Mesengenic Differentiation: Comparison of Human and Rat Bone Marrow Mesenchymal Stem Cells

Arianna Scuteri1,3,*
Elisabetta Donzelli1,3,*
Dana Foudah1,*
Cristina Caldara1
Juliana Redondo1
Giovanna D’Amico2
Giovanni Tredici1,3
Mariarosaria Miloso1,3

1Dipartimento di Chirurgia e Medicina Traslazionale, Università Milano-Bicocca, via Cadore,
2Centro Ricerca Tettamanti, Clinica Pediatrica, Università Milano-Bicocca, Monza,
3NeuroMi, Milan Center for Neurosciences, Milano, Italy

Background and Objectives: Cellular therapies using Mesenchymal Stem Cells (MSCs) represent a promising approach for the treatment of degenerative diseases, in particular for mesengenic tissue regeneration. However, before the approval of clinical trials in humans, in vitro studies must be performed aimed at investigating MSCs’ biology and the mechanisms regulating their proliferation and differentiation abilities. Besides studies on human MSCs (hMSCs), MSCs derived from rodents have been the most used cellular type for in vitro studies. Nevertheless, the transfer of the results obtained using animal MSCs to hMSCs has been hindered by the limited knowledge regarding the similarities existing between cells of different origins. Aim of this paper is to highlight similarities and differences and to clarify the sometimes reported different results obtained using these cells.

Methods and Results: We compare the differentiation ability into mesengenic lineages of rat and human MSCs cultured in their standard conditions. Our results describe in which way the source from which MSCs are derived affects their differentiation potential, depending on the mesengenic lineage considered. For osteogenic and chondrogenic lineages, the main difference between human and rat MSCs is represented by differentiation time, while for adipogenesis hMSCs have a greater differentiation potential.

Conclusions: These results on the one hand suggest to carefully evaluate the transfer of results obtained with animal MSCs, on the other hand they offer a clue to better apply MSCs into clinical practice.

Keywords: Mesenchymal Stem Cells, Human, Rat, Osteogenic Differentiation, Adipogenic Differentiation, Chondrogenic Differentiation

Introduction

Mesenchymal Stem Cells (MSCs) are undifferentiated multipotent stem cells residing mainly in the bone marrow but also in different adult tissues (1-4), with the function of replacing dying cells and preserving tissue homeostasis. Under appropriate culture conditions, MSCs are able to differentiate in vitro into mesengenic lineages (osteogenic, chondrogenic and adipogenic), but also to transdifferentiate into cells of nonmesodermal origin such as hepatocytes (5) and neural cells (6).

Recently, MSCs have been proposed as offering a prom-
ising therapeutic approach in regenerative medicine and tissue engineering because of their favorable biological characteristics: 1) MSCs can be easily isolated and expanded in vitro (7); 2) after in vivo administration MSCs can migrate to the sites of injury (8); 3) MSCs can be used autologously and their hypo-immunogenicity makes them suitable also for allogenic transplantation (9). Furthermore, other mechanisms could contribute to the therapeutic benefits of MSCs such as their immunoregulatory properties and their capacity to secrete a broad spectrum of bioactive macromolecules (10). Several preclinical and clinical studies have confirmed the therapeutic potential of MSCs and a number of phase I/II and III clinical trials have recently been completed or are underway (please see http://clinicaltrials.gov).

However, literature on MSCs often reported controversial results, partly due to different culture conditions, but surely also due to the intrinsic variability existing both between MSCs derived from different species, and also among donors/strains of the same species.

The different behavior between MSCs of human and non-human origin may be an hurdle, since animal models are used as first approach to study the feasibility of MSCs for treatment of human diseases. In particular, several studies have set up isolation and differentiation protocols to allow the use of rodent MSCs into transgenic animal models (11, 12).

Gene expression profiling of rodent bone marrow-derived MSCs compared with hMSCs has revealed a high degree of concordance (13, 14), but in spite of the shared expression of some common surface antigens such as CD29, CD90, CD105, used for MSC characterization, rat MSCs (rMSCs) and human MSCs (hMSCs) essentially differ for what concerns the genomic stability (15, 16), the spontaneous expression of neural markers (17, 18), and the immunoregulatory capacities (19), and therefore the translation of the results has not a foregone conclusion, since the biological analogies between different species are not sufficient to directly shift the results obtained in the different studies, and to better apply it in clinic.

