the fraction of mononuclear

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cells was separated by centrifugation on Ficoll density gradient (1.077 g/ml, Sigma-Aldrich, USA). The mononuclear cells collected from one mouse were plated on 25 cm² culture dish (Becton Dickinson, USA) and cultured in the DMEM LG medium supplemented with 20% serum, GlutaMAX (Gibco, Invitrogen, USA), 100 U/ml of penicillin (Sigma-Aldrich, USA), 0.1 mg/ml of streptomycin (Sigma-Aldrich, USA) and 10 ng/ml bFGF (Sigma-Aldrich, USA) in 37°C, 5% CO₂, 95% air atmosphere. The medium was changed twice a week. The special protocol was applied during each passage to propagate only the low-adherent subpopulation. The cells were washed with 0.25% trypsin/EDTA (PAA, Austria) twice and only the easily detached fraction was passaged. The cells that were still adherent after this step were not scraped or further trypsinized and were left on dish. Each time the cells were seeded at the density of 1×10^4 /cm² and cultured up to 10 passages. The cells at passage 10 were further investigated for endothelial or neuronal differentiation as well as sphere/cluster formation (Fig. 1).

Endothelial differentiation

For endothelial differentiation the cells were detached with Accutase (PAA, Austria), washed twice with PBS and seeded at 1×10^4 /cm² on 6-well plate coated with a mixture of 20 µg/ml fibronectin derived from human plasma (Sigma-Aldrich, USA) and 0.25% gelatin type B from bovine skin (Sigma-Aldrich, USA) (Fig. 1). The cells were cultured according to two distinct protocols, either in DMEM medium with 2% serum and 50 ng/ml of VEGF (Sigma-Aldrich, USA) or in defined EGM-2-MV medium (Lonza, USA) with 2% serum. The medium was changed every two days and the cells were cultured in endothelial differentiation conditions for one week. The experiment was repeated three times.

When the endothelial differentiation was performed after the sphere formation step (Fig. 1), the clusters were initially dissociated by gently pipetting and the sphere-derived cells were seeded and cultured in the same conditions as described above.

TNF α stimulation, VCAM-1 labeling and flow cytometric analysis

The cells were culture without serum for 12 h and then stimulated with 10 ng/ml of TNF α (Sigma-Aldrich, USA) for 4 h. After the stimulation the cells were detached with Accutase and examined for VCAM-1 expression by flow cytometry. The cells were incubated with the unconjugated primary rat anti-mouse VCAM-1 antibody (1:200, BD Bioscience, USA) or matched isotype control rat IgG_{2aK} antibody (1:200 dilution, BD Bioscience, USA). Secondary goat anti-rat Ig antibody conjugated with FITC was used in the next step (1:200 dilution, BD Bioscience, USA). The labeled cells were analyzed on FACS LSR II flow cytometer (Becton Dickinson, USA) using FACS Diva (Becton Dickinson, USA) and FlowJo software (Tree Star, USA).

Uptake of AcLDL and *Bandeiraea simplicifolia* lectins binding

Non-differentiated MSC and differentiated with DMEM + VEGF and EGM-2-MV media for one week were labeled with Dil-labeled AcLDL (Molecular Probes, USA) at 50 µg/ml for 24 h at 37°C. The AcLDL uptake was subsequently evaluated by fluorescence microscopy (Eclipse TS100, Nikon, Japan).

For lectin binding the cells were detached from culture plates with Accutase, washed twice with PBS, and incubated with biotinylated *Bandeiraea simplicifolia* isolectin B₄ for 1.5 h 4°C (1:100 dilution, Vector Laboratories, USA). After the incubation,

cells were washed twice with 2% serum in PBS and incubated with FITC conjugated egg avidine (1:200 dilution, Sigma-Aldrich, USA). The sample without lectin incubation acted as a negative control. The samples were analyzed by flow cytometry as described above.

Tube formation on matrigel assay

The 40 µl of matrigel (BD Bioscience, USA) were put per well of a 96-well plate. After the polymerization, 7×10^3 cells in 100 µl of DMEM LG with 1% serum were seeded to each well and incubated for 24 h in 37°C, 5% CO₂, 21% O₂ atmosphere. Tube formation was investigated by phase-contrast microscopy (Eclipse TS100, Nikon, Japan).

Neuronal differentiation

The expanded MSC were detached from culture plates with Accutase, washed twice with PBS and subsequently seeded at 2×10^4 /cm² density on a 6-well plate (Becton Dickinson, USA) coated with 50 µg/ml of poli-D-lysine (Sigma-Aldrich, USA) (Fig.1). The cells were cultured in the DMEM/F12 medium (PAA, Austria) containing B27 supplement (PAA, Austria), 100 ng/ml of bFGF (Sigma-Aldrich, USA), 20 ng/ml EGF (Sigma-Aldrich, USA), 1 µM retinoic acid (Sigma-Aldrich, USA) for one week. The medium was changed every two days.

When the differentiation was performed after the sphere formation step, the spheres/clusters were dissociated by gently pipetting and the sphere-derived cells were seeded in the same conditions as above.

Sphere formation

The expanded MSC were detached with Accutase, washed twice with PBS and seeded in DMEM/F12 medium containing B27 supplement, 20 ng/ml of bFGF, 20 ng/ml of EGF, 50 µg/ml of heparin at 2×10^4 /cm² density on a 6-well plate pre-coated with 50 µg/ml of poli-D-lysine. Half of the medium was changed each three days by careful removing the old medium with a pipette to avoid collecting the spheres. After one week, the spheres-derived cells were further differentiated in endothelial or neuronal conditions as described above.

Immunohistochemistry staining

Cells cultured in chamber slides in non-differentiated conditions and neuronal differentiation conditions were fixed with 4% PFA, blocked with 0.25% glycine and 1% BSA and then incubated with anti-neurofilament heavy antibody at 4°C overnight (dil. 1:1000, cat.: ab8135, Abcam, UK). The samples were incubated with secondary goat anti-rabbit antibody for 45 min. at room temperature (dil. 1:400, cat.: A11008, Molecular Probes, USA). The slides were analyzed with confocal microscopy (Leica, Germany).

