FOLIA HISTOCHEMICA
ET CYTOBIOLOGICA
Vol. 46, No. 3, 2008
pp. 307-314

# Adventage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow CD105$^+$ and CD271$^+$ cells

**Danuta Jarocha[1], Ewa Lukasiewicz[1,2], Marcin Majka[1,2]**

[1]Center of Transplantation, University Children's Hospital, Cracow, Poland,
[2]Department of Transplantation, Jagiellonian University Medical College, Cracow, Poland

**Abstract:** Mesenchymal Stem Cells (MSC) are employed in gene and cellular therapies. Routinely MSC are isolated from bone marrow mononuclear cells (MNC) by plastic adherence. Here we compared new isolation strategies of bone marrow MSC including immunodepletion of hematopoietic cells and immunomagnetic isolation of CD105$^+$ and CD271$^+$ populations. Four fractions were obtained: MNC MSC, RosetteSep-isolated MSC, CD105$^+$ and CD271$^+$ sorted MSC. We evaluated i) number of CFU-F colonies, ii) cell phenotype, iii) in vitro differentiation of expanded cells and iv) expression of osteo/adipogenesis related genes. Results: Average number of day 9 CFU-F colonies was the highest for CD271 positive fraction. Real-Time PCR analysis revealed expression of RUNX2, PPARγ and N-cadherin in isolated cells, particularly high in CD271$^+$ cells. Expression of CD105, CD166, CD44, CD73 antigens was comparable for all expanded populations (over 90%). We observed various levels of hematopoietic contamination with the highest numbers of CD45$^+$ cells in MNC-MSC fraction and the lowest in CD105$^+$ and CD271$^+$ fractions. Cells of all the fractions were CD34 antigen negative. Expanded CD105 and CD271 populations showed higher level of RUNX2, osteocalcin, PTHR, leptin, PPARγ2 and aggrecan1 genes except for α1 collagen. After osteogenic differentiation CD105$^+$ and CD271$^+$ populations showed lower expression of RUNX, PPARγ2 and also lower expression of osteocalcin and PTHR than MNC, with comparable α1-collagen expression. Chondrogenic and adipogenic gene expression was higher in MNC. More clonogenic CD105$^+$ and particularly CD271$^+$ cells, which seem to be the most homogenous fractions based on Real-Time PCR and immunostaining data, are better suited for MSC expansion.

**Key words:** MSC - Isolation - CD105$^+$ - CD271$^+$ - Expansion

## Introduction

Mesenchymal stem cells (MSC), or as proposed recently multipotent stromal cells [1], reside in small numbers in bone marrow (BM) and other tissues and have been shown to be relatively easy to expand ex vivo. MSC differentiate *in vitro* and *in vivo* into different connective tissue cells types including osteoblasts, adipocytes, chondrocytes, endothelial or myogenic cells [2-4]. This multidifferentiation potential of MSC raised a clinical interest to employ these cells for regeneration purposes *e.g.*, in Osteogenesis Imperfecta, stroke or heart infarct. MSC lack also MHC-II antigens and possess immunomodulatory properties which enable them, for example to ameliorate GvHD after hematopoietic transplantation [5-7].

Since their first description in the bone marrow by Friedenstein and co-workers [8], MSC have been routinely isolated based on their adherence to plastic. A disadvantage of this isolation strategy is the possibility of hematopoietic contamination and heterogeneity of starting population of adherent fraction of bone marrow mononuclear cells (BMMNC). This, in turn, will involve a development of more effective strategies to purify these cells directly from harvested BMMNC. The exact phenotype of MSC is not known, but as suggested in the recent publications they should be enriched in SSEA$^+$lin-CD45- fractions of BMMNC [9]. Furthermore, the most primitive BM-residing cells such as VSEL stem cells [10], MAPC [11] or MIAMI cells [12] could be precursors of BM-derived non-hematopoietic stem cells - including MSC.

**Correspondence:** M. Majka, Dept. of Transplantation, Polish-American Institute of Pediatrics, Jagiellonian University Medical College, Wielicka Str. 265, 30-663, Cracow, Poland; tel.: (+4812) 6591593, fax: (+4812) 6591594, e-mail: mmajka@cm-uj.krakow.pl


Folia Histochem Cytobiol. 2008:46(3): 307 (307-314)
doi: 10.2478/v10042-008-0046-z

In contrast, the phenotype of MSC expanded/differentiated in *in vitro* cultures from BM-derived precursors is better described and these expanded *ex vivo* cells were found to be Stro-1$^+$, CD105$^+$, CD73$^+$, CD29$^+$, CD166$^+$, CD106$^+$, CD44$^+$, HLA-ABC$^+$, CD90$^+$ and at the same time CD45$^-$, CD34$^-$, CD14$^-$, HLA-DR$^-$ [2,13,14]. They also express receptors for different growth factors such as PDGF-R, EGF-R, IGF-R or NGF-R [15,16].