**Materials and Methods**

When not otherwise indicated, materials were purchased from Sigma Co, Saint Louis, MO, USA.

**MSCs' isolation and culture**

rMSCs (p4-8) were collected according to a previously published protocol (20) and all animal procedures were conducted in accordance with the European Communities Council Directive 86/609/EEC. In brief, bone marrow was flushed from the femurs and tibias of 10-week old Sprague Dawley rats (n=5; Harlan, Udine, Italy) and red blood cells were lysed. The remaining cells were plated in 75 cm² culture flasks in culture medium, consisting of α-MEM (Lonza Group Ltd., Basel, Switzerland) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml fungizone and with 20% Fetal Bovine Serum (FBS, Hyclone, Logan, UT). All supplements were purchased from Lonza.

hMSCs (p4-8) were isolated after patients’ understanding and informed consent from aliquots of heparinized bone marrow obtained in excess from individuals undergoing marrow harvest for allogenic transplantation at the San Gerardo Hospital (n=5; Monza, Italy). In brief, mononuclear cells were collected after centrifugation in a Ficoll-Hypaque gradient and seeded 160.000 cells/cm² into culture flasks in culture medium, consisting of DMEM (Lonza) supplemented with 2 mM L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml fungizone and with 10% FBS.

rMSC and hMSC cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. After 48 hours the non-adherent cells were removed and, subsequently, the medium was changed every 3∼4 days. When rMSC and hMSC cultures respectively reached confluence, cells were detached by trypsinization and used in experiments.

**MSC differentiation**

hMSCs and rMSCs were analyzed for their capacity to differentiate towards osteogenic, adipogenic and chondrogenic lineages using specific protocols. hMSCs and rMSCs cultured in medium without any differentiating agent were used as a control.

**Osteogenic differentiation**

Cells were seeded at approximately 3,500∼4,000 cells/cm² onto culture dishes in culture medium, α-MEM (for
rMSCs or DMEM (for hMSCs), and supplemented with 10% FBS until subconfluence occurred. Afterwards, cells were grown in culture medium alone (control cells) or in osteogenic medium (OS cells) consisting of culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.05 mM ascorbic-2-phosphate acid. The osteogenic differentiation was evaluated by Alizarin Red S staining. At pre-established times, rMSC (7, 14, 21, 28 days) and hMSC (7, 14, 21, 28, 35, 42 days) cultures were fixed with 4% paraformaldehyde for 10 minutes. Then cells were incubated for 30 minutes at room temperature in 1% Alizarin Red S and 1% ammonium hydroxide. Following incubation, cultures were washed twice with water and air-dried. Alizarin Red S dye binds to calcium ions present in mineralized deposits resulting in a brilliant red staining. To perform a quantitative analysis, Alizarin Red S dye was solubilized with a solution of 5% SDS in 0.5M HCl and the optical density was measured with a spectrophotometer at 425 nm.