RNA extraction, RT-PCR, real-time RT-PCR analysis

Total cellular RNA was extracted by using RNeasy[®] Total RNA Isolation System (Promega, USA) according to the manufactures instructions. Reverse transcription reaction was performed with M-MLV reverse transcriptase (Promega, USA). PCR reaction was conducted with Taq polymerase (Promega USA) using the following conditions: 95°C for 5 min, 40 cycles of 95°C for 30 s, annealing temperature for 30 s, 72°C for 30 s and ended with 72°C for 5 min. The PCR products electrophoresis was performed according to standard laboratory protocols. Specific primer sequences, annealing temperatures and product lengths are summarized in Table 1. Quantitative real-time RT-PCR was carried out using the StepOne Plus cycler (Applied Biosystems, UK) and SYBR[®] Green JumpStart[™] Taq

Table 1.

Gene		Primer sequence (5' → 3')	Product length	Annealing temp.
bIII tubulin	for	TGGAACCYGGAACCATGGACAGTG	140 bp	60°C
	rev	TCCACCAGCTCCGCSCCCTC		
CD14	for	AAGCCCGTGGAACTGGAAGC	242 bp	58°C
	rev	GAAAGCGCTGGACCAATCTGGC		
CD29	for	GGACGCTTACTGCAGAAAGAG	241 bp	56°C
	rev	ACAGTCACAKGCRCTGCCAGTG		
CD31	for	CACCRGGTGCTGTTCTATAAGG	202 bp	54°C
	rev	CCAGTGTCACCYTGGGMMTGG		
CD34	for	GGAGCCACCAGAGCTAYTCC	172 bp	56°C
	rev	CCTGGCCTCCACCRITCTCC		
CD44	for	CCTCGTCACGTCCAACACCTCC	383 bp	58°C
	rev	TCGATGGTGGAGCCGCTGC		
CD45	for	GCATCCATCCTCGTCCACTGC	180 bp	54°C
	rev	GATAGATGCTGGCGATGATGTC		
CD105	for	CGCTTCAGCTTCCTCCTCCG	281 bp	59°C
	rev	CACCACGGGCTCCCGCTTG		
CD140b	for	TACGTGCCCATGYTGGACATG	175 bp	54°C
	rev	TGGTAGCTGAAGCCCACGAG		
CD166	for	TGGCGGCTTCAACGACCATCAC	136 bp	60°C
	rev	TCCACACCACAGTCGCGTTCCT		
EF2	for	GCGGTCAGCACAATGGCATA	218 bp	60°C
	rev	GACATCACCAAGGGTGTGCAG		
GFAP	for	ATC ACC AAG GGT GTG CAG	150 bp	54°C
	rev	ACCACGATGTTCTCTTGAGG		
KDR	for	CCTCACCTGTTTCTGTATGGAG	301 bp	60°C
	rev	GAKGCCACAGACTCCCTGC		
Nestin	for	GAAAAGTTCCAGCTGGCTGTG	112 bp	54°C
	rev	AGGGACATCTTGAGGTGYGC		
Neuro filament H	for	CTGCTCAAYGTCAAGATGGC	130 bp	60°C
	rev	GAGGGAATTTTKGGGAGTCCTTC		
Neuroglycan C	for	CCACCATTGCCGAGGGCTCTC	150 bp	60°C
	rev	CCTGGTCACCTTTGCTGCCACCCTC		
Tau	for	TGGCCAAGCAGGGTTTGATGATC	205 bp	60°C
	rev	CCCATCACTGATTTTGAAGTCCCG		
Tenascin R	for	GAACTGCCACCGGACCAAC	114 bp	60°C
	rev	CTTCATTTTCYACAAAGGGGATG		
vWF	for	CCCCATCAGCCACACTTGATGC	180 bp	60°C
	rev	GCACCAGCACAGGGTTCAGC		

ReadyMix™ (Sigma-Aldrich, USA). Elongation factor 2 (EF-2) was used as housekeeping gene control. To show the mRNA fold changes the results were calculated as $2^{-\Delta Ct}$ and the non-differentiated cells were used as reference sample.

Statistical analysis

Data are reported as means ± SEM. The data were analyzed with non-paired Student's *t*-test. A one-way ANOVA with post-hoc Tukey test was applied when more than two groups were compared. The graphs design and statistical analysis were

performed using GraphPad Prism 4 software (GraphPad Software, USA)

RESULTS

Long-term propagation of low-adherent fraction of BM-derived cells does not reduce their primitive characteristics and multipotentiality

In early stages of long-term cultures of BM-derived adherent cells, we observed their morphological heterogeneity persisting

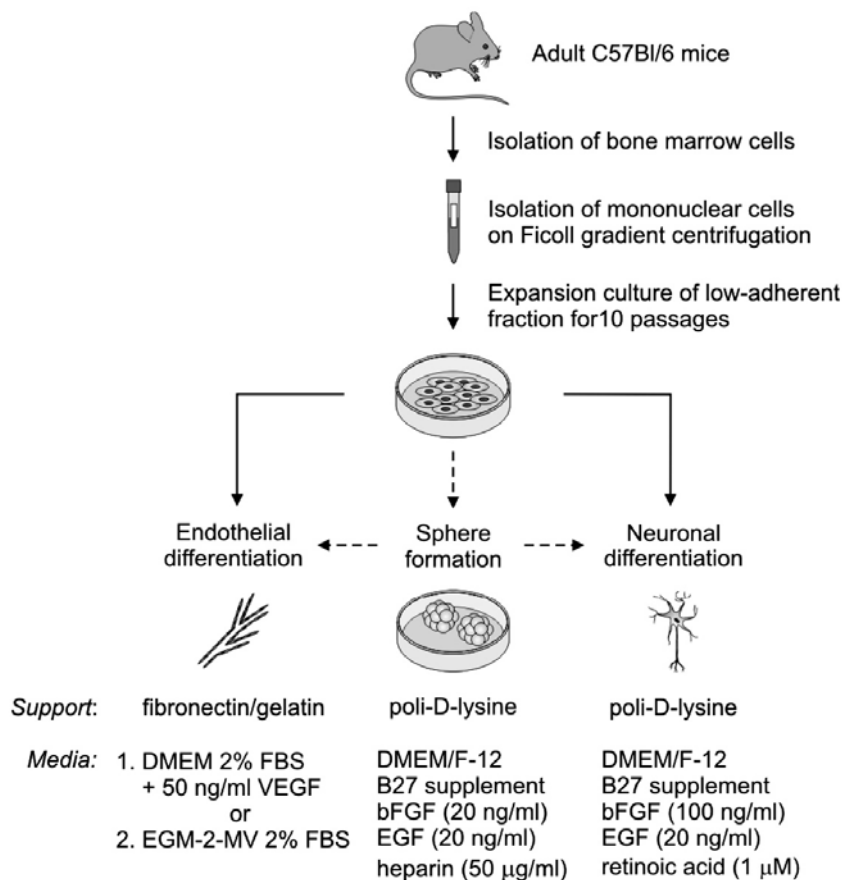


Fig. 1. The experimental scheme. Mononuclear BM-derived cells were isolated from C57Bl/6 adult mice following centrifugation on Ficoll gradient. The adherent fraction of MSC was initially expanded in long-term culture and subsequently differentiated into endothelial and neuronal lineages. Additionally, the ability to sphere formation was evaluated.