Based on these phenotypic analysis alternative isolation protocols were proposed to include negative and positive selection strategies or combination of both. Negative isolation strategies are based on enrichment of MSC by depletion of myeloid and lymphoid cells by immunomagnetic sorting [17-19]. In contrast, positive isolation strategies are based on the selection of the cells that express *e.g.* Stro-1, CD105, CD271 or CD34 antigen by immunomagnetic beads [15,20-22] or fluorescence-activated cell sorting (FACS) [23,24]. Recently, a strategy has been proposed to sort double positive population of cells which express Stro-1 and CD34 or SBA and CD34 [24]. Positive immunomagnetic selection could be also combined during single isolation strategy with FACS *e.g.* to isolate Stro-1$^+$CD106$^+$ cells [25] or D7-FIB$^+$ CD45$^+$ and CD271$^+$ cells [26]. Similarly, it has been proposed to deplete immunomagnetically CD45 and CD14 cells before FACS isolation of CD73, CD90 or CD105 positive cells [27].

In this study we have evaluated four fractions: i) non-purified BMMNC, ii) BM enriched for MSC by RosetteSep Isolation Kit, and immunemagnetically selected iii) CD105$^+$ and iv) CD271$^+$ cells for i) their efficacy to form CFU-F colonies, ii) expression of MSC-associated antigens (CD105, CD166, CD44, CD73, CD45, CD34) and genes (*e.g.* RUNX2, PPARγ2, osteocalcin, N-cadherin) and iii) their adipocyto/osteogenic potential *in vitro*.

## Materials and methods

**Bone marrow samples**. Bone marrow samples were aspirated from pelvic bones of healthy donors with their prior consent obtained according to the procedures approved by the Ethics Committee of the Jagiellonian University.

**Isolation of BM-derived MSCs**. Four isolation strategies (Fig. 1) starting from the same amount of bone marrow sample were applied to evaluate whether the way of isolation strategy influences MSC expansion efficacy and differentiation potential of expanded MSCs.

A population enriched in MSC were isolated by negative immunoselection (RosetteSep Isolation Kit, StemCell Technologies Inc., Vancouver, Canada no. 15128) according to manufacturer's instructions. In brief, cells were incubated for 25 minutes with the depletion cocktail of tetrameric antibodies at room temperature. Then the cells were diluted with PBS and isolated by density gradient centrifugation. A desired population, located in the interfase was collected and washed in PBS. To obtain LD-MNCs bone marrow sample was layered on a Lymphocytes Separation Medium (PAA Laboratories GmbH, Goetzis, Austria) [28]. For isolation of CD105 population the LD-MNCs were incubated with magnetic microbeads conjugated to human CD105 monoclonal antibody (Miltenyi Biotec, Germany) for 15 minutes at 4°C, rinsed and placed on a miniMACS column. For isolation of CD271 population the LD-MNCs were incubated with APC-conjugated ME20.4-1.H4 mAb labeling the LNGF receptor (or p75 NTR) (Miltenyi Biotec, Germany) for 10 minutes at 4°C, rinsed, incubated with anty-APC immunomagnetic beads for 15 minutes at 4°C, and placed on a miniMACS column. After isolation the cells were counted and assessed for viability. Their purity was determined by flow cytometry.

**Culture of BM MSCs**. BM mononuclear cells or cells from other fractions were plated into vented 25 cm2 tissue culture flask (Sarstedt, Newton USA) with Dulbecco's-Modified Eagles Medium (DMEM, Sigma-Aldrich Germany D5523) supplemented with 10% FBS (Stem Cell Technologies, Vancouver Canada 06472) and antibiotics (PAA Laboratories GmbH, Goetzis, Austria). Medium plating density were as follows: MNC MSC - 17 × 10$^6$; RosetteSep-isolated MSC - 2.4 × 10$^6$; CD105$^+$ - 0.29 x 10$^6$; CD271$^+$ - 0.22 × 10$^6$ per 25 cm$^2$ flask. Flasks were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ and after 7 days half of the medium was replaced with a fresh one. Than the cells were cultured with a half medium change every week until the fibroblast-like cells at the base of the flask reached confluence. On reaching confluence, the adherent cells were detached using 0.25% trypsin and re-seeded at 1 x 10$^5$ cells per 25 cm$^2$ flask - 4000 per 1 cm$^2$ (first passage). These cells were incubated again until confluence, and were once again trypsinized and re-seeded at 1 × 10$^5$ cells per 25 cm$^2$ flask - 4000 per 1 cm$^2$ (second passage). At the end of second passage when the cells reached confluence, they were trypsinized and either cryo-preserved or used immediately [29].

**Staining of CFU-F colonies**. For an assessment of capability of MSC to form CFU-F colonies cells were cultured 9 days [30]. On day 9 the flasks were rinsed, fixed with methanol and stained with Wright solution and the number of colonies was counted.

**Flow cytometry.** MSC phenotype was analyzed with the panel of mouse mAb specific for MSC - CD73, CD105, CD166 and hematopoietic cells - CD45 (Becton Dickinson). Briefly, to 1×10$^5$ cells suspended in 100 µl of staining buffer (PBS, 2% FBS) 20 µl of mouse mAb was added. Next, the cells were incubated in the dark for 30 min at 4°C. Stained cells were washed, collected using FACSCanto cytometer (Becton Dickinson; USA) and analyzed with FACS Diva software (Becton Dickinson; USA).