Adipogenic differentiation

rMSCs and hMSCs were seeded at approximately 15,000~20,000 cells/cm² onto dishes in culture medium (respectively α-MEM or DMEM) supplemented with 10% FBS. After 24 hours cells were induced by treatment with “Adipogenic Induction Medium” (AIM) consisting of 4.5 g/L glucose culture medium supplemented with 10% FBS and adipogenic supplements (10 µg/ml insulin, 500 µM isobutylmethylxanthine, 100 µM indomethacin and 1 µM dexamethasone). When lipid droplets were observed throughout the cell culture, the medium was switched to “Adipogenic Maintainance Medium” (AMM), consisting of 4.5 g/L glucose culture medium serum-free and supplemented with ITS+premix (1:100, Becton Dickinson, Franklin Lakes, NJ USA), 1 mM piruvate, 100 nM dexamethasone, 37.5 µg/ml ascorbic-2-phosphate acid and 10 ng/ml Transforming Growth Factor-β3 (TGF-β3, Peprotech, Rocky Hill, NJ, USA). hMSC’s “chondrogenic medium” consisted of 4.5 g/L glucose culture medium serum-free and supplemented with ITS+premix (1:100, Becton Dickinson, Franklin Lakes, NJ USA), 1 mM piruvate, 100 nM dexamethasone, 50 µg/ml ascorbic acid 2-phosphate, 10 ng/ml TGF-β3. Chondrogenic differentiation was evaluated by Safranin O staining. hMSC and rMSC pellets, treated with chondrogenic medium or with culture medium only, were fixed with 10% neutral buffered formalin for 30 minutes, paraffin embedded by standard methods and cut into 7 µm sections. Sections were stained with Hematoxylin-eosin and Safranin O 0.1%. The slides were mounted and photos were taken using a Nikon Coolscope instrument (Nikon Instruments S.p.A., Italy). Safranin O is a histological cartilage-specific staining that binds to proteoglycans and glycosaminoglycans resulting in a red/orange staining.

Results

MSCs’ characterization

In accordance with the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (21), and as previously demonstrated (15, 16, 22), the MSCs isolated from rat and human bone marrow used in our experiments, were: a) plastic-adherent and capable of extensive proliferation when maintained in their standard culture conditions; b) positive for several antigens such as CD29, CD90, CD105, CD73, and lacking in the

Statistical Analysis

Experiments for the quantitative assessment of osteogenic and adipogenic differentiation were performed three times in cells from different donors. The results were normalized to the absorbance of untreated control cells and they are expressed as mean±Standard Deviation. Statistical analysis was performed using the one-way ANOVA test and Tukey’s multiple-comparison test as a post-test with the Graph Pad Prism statistical package (GraphPad Software, San Diego, CA, USA).
expression of hematopoietic surface molecules CD34, CD45; c) able to differentiate into osteogenic, adipogenic and chondrogenic lineages under specific in vitro differentiating conditions. The different abilities of MSCs of human and rat origin to differentiate into the three mesenchymal lineages was compared.

**Osteogenic differentiation**

In rMSC cultures the Alizarin Red staining evidenced the presence of mineralized calcium only in osteogenic medium-treated cells after 14 days of treatment, and the intensity of the staining increased after 21 and 28 days of induction (Fig. 1A). These results were confirmed by the quantitative analysis of osteogenic differentiation, which evidenced a statistically significant increase in mineralized matrix deposition starting from 14 days of culture (Fig. 1B). On the contrary, untreated control cells resulted negative at all the examined time points for the Alizarin Red staining (Fig. 1A).

In hMSC cultures, the Alizarin Red positive staining was evident only after 28 days of treatment with osteogenic medium and the intensity of the staining increased at the later times examined, as also demonstrated by the quantitative analysis of mineralized matrix deposition (Fig. 1C and D). However, in some hMSC cultures from different donors, this Alizarin Red positive mineral deposition was observed later, after 35 or 42 days of osteogenic medium treatment. In control hMSC cultures Alizarin Red staining was negative (Fig. 1C).

**Adipogenic differentiation**

Small translucent Oil Red O positive lipid droplets were evident in the cytoplasm of some adipogenic treated rMSCs starting form 14 days of induction (Fig. 2A). The number of cells with intracellular lipid droplets increased slightly but not in a significant way after 21 days of treatment and it was estimated as being about 5% of the treated cells. The number of cells with lipid droplets did not
change at later times but, at 35 days post-induction, the droplets’ size increased in a few cells. The quantitative analysis of Oil Red O accumulation evidenced that, after the initial increased after 14 days of treatment, the dye amount reached a steady level which was maintained for all the observation time (Fig. 2B). In control rMSC cultures, no cells with Oil Red O positive lipid droplets were observed (Fig. 2A).