during few initial passages. As shown in Fig. 2A, several types of cells exhibiting different morphological characteristics could be distinguished in such cultures in early stages. Moreover, the existence of two cell fractions that differ with dish adherence was noticed. The subpopulation that was still adherent after short trypsinization (see "Materials and Methods") was not capable of being further propagated in long-term culture. Oppositely, due to prolonged propagation of the fraction of cells with low adhesive capacity, we were able to obtain more homogeneous population that exhibited broad plasticity. At passage 10, this population of cells presented more homogenous morphology and retained the spindle-like shape (Fig. 2A), typical for MSC.

The cultured cells were investigated for expression of several markers characteristic for MSC at passages 2 and 10. We checked the transcription of CD14, CD34 and CD45, which have been indicated to be absent on MSC as well as CD29, CD44, CD105 and CD166 which are typical markers expressed by MSC (Phinney and Prockop, 2007; Pittenger et al., 1999). Additionally, the presence of some neuronal (nestin, β III-tubulin) and glial marker (glial fibrillary acidic protein; GFAP) was also investigated.

The cultured adherent cells expressed markers typical for MSC such as CD29, CD44, CD105 and CD166 at both passage 2 and passage 10. Moreover, the cells expressed neuronal and glial markers when examined at these two time points (Fig. 2B). The adherent cells at passage 2 were enriched in a fraction of cells expressing CD14 and CD45 antigens indicating the presence of hematopoietic cells from monocytic/macrophages lineage in such culture (Fig. 2B). Importantly, the cul-

tured cells at passage 10 hardly expressed the hematopoietic marker CD45 and exhibited increased expression of CD34 antigen suggesting their primitive characteristics (Fig. 2B).

The expression of the markers CD14 and CD34 did not fulfill the mentioned general MSC criteria. Nevertheless, spontaneous adipocyte differentiation of the isolated population was observed what proofed its mesenchymal precursor character. The adipocyte cells possessing the typical morphology with fat droplets were detected in high numbers among the low-adherent population on P10, when cultured in confluence for prolonged time (Fig. 2C). In these cultures the expression of PPAR γ was checked as it is critical molecular sign used to characterize adipocyte differentiation (Chawla et al., 1994). We confirmed high expression of PPAR γ in the low-adherent population on P10 where adipocytes were observed, in contrast to low expression in initial culture of bone-marrow derived cells where adipocyte differentiation was not observed (Fig. 2C).

Therefore, we were able to establish the simple pre-plating culture protocol resulting in long-term propagation of BM-derived MSC with primitive features characterized by CD45⁻CD14⁺CD34⁺ phenotype.

Expanded low-adherent BM-derived MSC exhibit endothelial differentiation capacity *in vitro*

In the next step, we investigated the differentiation capacity of the expanded low-adherent MSC by testing their endothelial potential. For this purpose the cells were propagated up to passage 10 and subsequently exposed to growth factors stimulating endothelial differentiation. Two different strategies of endothelial differentiation were used. The first strategy relies on use

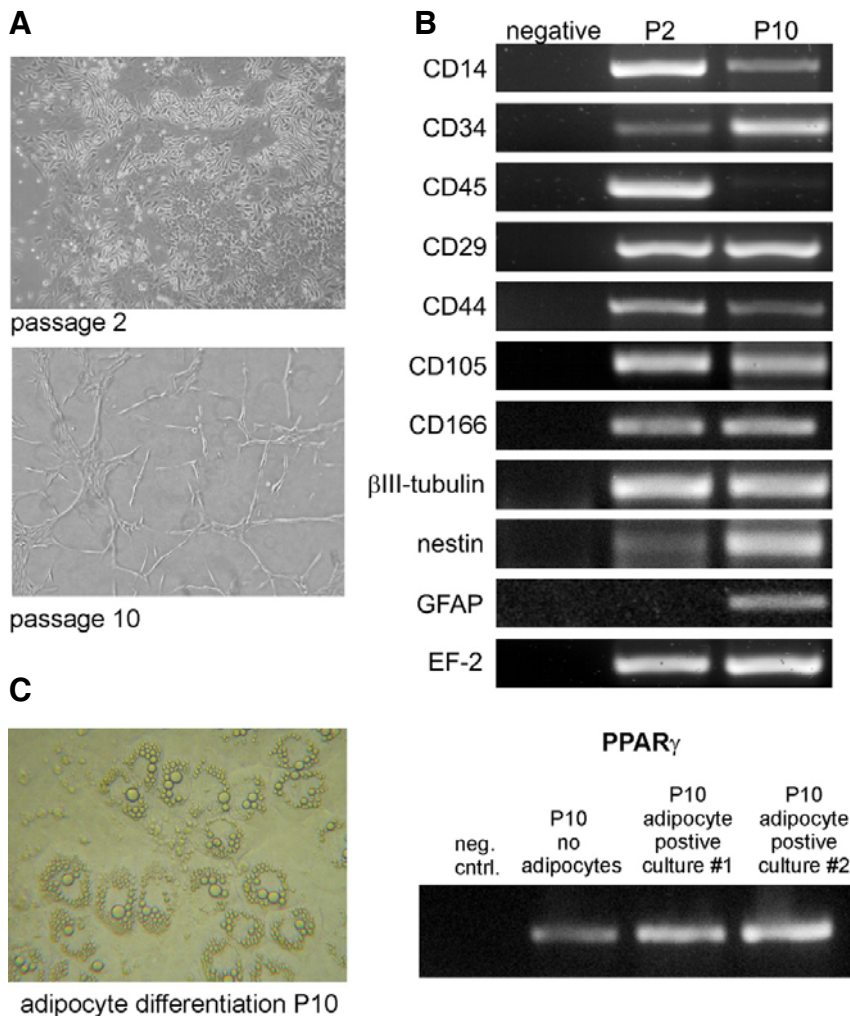


Fig. 2. Phenotype of expanded low-adherent MSC during culture. (A) Morphology of expanded cells under phase-contrast microscope (magnification 100 \times). At passage 2 adherent fraction exhibits significant heterogeneity and contains different types of cells, while at passage 10 cells are more homogeneous and uniform in their morphology. (B) The expression of markers characterizing MSC (CD29, CD44, CD105, CD166), neuronal progenitors (nestin, β III-tubulin, GFAP), hematopoietic cells (CD14, CD45) and primitive cells (CD34) in expanded low-adherent BM-derived cells. All the listed genes were expressed on both passage 2 (P2) and passage 10 (P10), except for CD45 that was present only on P2, and GFAP that was expressed only on P10. EF-2 - housekeeping gene; Negative - a sample without any template served as negative control. (C) Adipocyte morphology of low-adherent population at passage 10 in prolonged confluent cultures. The adipocyte differentiation was confirmed by high expression of PPAR γ in cultures with cells showing the adipocyte morphology compared to initial BM cultures where the adipocyte cells were not observed.