**Evaluation of *in vitro* differentiation potential of MSCs.**
**Osteogenic differentiation**. To induce osteogenic differentiation, 5×10$^4$ cultured cells were plated in 24-well plate (Sarstedt, Becton Dickinson) in Nonhematopoietic OsteoDifferentiation Medium (Miltenyi Biotec, Germany, order no.: 130-091-678). Cells were cultured for 30 days with twice weekly refeeds. To demonstrate osteogenic differentiation, the cultures were washed with phosphate-buffered saline, fixed with 4% paraphormaldehyde for 2 min., washed with distilled water, stained with alizaryn red S for 1 min. and finally washed with ethanol in order to assess calcium phosphate deposits.
**Adipogenic differentiation**. To induce adipogenic differentiation, 10×10$^4$ cultured cells were plated in 6-well plate (Sarstedt, Newton USA) in Nonhematopoietic AdipoDifferentiation Medium (Miltenyi Biotec, Germany, order no.: 130-091-677). Cells were cultured for 30 days with twice weekly refeeds. The cells containing lipid vacuoles were observed after 2 or 3 weeks.


Folia Histochem Cytobiol. 2008:46(3): 308 (307-314)
doi: 10.2478/v10042-008-0046-z

**Table 1.** Influence of MSC isolation method on CFU - F number. Bone marrow samples were divided into four portions. Each portion was subjected to MSC isolation with a different isolation strategy and cells were plated to grow CFU-F. Purified fractions were seeded in much lower initial density compared to BMMNC fraction. After 9 days the number of CFU-F colonies was scored under light microscope. Data are presented as a mean value and a range (n=3).

| | Isolation strategy | | | |
|---|---|---|---|---|
| | MNCs | RosetteSep | CD105 | CD271 |
| Number of cells seeded | 17 x $10^6$ (10.3-28) | 2.4 x $10^6$ (1.1-4.2) | 0.29 x $10^6$ (0.08-0.75) | 0.22 x $10^6$ (0.11-0.38) |
| Number of CFU-F colonies / $10^7$ | 25.7 (14.5-41.2) | 49.3 (21.2-97.1) | 104.8 (36.5-221) | 148.1 (55.3-211) |

**RNA Extraction and Reverse Transcription**. Total RNA was extracted using RNeasy Mini Kit (Quiagen) followed by DNAse treatment (Promega). The reverse polymerase transcription was performed using MMLV reverse transcriptase (Invitrogen) according to manufacturer's protocol.

**Quantitative Real-Time PCR Analysis**. Detection of mRNA levels was performed by real-time PCR assay on ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Specific primers-probe set were purchased from Applied Biosystems: N-cadherin Hs00169953_m1, RUNX2 Hs00231692_m1, PPAR$\gamma^2$ Hs01115513_m1, osteocalcin Hs01587814_m1, PTHR Hs00896835_g1, $\alpha^1$-collagen Hs01076780_m1, LEP Hs01084494_m1, LPL Hs01012569_m1, aggrecan Hs01048727_m1). The mRNA expression level for all samples was normalized to the housekeeping gene GAPDH.

**Statistical analysis**. Data for number of cells seeded, number of CFU-F colonies, expression level of chosen antigens are represented by the mean and a range. Data for mRNAs expression level are represented by the mean and standard deviation (SD).

## Results

### *Influence of isolation mode on CFU-F colonies number*

Bone marrow samples (n=3) were divided into two parts (3/4 and 1/4) and processed as described below (Fig. 1). Accordingly, the part containing 3/4 of marrow sample was enriched for BMMNC by Ficoll gradient centrifugation and subsequently the cells harvested from interphase were divided into three equal parts. One equal part of these cells was directly seeded into the culture flask whereas the second and third part were subjected to isolation of $CD105^+$ and $CD271^+$ cells, respectively. The remaining 1/4 of initially harvested BM samples were directly enriched for MSC by employing RosetteSep Isolation Kit.

Based on processing strategy four fractions of BMMNC were obtained: 1) unpurified BMMNC, 2) RossetteSep isolated BMMNC and purified 3) $CD105^+$ and 4) $CD271^+$ cells. These cells were subsequently counted before plating to assess their seeding density. The mean and range of seeding density was for BMMNC 17 × $10^6$ (10.3-28), for RosetteSep 2.4 × $10^6$ (1.1-4.2), for $CD105^+$ 0.29 × $10^6$ (0.08-0.75) and for $CD271^+$ cells 0.22 × $10^6$ (0.11-0.38) (Table 1). On average BMMNC seeding density was 7 times higher than RosetteSep and 58 and 77 times higher than for $CD105^+$ and $CD271^+$ populations, respectively. The four groups of isolated cells were seeded into 25 $cm^2$ flacks and cultured for 9 days.