Small translucent lipid droplets positive for Oil Red O staining were evident in the cytoplasm of some adipogenic treated hMSCs (<5%) after 7 days of treatment. The number of these cells increased at later days, reaching the maximum (about 20~25%) at day 14 (Fig. 2C). The number of cells with lipid droplets did not change at later times but their size increased, probably as a result of a process of droplet fusion. The quantitative analysis of Oil Red O staining demonstrated a progressive and significant increase of dye accumulation during the differentiation period (Fig. 2D). In control hMSC cultures, no cells with Oil Red O positive lipid droplets were observed at any time (Fig. 2C). Variations in terms of the timing of differentiation were observed depending on the donors, since in some cases the differentiation process started to be evident later (14 days rather than 7 days).

**Chondrogenic differentiation**

Analysis of paraffin-embedded rMSC pellets treated for 5 weeks with chondrogenic medium revealed the presence, in a few areas of the sections, of big oval or polygonal cellular structures with abundant cytoplasm (Fig. 3). After 6 weeks of chondrogenic treatment, similar structures were present throughout the sections. The matrix in which such cells were located stained positively for Safranin O confirming the presence of proteoglycans and glycosaminoglycans. Moreover, many cells were located in small areas that resembled cartilaginous lacunae, sometimes with the presence of more than one cell, similar to cartilage isogen groups. Control pellets did not show the presence of any
specific cellular structure and were negative for Safranin O staining (Fig. 3).

Cartilaginous-like structures positive for Safranin O were not observed in hMSC treated pellets up to 6 weeks of treatment with chondrogenic medium, and only after 7 weeks of chondrogenic induction, they became evident (Fig. 3). Control hMSC pellets were always Safranin O negative and did not show cartilaginous-like structures.

Discussion

In view of a therapeutic application of MSCs for tissue repair and regeneration, many studies have been performed to evaluate the differentiation potential by using MSCs from various species, mainly from rodents. Among rodents, the rat model has several advantages with respect to mouse (23) representing a valid and widely used experimental model for diseases affecting tissues of mesen- genic origin (24). However, the results obtained from MSCs of different species are not fully comparable, since intrinsic differences exist and often do not allow the transfer of the findings observed (25).

A dissimilar differentiation potential among MSCs of different species has already been reported (25): sheep- and rabbit-derived MSCs have been reported as having a greater tendency to differentiate towards chondrogenic lineage with respect to human MSCs which take a longer time to differentiate. The diversity among MSCs is not limited to the differentiative abilities, but it reflects a slightly different molecular pattern, since it has been reported that MSCs from different species do not express the same surface antigens (19), do not have the same alkaline phosphatase activity (26), do not respond to the growth factor BMP-2 in the same way (27), have a different proliferation rate (14) and have different immunoregulatory capacities (19), and they are also characterized by a different genomic stability (15, 16). Also in terms of isolation there are some differences between species since some MSCs are more difficult both to isolate from bone marrow and to expand in culture than human or rat MSCs (11).

The results emerging from our study also evidenced a certain degree of variation in terms of differentiation time among MSCs of human origin. This is probably due to the intrinsic variability of each individual (28), since many factors influence the differentiation process, such as age, sex of the donors (29), or simply a different “propensity” of some bone marrow to produce mesengetic-derived tissues. The biological individual variability of MSCs of human origin raises a potential problem for the clinical use of autologous cells, since the MSCs from one patient
may take longer to differentiate with respect to those from another donor, thus making it difficult to set up a standard protocol suitable for clinical application. Recently, many studies have been directed at speeding up the differentiation process. In particular, the use of certain chemicals or some trophic factors, such as FGF4, seems to improve the MSCs’ expansion rate (30), while other factors, such as FGF, PDGF and TGF β, support the mesengenic differentiation process (31). Moreover, several authors have demonstrated that culturing MSCs under hypoxic conditions can enhance their production, enrichment and their overall differentiation (32) towards osteogenic (33), adipogenic (34) and chondrogenic lineages (35). All these approaches may help to improve MSCs’ usability overcoming the donor-dependent variability of human MSCs.

There are great differences also among MSCs from different rodent strains in terms of growth and differentiation (12). However, the rat MSCs used in our study came from animals of the same age, sex and strain and no variability in differentiation ability was observed.