of defined endothelial EGM-2-MV medium with low serum content (2%). The second strategy, originally described by Oswald and co-workers (Oswald et al., 2004) employs culture in DMEM medium with low serum content (2%) and addition of VEGF (50 ng/ml). As a result of both applied differentiation protocols, MSC lost their fibroblast morphology and acquired a cobblestone shape. However, the cells cultured in defined EGM-2-MV medium occurred to be smaller and more rounded than cells cultured in the presence of VEGF, and resembled more endothelial-like phenotype (Fig. 3A).

The MSC differentiated in two distinct media were subsequently examined for the expression of markers characteristic for endothelial cells by employing multiple methods.

MSC express endothelial markers

The expanded and differentiated low-adherent MSC expressed mRNA for VEGFR2, CD31, vWF and CD105 indicating their ability to give rise into endothelial phenotype (Fig. 3B). Moreover, MSC cultured in EGM-2-MV media exhibited greater concentration of mRNA for VEGFR2, CD31 and vWF when compared to MSC cultured in the presence of only the VEGF or to non-differentiated cells (Fig. 3B). Importantly, mRNA levels for VEGFR2, CD31 and vWF highly increased after endothelial differentiation in EGM-2-MV media (9.22 ± 1.59 , 19.02 ± 0.88 and 36.82 ± 19.95 fold difference, respectively), while the ex-

pression of CD105 was not elevated (1.48 ± 0.41 fold difference, respectively) (Fig. 3B). On the other hand, the above mentioned markers were hardly up-regulated when the differentiation with DMEM and VEGF was applied (Fig. 3B).

MSC express VCAM-1 after TNF α stimulation

The expression of VCAM-1 after stimulation with TNF α has been proposed as one of the feature that can help to distinguish endothelial progenitor cells from other cell types (Fernandez Pujol et al., 2000; Ingram et al., 2004).

In the present study, flow cytometric analysis showed basal expression of VCAM-1 on the cells before and after differentiation with both media. The 4 h long incubation with TNF α was enough to enhance expression of VCAM-1 not only in differentiated MSC but also in non-differentiated cells (Fig. 4A). There were no significant differences between cells in either tested conditions.

MSC uptake AcLDL and bind *Bandeiraea simplicifolia* lectins

The phenomenon of the uptake of acetylated low density lipoproteins (AcLDL) has been described for endothelial cells and was successfully applied for their isolation (Pitas et al., 1985). Binding of various lectins to the cellular surface has been also described as another endothelial attribute (Fei et al., 1990).

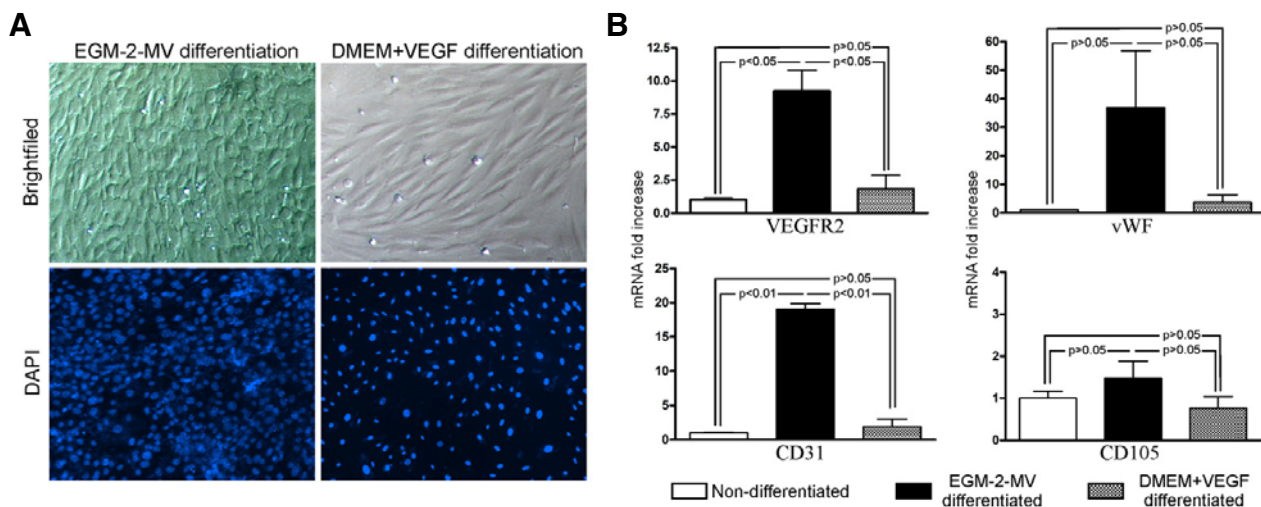


Fig. 3. Endothelial differentiation of expanded MSC. (A) Cell morphology after endothelial differentiation (magnification 200 \times). Additionally, DAPI staining of confluent cultures (magnification 100 \times) evidenced that EGM-2-MV differentiated cells were much smaller and condensed in the culture comparing to the cells differentiated with DMEM+VEGF. (B) Expression of endothelial markers evaluated by real-time RT-PCR following the differentiation of expanded MSC in defined EGM-2-MV medium or DMEM with VEGF, when compared to non-differentiated MSC. The level of expression of endothelial markers was evaluated in two independent representative experiments. Results are presented as mean \pm SEM.