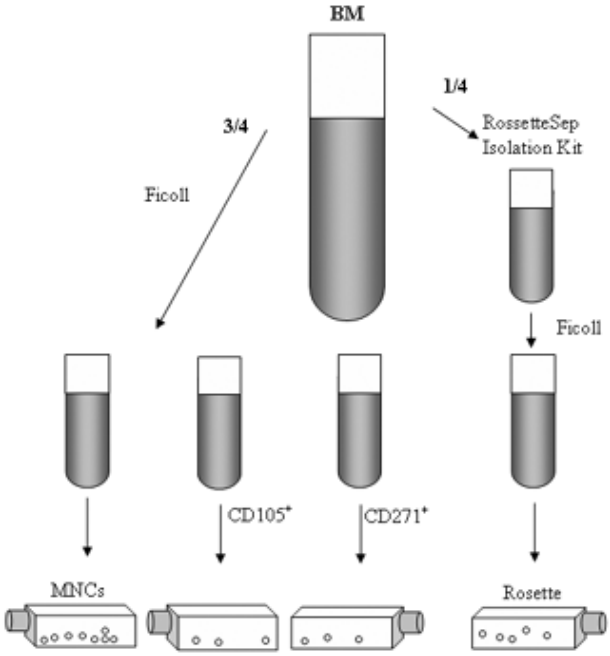


**Fig. 1.** Scheme of MSC isolation. Whole bone marrow aspirates were divided into two parts (3/4 and 1/4). Smaller part was incubated with RossettSep depletion (StemCell Technologies Inc. Canada) and subsequently MSC were isolated by density centrifugation. From larger part BMMNC were isolated by density centrifugation. Next, mononuclear cells were divided into 3 parts. One part was directly seeded into culture flasks and from other two parts CD105 and CD271 positive cells were isolated using immunomagnetic beads (Miltenyi Biotec, Germany)

We noticed that the $CD105^+$ and $CD271^+$ cells isolated by immunomagnetic beads yielded the highest number of CFU-F colonies (Table 1). Accordingly, the number of CFU-F colonies was 3-4 times higher as compared to unpurified BMMNC. Interestingly, CFU-F formed by purified CD271 and CD105 proliferated most rapidly and the cultures initiated by these cells became confluent faster in comparison to unpurified BMMNC or RosetteSep-isolated MSC (data not shown).


Folia Histochem Cytobiol. 2008:46(3): 309 (307-314)
doi: 10.2478/v10042-008-0046-z

**Table 2.** Characterisation of MSC obtained by different isolation strategies. Cells were cultured for three passages and antigen expression was determined by FACS analysis. Data are presented as a mean value and a range (n=3).

| Isolation strategy | Expression level of chosen antigens | | | | |
|---|---|---|---|---|---|
| | CD105 | CD166 | CD73 | CD44 | CD45 |
| MNCs | 91.3 (82-97) | 87 (79-92) | 90.8 (87-93.5) | 79 (70-88) | 7.2 (5-9) |
| RosetteSep | 92.8 (90.3-95) | 87 (81.6-90) | 89.3 (83-98) | 86.5 (80-95) | 6.4 (0.1-15) |
| CD105 | 90.3 (83-99) | 95.7 (94-97) | 97.9 (96.8-99) | 89.7 (86-95) | 6.1 (0.1-16) |
| CD271 | 91 (83-99) | 95.7 (91-99) | 97 (94-99) | 91.7 (88-95) | 1.6 (0.3-3) |

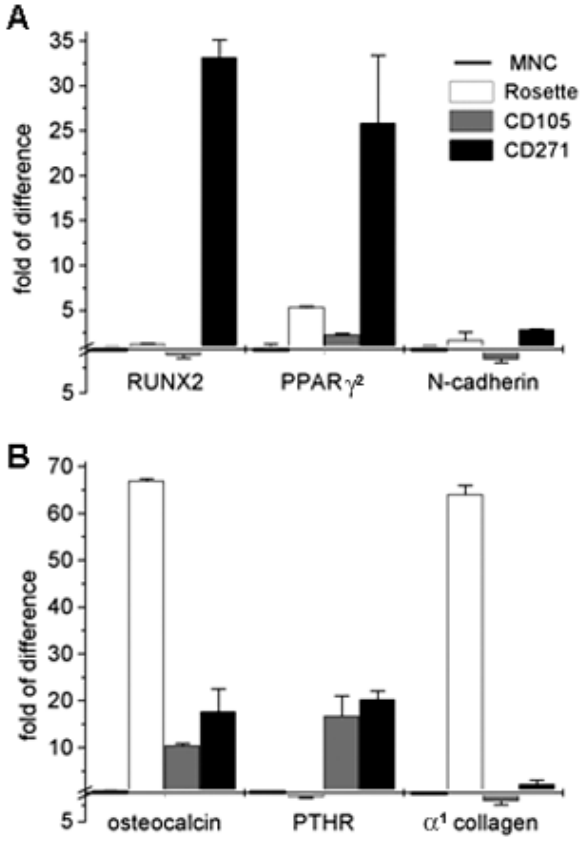


**Fig. 2.** RQ-PCR analysis of mRNA expression for osteo, adipo and chondrogenic genes on freshly isolated cells. **A**. early osteo and adipogenesis related genes. **B**. late osteo and adipogenesis related genes. A representative result is shown out of two independent experiments.