In conclusion, on the basis of our data, it is important to carefully evaluate the results of pre-clinical studies performed with animal MSCs before attempting to transfer them into clinic. The knowledge of the peculiar differentiative features of MSCs derived from different sources can help to select the populations the more fitting to reach the proposed aim, since the different cellular source may be a misleading factor, and to better apply the results into clinical practice.

Acknowledgments
We are grateful to Dr. E. Genton for her language assistance. This work was supported by MIUR - FIRB Futuro in Ricerca 2008 Prot. N° RBFR08VSVI_001.

Potential conflict of interest
The authors have no conflicting financial interest.

References
1. Grigolo B, Lisignoli G, Desando G, Cavallo C, Marconi E, Tschon M, Giavaresi G, Fini M, Giardino R, Facchini A. Osteoarthritis treated with mesenchymal stem cells on hyaluronan-based scaffold in rabbit. Tissue Eng Part C Methods 2009;15:647-658
2. Abdallah BM, Kassem M. The use of mesenchymal (skeletal) stem cells for treatment of degenerative diseases: current status and future perspectives. J Cell Physiol 2009;218:9-12
3. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. Nat Rev Immunol 2008;8:726-736
4. Orciani M, Di Primio R. Skin-derived mesenchymal stem cells: isolation, culture, and characterization. Methods Mol Biol 2013;989:275-283
5. Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK. In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 2004;40:1275-1284
6. Tondreau T, Dejeneffe M, Meuleman N, Stamatopoulos B, Delforge A, Martiat P, Bron D, Lagneaux L. Gene expression pattern of functional neuronal cells derived from human bone marrow mesenchymal stromal cells. BMC Genomics 2008;9:166
7. Prockop DJ. Marrow stromal cells as stem cells for non-hematopoietic tissues. Science 1997;276:71-74
8. Satake K, Lou J, Lenke LG. Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue. Spine (Phila Pa 1976) 2004;29:1971-1979
9. Tse WT, Pendleton JD, Beyer WM, Egalal MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003;75:389-397
10. Zhukareva V, Obrocka M, Houle JD, Fischer I, Neuhuber B. Secretion profile of human bone marrow stromal cells: donor variability and response to inflammatory stimuli. Cytokine 2010;50:317-321
11. Phinney DG, Kopen G, Isaacson RL, Prockop DJ. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 1999;72:570-585
12. Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 2004;103:1662-1668
13. Wieczorek G, Steinhoff C, Schulz R, Scheller M, Vingron M, Ropers HH, Nuber UA. Gene expression profile of mouse bone marrow stromal cells determined by cDNA microarray analysis. Cell Tissue Res 2003;311:227-237
14. Zavan B, Giorgi C, Bagnara GP, Vindigni V, Abatangelo G, Cortivo R. Osteogenic and chondrogenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells cultured into polymeric scaffolds. Eur J Histochem 2007;51 Suppl 1:1-8
15. Foudah D, Redaeli S, Donzelli E, Bentivegna A, Miloso M, Dalprà L, Tredici G. Monitoring the genomic stability of in vitro cultured rat bone-marrow-derived mesenchymal stem cells. Chromosome Res. 2009;17:1025-1039
16. Redaeli S, Bentivegna A, Foudah D, Miloso M, Redondo J, Riva G, Baronchelli S, Dalprà L, Tredici G. From cyogenomic to epigenomic profiles: monitoring the biologic behavior of in vitro cultured human bone marrow mesenchymal stem cells. Stem Cell Res Ther 2012;3:47
17. Foudah D, Redondo J, Caldara C, Carini F, Tredici G, Miloso M. Expression of neural markers by undifferentiated rat mesenchymal stem cells. J Biomed Biotechnol 2012;2012:820821
18. Foudah D, Redondo J, Caldara C, Carini F, Tredici G, Miloso M. Human mesenchymal stem cells express neuronal markers after osteogenic and adipogenic differentiation. Cell Mol Biol Lett 2013;18:163-186

19. Ren G, Su J, Zhang L, Xiao Q, Ling W, L’huillery A, Zhang J, Lu Y, Roberts AL, Ji W, Zhang H, Rabson AB, Shi Y. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells 2009;27:1954-1962