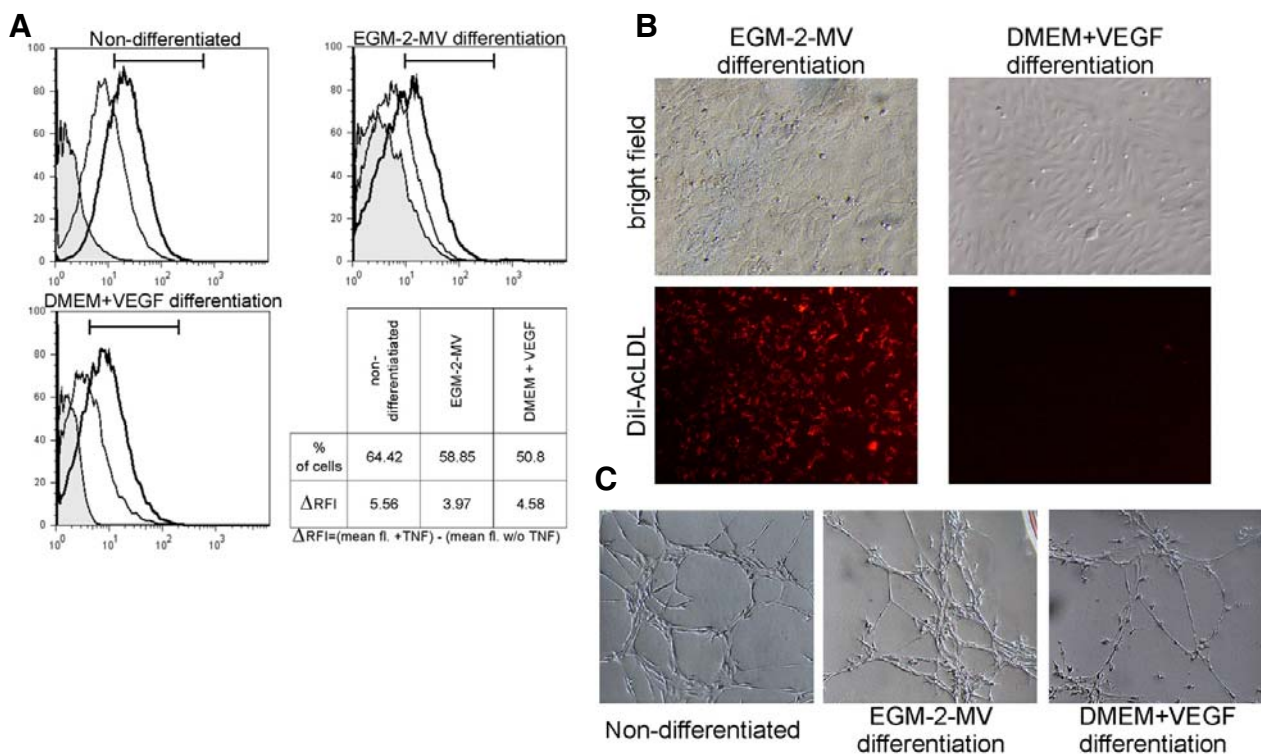


Fig. 4. The endothelial characteristic of non-differentiated and differentiated cells. (A) Flow cytometry analysis of VCAM-1 expression before and after stimulation with TNF α . Curves: gray - isotype control; thin line - VCAM-1 expression without TNF α stimulation; bold line - VCAM-1 expression after TNF α stimulation. (B) Expanded MSC differentiated with EGM-2-MV medium incorporated Dil-AcLDL, in opposite to cells differentiated with DMEM+VEGF (magnification 100 \times). (C) Both the non-differentiated and differentiated cells efficiently formed tubes on matrigel support (magnification 100 \times).

In this study, the uptake of Dil-labeled AcLDL by expanded low-adherent MSC was investigated before and after culture in differentiating conditions. As shown in Fig. 4B, only MSC differentiated in EGM-2-MV medium were capable to uptake AcLDL

exhibiting endothelial properties. Moreover, MSC differentiated accordingly to both culture protocols, in opposite to non-differentiated cells, bound *Bandeiraea simplicifolia* lectins when examined by flow cytometry (data not shown).

Table 2.

	Non-differentiated	EGM-2-MV differentiation	DMEM+ VEGF differentiation
VEGFR2	+/-	++	+/-
vWF	+/-	++	+/-
CD31	+/-	++	+/-
CD105	+	+	+
VCAM after TNFa stimulation	+	+	+
acLDL incorporation	-	+	-
B.s lectin binding	-	+	+
Tube formation on Matrigel	+	+	+

MSC form tubes in matrigel assay

The tube formation test employing the culture of cells on matrigel matrix has been proposed for studying the angiogenic potential of various cells. In this study, both differentiated and non-differentiated MSC showed the capacity of tube formation proving their functional angiogenic potential (Fig. 4C).

In sum, the data confirmed by employing various assays testing the endothelial phenotype and function, indicate that low-adherent MSC expanded in long-term culture preserves their angiogenic capacity *in vitro* (Table 2).

Expanded low-adherent BM-derived MSC exhibit neuronal differentiation potential *in vitro*

The expression of neuronal markers, such as neurofilament heavy, tau, neuroglycan C and tenascin-R, changed during neuronal differentiation of analyzed MSC. The tested genes were significantly up-regulated after differentiation proving the neuronal potential of expanded MSC (Fig. 5A). The mRNA levels for neurofilament heavy and tau protein highly increased after differentiation (27.4 ± 6.8 and 111.2 ± 21.7 fold difference, respectively), while the expression of neuroglycan C and tenascin-R was also elevated, but to a lower extent (3.0 ± 0.4 and 5.7 ± 0.1 fold difference, respectively) (Fig. 5A).

At the same time, the morphological changes of the cells after the neuronal differentiation accompanied the changes in expression of above mentioned genes, as evidenced by more elongated and needle-like cells resembling neurons (Fig. 5B). Moreover, in these cells the neurofilament heavy protein was present as demonstrated by immunohistochemical staining in contrast to non-differentiated cells that were negative for this antigen (Fig. 5C).

These observations suggest that the low-adherent fraction of MSC expanded in our culture system exhibits multipotent differentiation capacity.