## *Quantification of osteogenic, adipogenic and chondrogenic gene expression in freshly isolated cell fractions*

We employed real time RT-PCR to study expression of early (RUNX2, PPARγ2) and late (osteocalcin, PTHP, α1collagen, aggrecan1, LEP) osteogenic, adipogenic and chondrogenic genes in freshly isolated cell fractions. Figure 2 panel A shows that the expression of RUNX2 was the highest in purified CD271$^+$ cells. The CD271$^+$ and RosetteSep$^+$ cells displayed also the highest expression of N-cadherin. Furthermore, CD271, RosetteSep and CD105 cells expressed mRNA for PPARγ at higher level than BMMNC. BMMNC expressed also the lowest level of late osteogenic genes (Fig. 2 panel B). Worthy of note is the fact that we did not detect expression of aggrecan1 and LEP in freshly isolated cell fractions (the data not shown).

## *Phenotypic analysis of CFU-F-derived cells*

All four cell fractions were plated to grow CFU-F and subsequently CFU-F derived MSC were expanded during three passages. For analysis of MSC-related cell surface markers expression the cells were harvested and stained with antibodies against CD73, CD105, and CD166. CFU-F derived cells were also stained for expression of hematopoietic antigen CD45. Table 2 shows that expression pattern of evaluated MSC markers between cells from all four fractions was similar. Some of CFU-F derived cells expanded from BMMNC, RosetteSep and CD105$^+$ selected cells expressed CD45 antigen, and the cells isolated with anti CD271 antibodies were highly depleted from contaminating CD45$^+$ hematopoietic cells (Table 2).

## *Osteogenic and adipogenic differentiation potential of MSC*

CFU-F cells expanded for three passages were subsequently plated in the cultures promoting osteogenic and adipogenic differentiation and after 30 days stained with Alizarin S and Oil Red to visualize calcium-phosphate deposits and presence of lipid droplets, respectively. As shown in Figure 3 no differences in

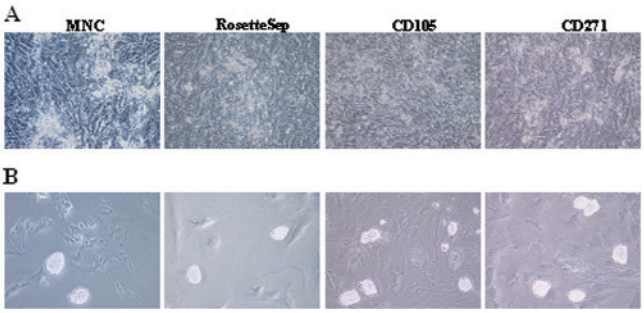


**Fig. 3.** Assessment of *in vitro* differentiation potential. Expanded cells after the third passage were differentiated for 30 and 20 days, into osteo- or adipocytic lineage respectively. Calcium phosphate deposits and presence of lipid droplets were evaluated microscopically (magnification ×400).

differentiating potential were observed among MSC cells obtained through different isolation procedures.

### *Osteogenic and adipogenic mRNA expression pattern in expanded and differentiated toward osteogenesis MSC*

The analysis of gene expression profile performed on expanded for three passages MSC revealed differences in expression of early osteogenic (RUNX2) and adipogenic (PPARγ2) genes. Accordingly, MSC expanded from CD105$^+$ and CD271$^+$ cells displayed a higher expression of RUNX2 and PPARγ2 as compared to MSC derived from BMMNC and RossetteSep cells (Fig. 4 panel A). Osteocalcin, PTHR and α1collagen were expressed at higher level in MSC-expanded from RossetteSep-, CD105$^+$- and CD271$^+$-derived cells as compared to BMMNC-derived MSC (Fig. 4 panel B). Similarly, these fractions exhibited higher expression of aggrecan1 and LEP (Fig. 4 panel C).

After osteogenic differentiation of MSC we observed only small differences in gene expression profiles (Fig. 5).

Accordingly, the levels of mRNA for early osteogenic RUNX2 and early adipogenic PPARγ2 (Fig. 5 panel A) as well as late osteogenic osteocalcin and PTHR (Fig. 5 panel B) and late chondrogenic aggrecan1 (Fig. 5 panel C) genes were comparable with differences oscillating around two folds between the fractions.

The only higher difference was observed for CD271$^+$- and RosetteSep-derived MSC which presented lower expression of mRNA for LEP as compared to MSC expanded from BMMNC and CD105$^+$ cells (Fig. 5 panel C).