20. Donzelli E, Salvadè A, Mimo P, Viganò M, Morrone M, Papagna R, Carini F, Zapo A, Miloso M, Baldoni M, Tredici G. Mesenchymal stem cells cultured on a collagen scaffold: In vitro osteogenic differentiation. Arch Oral Biol 2007;52:64-73

21. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317

22. Salvadè A, Belotti D, Donzelli E, D’Amico G, Gaipa G, Renoldi G, Carini F, Baldoni M, Pogliani E, Tredici G, Biondi A, Biagi E. GMP-grade preparation of biomimetic scaffolds with osteo-differentiated autologous mesenchymal stromal cells for the treatment of alveolar bone resorption in periodontal disease. Cytotherapy 2007;9:427-438

23. Martínez-Lorenzo MJ, Royo-Cañas M, Alegre-Aguarón E, Desportes P, Castiella T, García-Alvarez F, Larrad L. Phenotype and chondrogenic differentiation of mesenchymal cells from adipose tissue of different species. J Orthop Res 2009;27:1499-1507

24. Chu CR, Szczodry M, Bruno S. Animal models for cartilage regeneration and repair. Tissue Eng Part B Rev 2010;16:105-115

25. Martínez-Lorenzo MJ, Royo-Cañas M, Alegre-Aguarón E, Desportes P, Castiella T, García-Alvarez F, Larrad L. Phenotype and chondrogenic differentiation of mesenchymal cells from adipose tissue of different species. J Orthop Res 2009;27:1499-1507

26. Reilly GC, Radin S, Shen AT, Ducheyne P. Differential alkaline phosphatase responses of rat and human bone marrow derived mesenchymal stem cells to 45S5 bioactive glass. Biomaterials 2007;28:4091-4097

27. Diefenderfer DL, Oszczka AM, Reilly GC, Leboy PS. BMP responsiveness in human mesenchymal stem cells. Connect Tissue Res 2003;44 Suppl 1:305-311

28. Montzka K, Lassonczyk N, Tschöke B, Neuss S, Führmann T, Franzen R, Smeets R, Brook GA, Wöltle M. Neural differentiation potential of human bone marrow-derived mesenchymal stromal cells: misleading marker gene expression. BMC Neurosci 2009;10:16

29. Coipeau R, Rosset P, Langonne A, Gaillard J, Delorme B, Rico A, Domenech J, Charbord P, Sensebe L. Impaired differentiation potential of human trabecular bone mesenchymal stromal cells from elderly patients. Cytotherapy 2009;11:584-594

30. Farré J, Saura L, Prat-Vidal C, Soler-Botija C, Llach A, Molina CE, Hove-Madsen L, Cairó JJ, Gódia F, Bragós R, Cinca J, Bayes-Genis A. FGF-4 increases in vitro expansion rate of human adult bone marrow-derived mesenchymal stem cells. Growth Factors 2007;25:71-76

31. Ng F, Boucher S, Koh S, Sastry KS, Chase L, Lakshmipathy U, Choong C, Yang Z, Vemuri MC, Rao MS, Tanavde V. PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. Blood 2008;112:295-307

32. Dos Santos F, Andrade PZ, Saura L, Abecasis MM, da Silva CL, Cabral JM. Ex vivo expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. J Cell Physiol 2010;223:27-35

33. Nagano M, Kimura K, Yamashita T, Ohneda K, Nozawa D, Hamada H, Yoshikawa H, Ochiai N, Ohneda O. Hypoxia responsive mesenchymal stem cells derived from human umbilical cord blood are effective for bone repair. Stem Cells Dev 2010;19:1195-1210

34. Valorani MG, Germani A, Otto WR, Harper L, Biddle A, Kho CP, Lin WR, Hava MI, Tropel P, Patrizi MP, Pozzilli P, Alison MR. Hypoxia increases Sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential. Cell Tissue Res 2010;341:111-120

35. Kanchi M, Ferguson D, Prendergast PJ, Campbell VA. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1alpha. J Cell Physiol 2008;216:708-715