The low-adherent BM-derived MSC form spheres/clusters *in vitro*

Interestingly, low-adherent MSC, expanded in the long-term culture exhibited capacity to form spherical structures *in vitro*, when seeded on poly-D-lysine coated dish in the medium designated for neurosphere formation. The process of sphere formation was initiated very early (24 h after initial seeding), when the loosely attached cells started to migrate to form few-cell aggregates (Fig. 6A, day 1). The cells rapidly proliferated and formed larger clusters during next 3-4 days (Fig. 6A, day 3 and

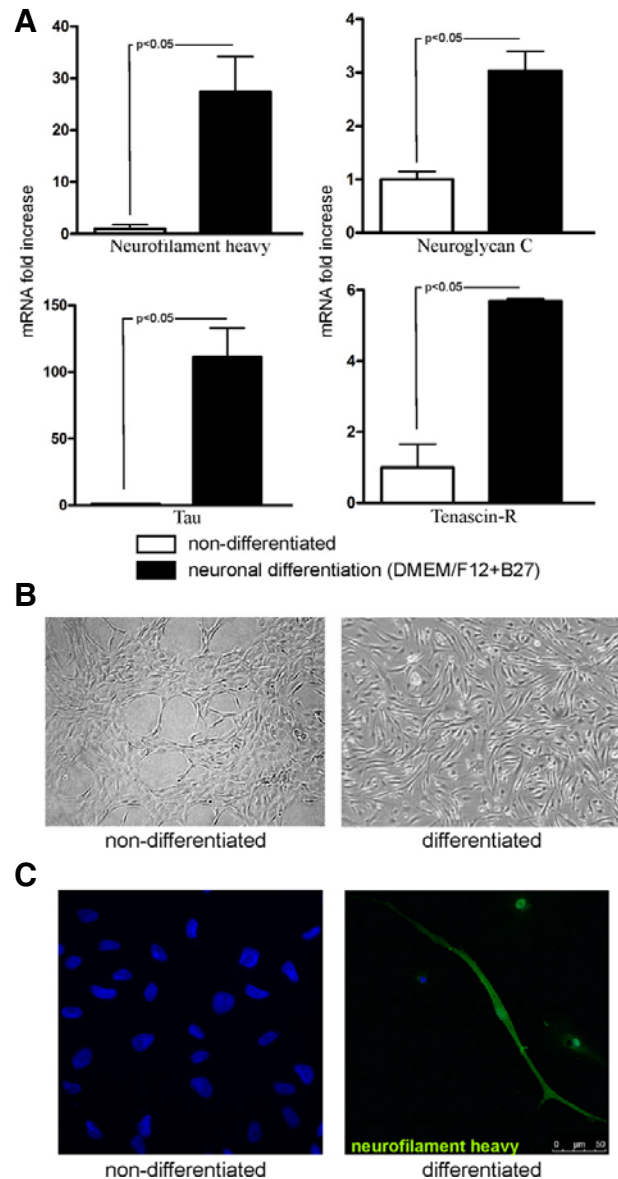


Fig. 5. Neuronal differentiation of expanded MSC. (A) Expression of neuroglycan C, neurofilament heavy, tau and tenascin-R evaluated in expanded MSC after neuronal differentiation by real-time RT-PCR, when compared to non-differentiated MSC. The expression levels of neuronal markers were evaluated in two independent representative differentiation experiments. Results are presented as mean \pm SEM. (B) Morphology of MSC undergoing neuronal differentiation (magnification 100 \times). The morphology of cells changed during culture resulting in elongated, needle-like phenotype of cells after one week of culture in neuronal conditions. (C) Confocal microscopy analysis confirmed the neurofilament heavy protein expression in cells acquiring the neuron-like morphology after neuronal differentiation.

5). Eventually, after about one week, the cultures predominantly contained large round cellular clusters/spheres (Fig. 6A, day 7 and 10) which often detached from the bottom of the plate, floated in the culture medium and fused together during prolonged incubations. Such clusters contained normal living cells

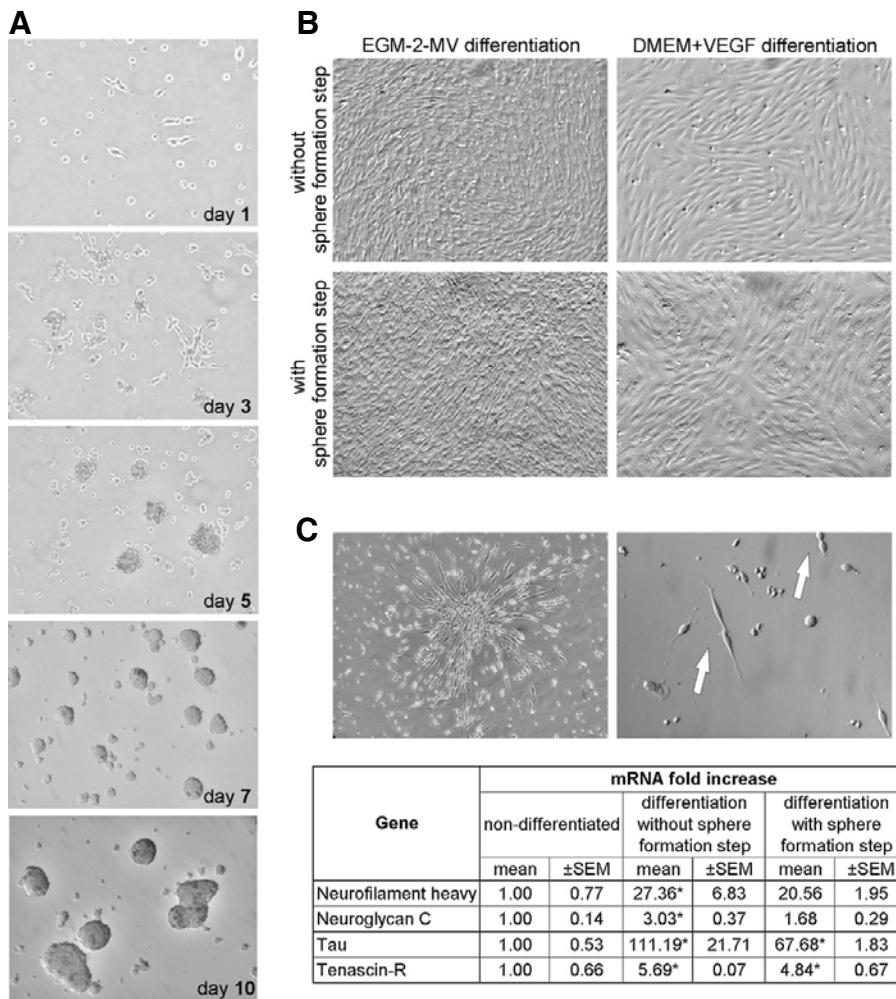


Fig. 6. Sphere formation and differentiation of sphere-derived cells. (A) Process of sphere/cluster formation. At 24 h after seeding few cells adhered to the poli-D-lysine coated dish (day 1). The cells migrated during next 3-4 days forming cellular aggregates initiating spheres (day 3). Rapid proliferation of these cells resulted in increased size of clusters (day 5). Finally, around one week after seeding some of the large clusters spontaneously detached from the culture dish and grew into floating round spheres (day 7). When the culture was prolonged the fusion of floating spheres was observed (day 10) (magnification 100×). (B) Morphology of expanded MSC without or with sphere formation step following endothelial differentiation (magnification 100×). The cells were culture in EGM-2-MV defined medium or DMEM supplemented with VEGF. (C) The neuronal differentiation of MSC with and without the sphere formation step. The cells acquired the elongated, needle-like bipolar morphology (magnification 100×). Proliferating cells are indicated by arrows (magnification 200×). Real-time RT-PCR analysis showed the changes in expression of neuronal markers on mRNA level in differentiated MSC (without and with sphere formation step) when compared to non-differentiated cells. The expression levels of indicated markers were evaluated

in two representative differentiation experiments. Results are presented as mean ± SEM. (*) $p < 0.05$ vs. non-differentiated cells.