## Discussion

Since their initial discovery, MSC isolation procedure is still based on their plastic adherence properties [8,29,31,32]. In this study we compared side by side

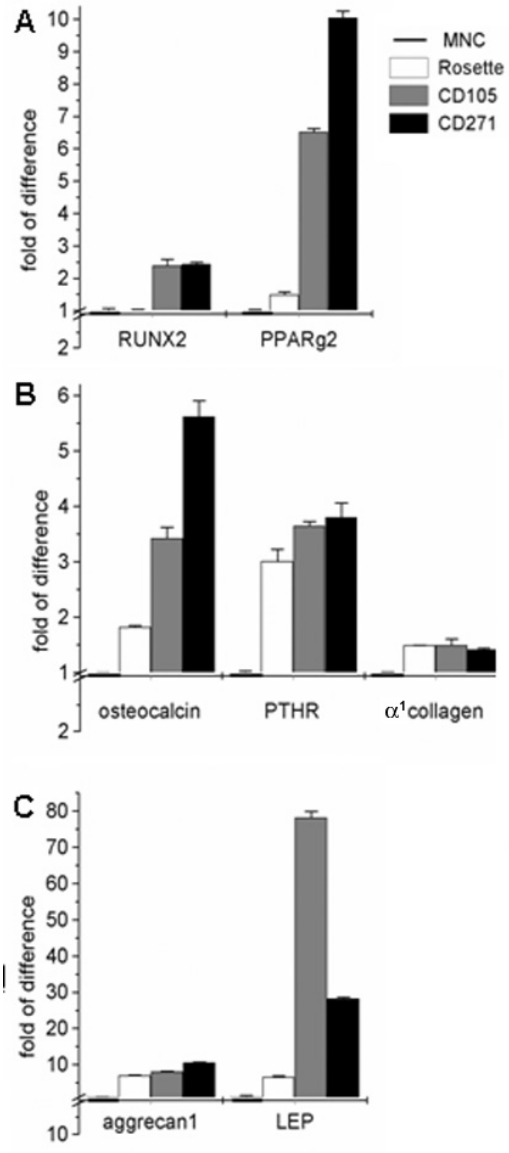


**Fig 4.** RQ-PCR analysis of mRNA expression for osteo, adipo and chondrogenic genes after three passages. Panel A - early osteo and adipogenesis related genes. Panel B - late osteo and adipogenesis related genes. A representative result is shown out of two independent experiments.

expansion and differentiation efficacy of MSC isolated by employing three different commercially available isolation strategies to adherence based protocol. Our data reveal that MSC purification using various isolation strategies results in different proliferation capacity of these cells and different patterns of gene expression.

Positive selection strategies based on use of CD105 and CD271 antibodies yielded four and five times more CFU-F colonies respectively in comparison to BMMNC and population enriched for MSC by negative selection. Consequently, cultures started from CD105$^+$ and CD271$^+$ cells reached confluence and proliferated faster than those initiated from BMMNC


Folia Histochem Cytobiol. 2008:46(3): 311 (307-314)
doi: 10.2478/v10042-008-0046-z

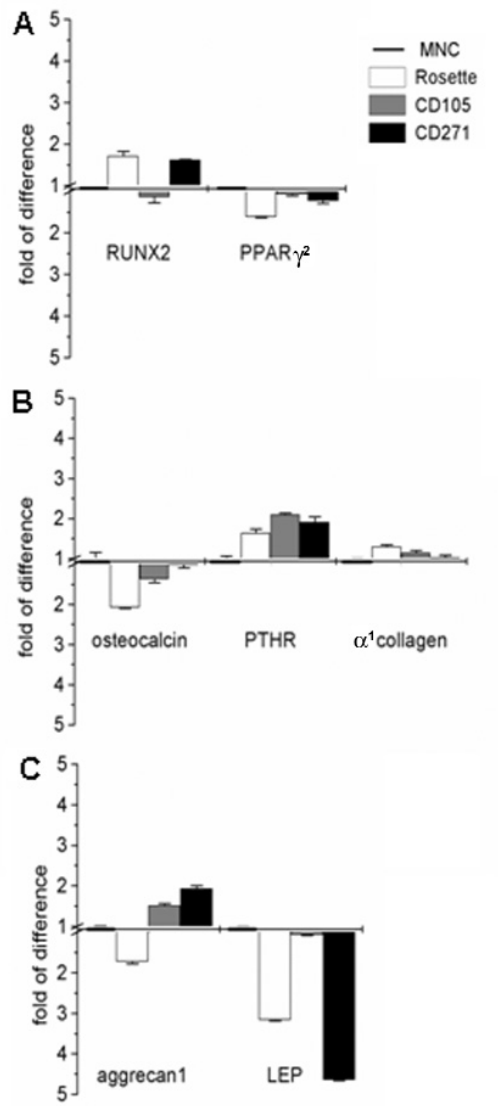


**Fig 5.** RQ-PCR analysis of mRNA expression for osteo, adipo and chondrogenic genes after osteodifferentiation process. Panel A - early osteo and adipogenesis related genes. Panel B - late osteo and adipogenesis related genes. A representative result is shown out of two independent experiments.

or cells enriched by RosetteSep Isolation Kit. The possible explanation for this kind of difference is the fact that when cultured without accompanying hematopoietic cells present in MNC and RosetteSep fractions, proliferation potential of CD105 [20,27,33] and CD271 cells [22,26] is unlashed. This suggests that the growth of these cells is inhibited by hematopoietic cells present in MNC and RosetteSep fractions. It is also possible that lower seeding density is advantageous in MSC expansion. Such phenomenon was already described [35,36]. Positively isolated fractions were seeded at much lower density than MNC, which may be the reason for observed higher number of CFU-F colonies. Furthermore, in MSC cultures started from BMMNC we sometimes observed appearance of round, strongly proliferated cells attached to adherent cells, which transferred with MSC to the next passage. Such cultures usually stopped proliferating. At the same time such phenomenon was not observed in cultures started from other fractions isolated from the same bone marrow sample