capable of forming secondary spheres when dissociated to a single-cell suspension and re-seeded on a new plate. Interestingly, all expanded low-adherent MSC cultured under the sphere formation conditions, uniformly participated in the phenomenon of the clusters formation. The experiment was repeated several times, always leading the MSC to the sphere formation.

Sphere-derived cells preserve the multipotent differentiation capacity

In order to investigate the endothelial differentiation capacity, the cells isolated from spheres/clusters were cultured either in defined EGM-2-MV medium or DMEM supplemented with VEGF as described before. Independently of the applied endothelial differentiation media, the cells derived from spheres became more heterogeneous when compared to the initially expanded population of low-adherent MSC with uniform morphology (Fig. 6B). Moreover, despite the initial differentiation of cells in the endothelial medium, we observed a great decrease in the tube formation ability by the MSC derived from spheres (Fig. 7) when compared to the expanded MSC which did not undergo the sphere formation step (Fig. 7). Interestingly, the MSC derived from spheres/clusters formed on matrigel unorganized

groups of cells which morphologically resembled oligodendrocytes (Fig. 7). These observations indicate diminished endothelial differentiation potential of MSC which were cultured through the sphere formation stage.

Parallely, the neuronal differentiation capacity of MSC derived from spheres was investigated. The cells isolated from such clusters and re-seeded in the culture medium promoting neuronal differentiation changed their morphology to bipolar needle-like shape, resembling neuronal phenotype (Fig. 6C). Moreover, expressions of mRNA for neuronal markers such as neuroglycan C, tenascin-R, neurofilament heavy and tau were increased in the differentiated sphere-derived MSC in comparison to non-differentiated cells (Fig. 6C). However, the up-regulation of neuronal markers was less pronounced in MSC derived from spheres, than in cells which did not undergo the step of sphere formation (Fig. 6C). Thus, it appears that low-adherent MSC cultured through the sphere formation step preserve neuronal capacity, however at a lower level than the expanded MSC propagated without this culture step.

DISCUSSION

In this study, we isolated murine bone-marrow derived primitive

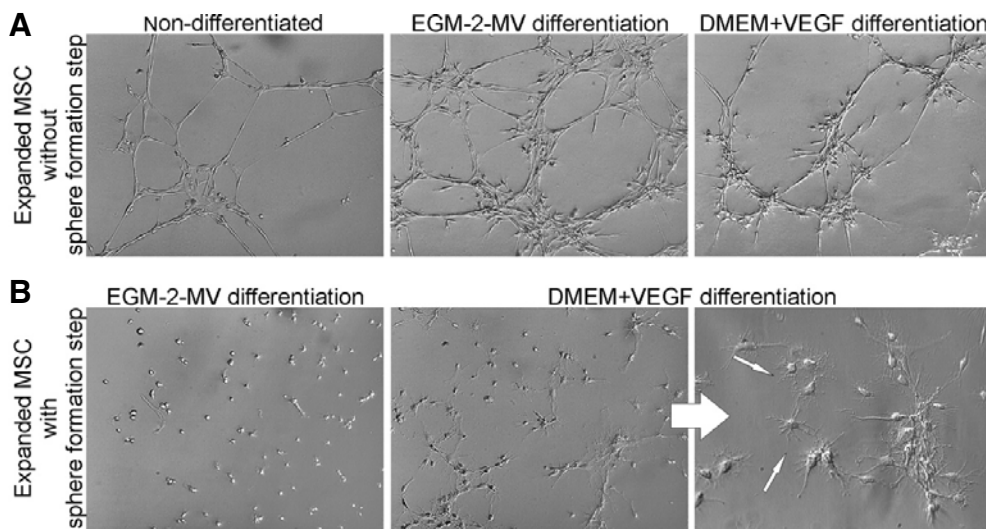


Fig. 7. Tube formation on Matrigel. (A) Both non-differentiated and differentiated MSC (in EGM-2MV medium or DMEM with VEGF) efficiently formed tubes without the sphere formation step (magnification 100 \times). (B) Tube formation by MSC which undergo differentiation in endothelial media following sphere formation step, was not observed (magnification 100 \times). Interestingly, they acquired more neuronal-like morphology indicated by arrows on magnified image (magnification 200 \times).

cell population that retains mesenchymal stem cells. Importantly, this fraction of BM cells exhibited low-adhesive properties and express CD34 marker and monocytic antigen CD14. However, taking into consideration our results and other literature reports, we proposed to classify these cells as subpopulation of MSC, despite the confusing expression of CD14 and CD34. Firstly, they express several MSC positive markers CD29, CD44, CD105, CD166, showed plastic adherence and fibroblast-like morphology. It is also possible that this CD14⁺CD34⁺ subpopulation is initially much more infrequent among the major CD14⁺CD34⁺ MSC population and could be expanded only in long-term culture with the application of the novel protocol. Moreover, there are reports indicating the CD14⁺ population as origin of the MSC (Kuwana et al., 2003), as well as, describing CD34⁺ cells as precursors of human (Kopher et al., 2010) and murine (Kaiser et al., 2007) MSC. Interestingly, the CD34 expression in long-term MSC culture was observed (Li et al., 2008), in addition to report demonstrating higher angiogenic potential of CD34⁺ subpopulation of MSC (Copland et al., 2008), what is altogether consistent with our study. Finally, the spontaneous differentiation to adipocytes confirmed the potential to differentiate also toward mesenchymal lineages, what is intrinsic MSC feature (Pittenger et al., 1999).

The study shows that at initial stages of *in vitro* culture, the adherent fraction derived from BM mononuclear cells contained two distinct subpopulations according to their adhesiveness. The low-adherent subpopulation which was eventually long-term expanded during our study and showed fibroblastic appearance typical for MSC. Importantly, endothelial and neuronal differentiation potential was not only preserved in low-adherent expanded MSC, but was exclusively exhibited by this long-term cultured population with fibroblastic shape and proliferative capacity. It was not possible to expand in long term-culture the cells that showed strong adherence characteristic. Moreover, we did not observe any endothelial or neuronal differentiation in these cultures (data not shown). Thus, highly adherent fraction did not fulfill the experiment criteria being unable to long-term propagation, showed no plasticity and therefore it was excluded from further analysis.

The existence of murine bone marrow cells with different adherence to culture plate was also described by Zhang and colleagues (Zhang et al., 2007). Moreover, the same authors found a CD14⁺ subpopulation of adherent cells able to differentiate into endothelial cells, but only in early stages of culture suggesting their monocytic origin (Zhang et al., 2007).

The obtained results indicate that the expression of CD14 by subpopulation of low-adherent MSC expanded in our study is not only related to the presence of transient, non-proliferating monocytes that mimic endothelial cells and this population is distinct from the cells described by Zhang et al. (2007). The examined low-adherent CD14⁺CD34⁺ MSC population were capable to differentiate into endothelial phenotype as demonstrated at mRNA and functional levels. Simultaneously, the upregulation of neuronal markers was also observed what indicates that the presented population is not only restricted to endothelial phenotype, but possesses broader plasticity. Interestingly, Romagnani and co-workers established the presence of similar double-positive (CD14⁺CD34⁺) fraction of cells, capable to differentiate into both endothelial as well as neuronal cells, in human bone marrow and blood (Romagnani et al., 2005). The cells isolated in this study, with comparable differentiation plasticity and expression of CD14 along with CD34 may potentially represent murine population that corresponds to this described by Romagnani et al. in humans (Romagnani et al., 2005). Moreover, the population of murine cells could be expanded in long-term culture.

The potential of MSC to differentiate toward endothelial cells was previously shown by Oswald and co-workers (Oswald et al., 2004) and confirmed by other groups (Alviano et al., 2007; Gang et al., 2006), although some papers speculate about MSC endothelial differentiation potential (Wang et al., 2008; Zhang et al., 2007). The current study indicates the existence of CD14⁺CD34⁺ subfraction of MSC, that is capable to differentiate to endothelial cells and additionally shows neuronal differentiation.

Different isolation approaches and culture conditions applied by different investigators may explain the discrepancies between the results coming from different studies. We employed

the culture strategy propagating only the low-adherent fraction of MSC in medium with high serum content, whereas majority of other protocols expands whole fraction of adherent BM cells (Beyer Nardi and da Silva Meirelles, 2006; Brunt et al., 2007; Gneccchi and Melo, 2009). This fact may significantly change characteristics of the output population eventually obtained due to prolonged culture.

The effective endothelial differentiation of expanded MSC was observed when EGM-2-MV medium containing several growth factors such as VEGF, bFGF, IGF-1, hydrocortisone, heparin and ascorbic acid, was used in culture. EGM-2-MV medium occurred to be significantly more efficient in promoting endothelial differentiation of expanded low-adherent CD14⁺CD34⁺ MSC than DMEM medium supplemented with VEGF that is widely used culture medium (Alviano et al., 2007; Chen et al., 2009; Oswald et al., 2004). Additionally to morphological changes, the up-regulation of typical endothelial markers such as vWF, VEGFR2 and CD31 occurred only in the MSC which were differentiated in EGM-2-MV medium, but not in DMEM with VEGF. The endothelial potential of expanded low-adherent and differentiated MSC was finally confirmed by the up-regulation of VCAM-1 expression upon TNF α stimulation, incorporation of Dil-labeled AcLDL and binding of *Bandeiraea simplicifolia* lectins that may distinguish the functional endothelial progenitor cells from other cells (Fei et al., 1990; Ingram et al., 2004; Pitas et al., 1985).

Possibly, because of the presence of many growth factors in EGM-2-MV medium, it activates endothelial differentiation more efficiently via molecular signaling pathways involved in endothelial development *in vivo* (Cleaver and Melton, 2003; Jain, 2003).

Interestingly, the low-adherent CD14⁺CD34⁺ fraction of MSC was capable to form spheres/cellular clusters in the specific culture conditions. The sphere formation has been initially described as a feature of neural stem cells (Reynolds and Weiss, 1992), but other reports indicated this phenomenon as appropriate method for isolation of multipotent stem cells from a multitude of sources and differentiating them to various cell lineages (Shiota et al., 2007). Since the CD14⁺CD34⁺ MSC exhibited multipotentiality by differentiating into both endothelial and neuronal phenotype, we believe that the sphere formation may be related to the primitive characteristics of these cells.

Recent study by Shiota and co-workers showed the impact of sphere formation on following differentiation of murine MSC towards several lineages, but not into endothelium (Shiota et al., 2007). Similarly, significant change in differentiation potential of low-adherent MSC after sphere formation was observed in the current study, which, however, included also endothelial differentiation. MSC differentiated towards endothelial cells after sphere formation step lost their ability to form tubes on Matrigel assay, indicating that the sphere formation step decreases the angiogenic differentiation capacity of these cells. Depending on the endothelial differentiation medium, these cells either did not grow at all on Matrigel or acquired the oligodendrocyte-like morphology. However, the neuronal differentiation of MSC in a specific medium was also diminished due to the sphere formation stage as shown by decreased level of expression of neuronal markers. Taking these observations together, the sphere formation step was not beneficial for endothelial and neuronal differentiation of MSC.

Importantly, this work confirmed possible usage of dish adherence criterion to obtain more homogenous and primitive population from initial heterogeneous culture. However, we did not aimed to sort directly the double positive CD14⁺CD34⁺ cells to ensure completely pure population, we observed all the expanded low-adhesiveness restricted cells uniformly react to

applied differentiation conditions.

In summary, we isolated a novel population of MSC with a unique capacity. To our best knowledge, this is the first study showing the isolation and long-term expansion culture conditions for multipotent bone marrow-derived primitive cells that are non-hematopoietic (CD45⁻) and characterized by the presence of typical markers of MSC as well as the expression of CD14 and CD34 antigens. This population possesses endothelial and neuronal differentiation potential *in vitro* which may be decreased by sphere formation.

Such a relatively easily accessible and expandable population of BM-derived cells with the functional endothelial differentiation capacity may be applied for cell-based therapies of vascular diseases in the future. Furthermore, if next studies confirm that the neuronal potential of these cells can be used to obtain truly functional neuronal cells, the possibility for cell-based therapies of neurological disorders can be also considered.

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