Most of the data on MSC are obtained after prolonged culture *in vitro*. Thus, we also cultured purified cells for three passages to perform further evaluation of each fraction. Phenotype evaluation revealed that $CD271^+$ population contained the lowest hematopoietic contamination as judged by presence of CD45 positive cells. Also CD105 fraction has on average lower number of $CD45^+$ cells in comparison to MNC and RosetteSep populations. Lack of hematopoietic contamination persisting until the third passage might be at least in part responsible for observed faster $CD271^+$ and $CD105^+$ populations' proliferation.

In addition to the highest number of CFU-F colonies observed in $CD271^+$ fraction, these cells, in comparison to other populations, had higher expression level of RUNX2 and PPARγ2, transcription factors pivotal in the early differentiation of bone, and adipose tissues [37-39].

Freshly isolated CD271 cells and RosetteSep cells also highly expressed mRNA for N-cadherin. At the same time CD105 cells seem to be depleted of N-cadherin expressing cells when compared to MNC. Presence of N-cadherin positive cells in CD271 and RosetteSep fractions is intriguing in the light of recent reports showing that spindle-shaped, $N\text{-cadherin}^+$ osteoblasts (SNO) form niches supporting hematopoietic cells development [40]. Preservation or even enrichment of SNO precursor cells before subsequent transplantation could be of great importance for reconstitution of hematopoietic niches after myeloablative therapies which negatively affect bone marrow microenvironment [41-43].

Evaluation of gene expression profile of MSC after third passage, but before differentiation, revealed that differences in expression of early and late osteogenic, adipogenic and chondrogenic genes are still present; however, they are much lower than those observed for freshly isolated cells. Cultures started from $CD271^+$ and $CD105^+$ populations have higher enrichment in molecular markers of early osteo and adipo progenitors. Similarly, these populations have higher enrichment in late molecular markers for osteogenic progenitors (based on osteocalcin and PTHR level). Molecular markers of late adipo (LEP) and chondrogenic (aggrecan1) progenitors are expressed at higher level in enriched fractions when compared to MNC. In the light of these observations we conclude that differences between initial populations, although still seen after third passage, decrease during culture.


Folia Histochem Cytobiol. 2008:46(3): 312 (307-314)
doi: 10.2478/v10042-008-0046-z

Osteogenic and adipogenic differentiation potential of MSC expanded from MNC, RossetteSep, CD105 or CD271 populations was already demonstrated [18,20-22,26] but no parallel comparison of these cell fractions was reported. In this study we show that osteo and adipogenic differentiation potential of expanded cells, as assessed by microscopic evaluation of calcium-phosphate deposits and lipid droplets appearance, did not differ between fractions.

Evaluation of gene profile analysis after osteogenic differentiation process confirmed microscopic observation. Level of molecular markers for early and late osteo, adipo and chondrogenic progenitors showed only small differences, which probably did not influenced osteodifferentiation process. The only differences in higher expression (5 folds and 3 folds) were observed for LEP mRNA expression for $CD271^+$ and RosetteSep populations when compared to MNC and $CD105^+$ cells.

Our data indicate that isolation strategy based on CD271 expression results in obtaining a cell population which is highly enriched for molecular markers of early osteo and adipo progenitors. Furthermore, this strategy of bone marrow MSC purification led to the highest number of CFU-F colonies after 9 days of culture. Cultures started from cells isolated based on CD271 expression after three passages contained considerably lowest hematopoietic contamination in comparison to cultures started from MSC isolated by other strategies. Populations of these cells after three passages retained the highest level of molecular markers for early osteo and adipo progenitors and together with cells of other alternatively isolated populations expressed higher level of molecular markers for late osteo, adipo and chondrogenic progenitors.

**Acknowledgments:** This study was supported by research grant PZB-KBN 2/P05C/029/26 of the Polish State Committee of Scientific Research.

## References

[ 1] Keating A. Mesenchymal stromal cells. *Curr Opin Hematol.* 2006;13:419-425.
[ 2] Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143-147.
[ 3] Goshima J, Goldberg VM, Caplan AI. The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. *Clin Orthop.* 1991;269: 274-283
[ 4] Fibbe W. Mesenchymal stem cells. A potential source for skeletal repair. *Ann Rheum Dis.* 2002;61(Suppl II):ii29-ii31.
[ 5] Smits AM, van Vliet P, Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. *J Cell Mol Med.* 2005;9:25-36.
[ 6] Horwitz EM, Prockop DJ, Fitzpatrick LA *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med.* 1999;5:309-313.
[ 7] Bobis S, Jarocha D, Majka M. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem Cytobiol.* 2006;44:215-230.
[ 8] Friedenstein A.J, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* 1976;4:267-274.
[ 9] Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood.* 2007;109:1743-1751.
[10] Kucia M, Reca R, Campbell FR *et al.* A population of very small embryonic-like (VSEL) $CXCR4(^+)SSEA\text{-}1(^+)Oct\text{-}4^+$ stem cells identified in adult bone marrow. *Leukemia.* 2006;20:857-869.
[11] Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol*. 2002;30:896-904.
[12] D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci.* 2004;117:2971-2981.
[13] Conget PA, Minguell JJ (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol.* 1999;181:67-73.
[14] Haynesworth SE, Baber MA, Caplan A. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone.* 1992;13:69-80.
[15] Gronthos S, Simmons P. The Growth Factor Requirements of STRO-1 - Positive Human Bone Marrow Stromal Precursors Under Serum-Deprived Conditions *in vitro*. *Blood.* 1995;85: 929-940.
[16] Thomson TM, Rettig WJ, Chesa PG, Green SH, Mena AC, Old LJ. Expression of human nerve growth factor receptor on cells derived from all three germ layers. *Exp Cell Res.* 1998; 174:533-539.
[17] Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood.* 2001;98:2615-2625.
[18] Tondreau T, Lagneaux L, Dejeneffe M *et al.* Isolation of BM mesenchymal stem cells by plastic adhesion or negative selection: phenotype, proliferation kinetics and differentiation potential. *Cytotherapy.* 2004;6:372-379.
[19] Lagar'kova MA, Lyakisheva AV, Filonenko ES, *et al.* Characteristics of human bone marrow mesenchymal stem cells isolated by immunomagnetic selection. *Bull Exp Biol Med.* 2006;141:112-116.
[20] Majumdar MK, Banks V, Peluso DP, Morris EA. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *J Cell Phys.* 2000;185:98-106.
[21] Roura S, Farre J, Soler-Botija C *et al.* Effect of aging on the pluripotential capacity of human $CD105^+$ mesenchymal stem cells. *Eur J Heart Fail.* 2006;8:555-563.
[22] Quirici N, Soligo D, Bossolasco P, Servida F, Lumini C, Lambertenghi deliliers G. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol.* 2002;30:783-791.
[23] Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood.* 1991;78:55-62.
[24] Simmons PJ, Torok-Storb B. CD34 Expression by Stromal Precursors in Normal Human Adult Bone Marrow. *Blood.* 1991;11:2848-2853.
[25] Gronthos S, Zannettino AC, Hay SJ *et al.* Molecular and cellular characterization of highly purified stromal stem cells derived from human bone marrow. *J Cell Science*. 2003;116:1827-1835.

[26] Jones EA, Kinsey SE, English A *et al.* Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum.* 2002;46:3349-3360.
[27] Boiret N, Rapatel C, Veyrat-Masson R *et al.* Characterization of nonexpanded mesenchymal progenitor cells from normal adult human bone marrow. *Exp Hematol.* 2005;33:219-225.
[28] Friedenstein AJ, Gorskaja U, Kalugina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* 1976;4:267-274.
[29] Angelopoulou M, Novelli E, Grove JE *et al.* Cotransplantation of human mesenchymal stem cells enhances human myelopoiesis and megakaryocytopoiesis in NOD/SCID mice. *Exp Hematol*. 2003;31:413-420.
[30] Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. *Vet Surg.* 2006;35:601-610.
[31] Maccario R, Podesta M, Moretta A, *et al.* Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of $CD4^+$ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica.* 2005;90:516-525.
[32] Tolar J, Nauta AJ, Osborn MJ *et al.* Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells.* 2007;25:371-379.
[33] Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br J Haematol.* 2003;121:368-374.
[34] Aslan H, Zilberman Y, Kandel L. Osteogenic differentiation of noncultured immunoisolated bone marrow-derived $CD105^+$ cells. *Stem Cells.* 2006;24:1728-1737.
[35] Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells*. 2006;24:462-471.
[36] Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci USA.* 2000;28:3213-3218.
[37] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.* 1997;89:747-754.
[38] Nakashima K, Zhou X, Kunkel G *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 2002;108: 17-29.
[39] Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell.* 1994;79:1147-1156.
[40] Yin T, Li L. The stem cell niches in bone. *J Clin Invest.* 2006;116:1195-1201.
[41] Corazza F, Hermans C, Ferster A, Fondu P, Demulder A, Sariban E. Bone marrow stroma damage induced by chemotherapy for acute lymphoblastic leukemia in children. *Pediatr Res.* 2004;55:152-158.
[42] Li J, Law HK, Lau YL, Chan GC. Differential damage and recovery of human mesenchymal stem cells after exposure to chemotherapeutic agents. *Br J Haematol*. 2004;127:326-334.
[43] Schwartz GN, Warren MK, Rothwell SW *et al.* Post-chemotherapy and cytokine pretreated marrow stromal cell layers suppress hematopoiesis from normal donor $CD34^+$ cells. *Bone Marrow Transplant.* 1998;22:457-468.

*Submitted: 20 April, 2008*
*Accepted after reviews: 2 May, 2008*


Folia Histochem Cytobiol. 2008:46(3): 314 (307-314)
doi: 10.2478/v10042-008-0046-z